FERTILITY OF RAM SPERMATOZOA FROZEN BY THE PELLET METHOD

III. THE EFFECTS OF INSEMINATION TECHNIQUE, OXYTOCIN AND RELAXIN ON LAMBING

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Summary. A series of five experiments was conducted to examine ewe fertility following insemination with ram spermatozoa frozen by the pellet method.

With thawed semen of low concentration and relatively poor quality, fertility was very poor with an overall non-return rate of 5.6% (269 ewes). Following insemination with thawed semen of low and high sperm concentration (0.5 versus 1.5×10^9 motile cells/ml) the proportions of ewes lambing were 23.7 and 43.8% respectively. There was no difference in the proportion of ewes lambing when the inseminate volume was reduced from 0.3 to 0.1 ml (Exp. 2, dilute semen) or from 0.15 to 0.05 ml (Exp. 3, concentrated semen). Two inseminations gave higher fertility than a single insemination (Exp. 2, 38.8% versus 22.6%; Exp. 3, 53.0% versus 39.7%) but the magnitude of the response varied according to both the time of insemination and the concentration and volume of the inseminate.

High doses of oxytocin (5 or 10 i.u., i.m.) depressed the proportion of ewes lambing and relaxin (100 to 12,500 guinea-pig units/ewe) did not affect the depth to which the inseminating pipette could be inserted into the cervix.

Overall lambing results (percentage of ewes lambing) for Exps 2, 3, 4 and 5 were 30.5% (266 ewes), 46.3% (231), 49.3% (69) and 27.5% (291) respectively, whereas results for the best treatment combinations in these experiments were 61.9%, 56.7%, 64.7% and 40.4%.

INTRODUCTION

Lambing results following cervical insemination with ram spermatozoa frozen rapidly by the pellet method are generally low. Salamon (1967) obtained only 6% ewes lambing, Platov (1968) 2% to 24%, Loginova & Zeltobryuk (1968) 27%, Fraser (1968) 31% and Volkov (1968) 16%.

The causes of reproductive failure following insemination with ram spermatozoa frozen by the pellet method were investigated by Lightfoot & Salamon (1970a) who found that the transport of spermatozoa through the ewe's genital

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tract was primarily affected. The results demonstrated a failure of the establishment and maintenance of the cervical population of spermatozoa which could be partly overcome by use of inseminates with a high concentration of motile spermatozoa. The work also showed (Lightfoot & Salamon, 1970b) that embryonic mortality following cervical insemination with pellet frozen spermatozoa was of similar magnitude to that obtained with fresh semen, confirming that most reproductive wastage occurred before fertilization. Using double cervical insemination with thawed semen of high concentration, a lambing rate of 50% was obtained.

In view of these results, the experiments presented here were conducted to examine procedures that might enhance the transport of spermatozoa through the ewe's genital tract and therefore increase fertilization and lambing rates. The hormones, oxytocin and relaxin were also included in the study as there was reason to suspect that suitable therapy might influence sperm transport.

MATERIALS AND METHODS

Experimental designs

Details of design and treatment comparisons for each of the five experiments are presented in Experimental Designs and Results.

Sheep and management

Mature Merino ewes were used in all experiments and were allocated at random to treatments on a 'within draft' basis. The methods adopted for testing vasectomized rams before using them to identify ewes in oestrus, were as described previously (Lightfoot & Salamon, 1970a).

Semen

Semen was collected from mature Merino rams by artificial vagina. Ejaculates of good initial motility were pooled and diluted 1 : 1 (Exp. 1) or 1 : 3 (Exps 2 to 5) at 30° C with a diluent consisting of 166.5 mM-raffinose, 102 mM-sodium citrate, 15% v/v egg yolk to which glycerol (6% v/v, Exp. 1; 5%, Exps 2 to 5) was added. The diluted semen was cooled to 5° C over a period of 1½ hr, then held at that temperature for an additional 3 to 6 hr (Exps 1 to 4) or 10 to 12 hr (Exp. 5) before pellet freezing (0.035 ml, Exp. 1; 0.35 ml, Exps 2 to 5) on dry ice. The pellets were thawed in test tubes containing 88.4 mM-sodium citrate (2 : 1, pellets: thawing solution, v/v; Exp. 1) or 80.6 mM-sodium citrate-44.4 mM-glucose solution (1 : 3; Exps 2 to 5) held in a water bath at 37° C. Following thawing in Exps 2 to 5, the semen was centrifuged at 2500 rev/min for 15 min and the supernatant discarded to achieve the concentration of motile spermatozoa required for insemination.

Insemination

Cervical and cervical traction ('deep cervical') inseminations were as described previously (Salamon & Lightfoot, 1967).

In Exp. 2, a series of parameters termed 'classification factors', to distinguish them from true experimental factors, were recorded for each ewe at the time of

insemination. These classification factors were: time of detecting ewes in oestrus (18.00 hours or 08.00 hours draft), degree of vaginal constriction (normal, moderate constriction, marked constriction), necessity to drain mucus before insemination (mucus abundant or scarce), number of cervical papillae and the depth to which the inseminating pipette could be inserted into the cervical canal. For the latter observation, inseminating pipettes were calibrated at 1-cm intervals from the tip.

Oxytocin and relaxin

Oxytocin (Syntocinon, Sandoz Australia Pty Ltd) and relaxin (Releasin, Lot 10102.46, 20 mg relaxin reference standard/ml in sesame oil, Warner Lambert Pty Ltd, Australia) were administered by injection into the muscle of the hind leg. Oxytocin was administered at the time of insemination in Exp. 1 and immediately after either the first or second insemination in Exp. 5. In Exp. 4, relaxin was injected immediately after drafting, i.e. between 1 and 12 hr after the onset of oestrus.

Non-returns to service and lambing

The experimental ewe flocks were tested with vasectomized rams for 40 to 50 days after insemination to identify ewes that did not return to service. The non-return data for individual ewes was used to assist in the determination of lambing record (Exps 2 to 5) by udder examination (Dun, 1963) immediately after the conclusion of lambing.

Statistical analyses

The statistical significance of treatment comparisons in all experiments was tested by either analysis of variance, or χ^2 (Claringbold, 1961).

EXPERIMENTAL DESIGNS AND RESULTS

EXPERIMENT 1

The effects of method and time of insemination with frozen ram spermatozoa and the administration of oxytocin on fertility (non-returns to service) were examined in Exp. 1.

The experiment was of factorial design: $2 \times 2 \times 2$; $n = 33$ or 34 , $N = 269$.

- (1) Method of insemination—cervical versus cervical traction
- (2) Time of insemination—12 to 24 versus 24 to 36 hr after onset of oestrus
- (3) Administration of oxytocin—0 versus 10.0 i.u., i.m. at the time of insemination.

Ewes in oestrus were drafted from the flock twice daily at 06.00 hours and 18.00 hours, and held for either 12 or 24 hr before receiving a single insemination with 0.3 ml of thawed semen containing approximately 120×10^6 motile spermatozoa (0.4×10^9 motile cells/ml). The ewes not returning to service were recorded 38 to 41 days after insemination.

Owing to the sub-optimal methods used for freezing and thawing semen in this experiment (see Materials and Methods), the revival rate was low (25 to

30%) and the subsequent *in vitro* viability of the thawed spermatozoa was poor in relation to the semen used in Exps 2 to 5. Partly for this reason, the fertility was very low, with an overall non-return rate of 5.6%. The cervical traction method of insemination, however, resulted in significantly higher fertility than normal cervical insemination (9.7 versus 1.5%, $P < 0.01$). There was also an interaction between the administration of oxytocin and method of insemination ($P < 0.05$). The percentages of non-returns to service for control and oxytocin-injected ewes were 0.0 and 2.0%, respectively, following cervical insemination, compared with 14.9% and 4.5% after insemination by the cervical traction method. However, in view of the generally low fertility, this interaction is considered to be of little biological significance.

Results were similar whether ewes were inseminated either 12 to 24 or 24 to 36 hr after the onset of oestrus (5.3% and 5.9% respectively). The optimal treatment was insemination by cervical traction 24 to 36 hr after the onset of oestrus without an injection of oxytocin, which resulted in a non-return rate of 17.6%.

EXPERIMENT 2

The effects of inseminate type, and both method of insemination and number of inseminations with frozen ram spermatozoa on lambing were examined in Exp. 2.

The experiment was of factorial design: $3 \times 2 \times 2$; $n = 21$ to 23 , $N = 266$.

(1) Inseminate type—	Concentration of
motile spermatozoa
($\times 10^9/\text{ml}$)	Inseminate
volume
(ml)
(i)	1.5	0.1
(ii)	0.5	0.1
(iii)	0.5	0.3
- (2) Method of insemination—cervical versus cervical traction
 (3) Number of inseminations—1 versus 2

Ewes in oestrus were drafted from the flock both morning (08.00 hours) and evening (18.00 hours). Ewes detected in oestrus at the evening draft were inseminated for the first time at approximately 09.00 hours the following morning, together with the oestrous ewes from that morning's draft. The second insemination, when required, was performed approximately 12 hr later.

The overall results and χ^2 analysis are presented in Table 1. The concentration of motile spermatozoa in the inseminate had a marked effect on lambing ($P < 0.001$). Of the ewes inseminated, 43.8% and 23.7% lambled following insemination with concentrated ($1.5 \times 10^9/\text{ml}$) and dilute ($0.5 \times 10^9/\text{ml}$) semen respectively. Insemination by the cervical traction method resulted in significantly lower fertility than normal cervical insemination (23.9% versus 37.1%, $P < 0.05$). Two inseminations gave a higher lambing rate than a single insemination (38.8% versus 22.6%, $P < 0.01$) but there was an interaction ($P < 0.05$) between number of inseminations and inseminate type. The effect shown in

TABLE 1
EFFECTS OF TYPE OF INSEMINATE, METHOD OF INSEMINATION AND
NUMBER OF INSEMINATIONS WITH FROZEN RAM SPERMATOZOA
ON LAMBING

Treatment	No. of ewes		% of ewes lambing
	Inseminated	Lambing	
Type of inseminate			
Concentration of motile spermatozoa ($\times 10^9/\text{ml}$)			
(i) 1.5	89	39	43.8
(ii) 0.5	88	19	21.6
(iii) 0.5	89	23	25.8
Method of insemination			
Cervical	132	49	37.1
Cervical traction	134	32	23.9
Number of inseminations			
1	137	31	22.6
2	129	50	38.8
Overall	266	81	30.5

Analysis of χ^2

Effect	d.f.	χ^2
Type of inseminate (A)		
(i) versus (ii and iii)†	1	11.29***
(ii) versus (iii)‡	1	0.38
Method of insemination (B)	1	5.51*
Number of inseminations (C)	1	8.16**
A $\times$ B	2	1.06
A $\times$ C		
(i) versus (ii and iii) $\times$ C	1	1.92
(ii) versus (iii) $\times$ C	1	4.05*
B $\times$ C	1	1.41
A $\times$ B $\times$ C	2	1.09

† Concentrated versus dilute.

‡ 0.1 ml versus 0.3 ml.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 2
THE RELATIONSHIP BETWEEN THE TYPE OF INSEMINATE AND NUMBER OF INSEMINATIONS
ON LAMBING

Type of inseminate		One insemination			Two inseminations		
Concentration of motile spermatozoa ($\times 10^9/\text{ml}$)	Volume (ml)	No. of ewes		% of ewes lambing	No. of ewes		% of ewes lambing
		Inseminated	Lambing		Inseminated	Lambing	
1.5	0.1	46	19	41.3	43	20	46.5
0.5	0.1	45	8	17.8	43	11	25.6
0.5	0.3	46	4	8.7	43	19	44.2

Table 2 was due to the comparatively large increase in the proportion of ewes lambing following double insemination with 0.3 ml of dilute (0.5×10^9 motile cells/ml) semen. The best treatment was two cervical inseminations with 0.1 ml of concentrated (1.5×10^9 motile spermatozoa/ml) semen in which thirteen (61.9%) of the twenty-one inseminated ewes lambled.

Several classification factors were found to be significantly related to fertility. The results suggested the occurrence of an interaction ($\chi^2_1 = 3.71$; $0.05 < P < 0.1$) between time of drafting and number of inseminations on lambing (Table 3). In the single insemination treatment, a higher proportion of evening-draft ewes (inseminated approximately 15 to 25 hr after the onset of oestrus) lambled, compared with the ewes detected in oestrus at the morning draft (inseminated approximately 1 to 15 hr after the onset of oestrus). When a second insemination was given 12 hr after the first, a higher proportion of morning- than evening-draft ewes lambled.

TABLE 3
THE RELATIONSHIP BETWEEN TIME OF DRAFTING AND NUMBER OF INSEMINATIONS ON LAMBING

No. of inseminations	Time of drafting				
	1 hr before first insemination (08.00 hours)			15 hr before first insemination (18.00 hours)	
	No. of ewes		% of ewes lambing	No. of ewes	
	Inseminated	Lambing		Inseminated	Lambing
1 (at 09.00 hours)	77	14	18.2	60	17
2 (at 09.00 hours and 20.00 hours)	65	29	44.6	64	21
Overall	142	43	30.3	124	38
					30.6

Increases in both the number of cervical papillae ($\chi^2_2 = 10.73$; $P < 0.01$) and depth of insemination ($\chi^2_1 \text{ lin.} = 3.17$; $0.05 < P < 0.1$) were associated with higher lambing rates. Further analysis, however, revealed that these classification factors were not independently distributed. Depth of insemination increased markedly as the number of cervical papillae increased ($\chi^2_4 = 23.26$; $P < 0.001$). The depth of insemination was also related to the degree of vaginal constriction recorded ($\chi^2_4 = 13.74$; $P < 0.01$) due to the fact that a high proportion of ewes with constricted vaginae (indicative of barrenness) permitted only shallow insemination.

Although the cervical traction method of insemination resulted in a poorer lambing than the normal cervical method, it nevertheless achieved a greater depth of insemination. The proportions of ewes in which the depth of insemination was recorded as either less than or equal to 1.0 cm, 1.5 to 2.0 cm, or greater than 2.0 cm, were 27, 60 and 13%, compared with 49, 49 and 2% for the cervical traction and cervical methods of insemination, respectively ($\chi^2_2 = 21.91$; $P < 0.001$).

Perhaps the most important interaction involving classification factors and

TABLE 4
THE RELATIONSHIP BETWEEN ABUNDANCE OF MUCUS IN THE VAGINA AND DEPTH OF INSEMINATION ON LAMBING

	Depth of insemination into cervical canal (cm)										Overall			
	≤ 1				> 1 to 2				> 2					
	No. of ewes		% of ewes lambing	No. of ewes		% of ewes lambing	No. of ewes		% of ewes lambing	No. of ewes		% of ewes lambing		
	Inseminated	Lambing		Inseminated	Lambing		Inseminated	Lambing		Inseminated	Lambing			
Vaginal mucus														
Not abundant	58	8	13.8		69	26	37.7		10	4	40.0	137	38	27.7
	43	17	39.5		77	22	28.6		9	4	44.4	129	43	33.3
Abundant (required draining before insemination)														
Overall	101	25	24.8		146	48	32.9		19	8	42.1	266	81	30.5

fertility was that between depth of insemination and abundance of mucus in the vagina ($\chi^2 = 8.74$; $P < 0.05$; Table 4). Among ewes in which a depth of insemination of only 1 cm or less could be achieved, the lambing rate was much lower when mucus was scarce than when it was abundant (necessitating draining before insemination). Lack of mucus made little difference to fertility among ewes in which insemination depths of greater than 1 cm were achieved.

EXPERIMENT 3

The effects of method of semen collection, inseminate volume and number of inseminations with frozen ram spermatozoa on lambing were examined in Exp. 3.

The experiment was of factorial design: $2 \times 2 \times 2$; $n = 27$ to 30 , $N = 231$.

- (1) Method of semen collection—artificial vagina versus electro-ejaculation
- (2) Inseminate volume—0.05 versus 0.15 ml
- (3) Number of inseminations—1 versus 2.

The drafting procedure and times of insemination (cervical) were as described for Exp. 2, while the concentration of motile spermatozoa in the thawed semen used for insemination was approximately 1.6×10^9 cells/ml.

TABLE 5
EFFECT OF METHOD OF SEMEN COLLECTION, INSEMINATE
VOLUME AND NUMBER OF INSEMINATIONS ON LAMBING

Treatment	No. of ewes		% of ewes lambing
	Inseminated	Lambing	
Method of semen collection			
Artificial vagina	114	55	48.2
Electro-ejaculation	117	52	44.4
<i>P</i>			NS
Inseminate volume			
0.05 ml	116	52	44.8
0.15 ml	115	55	47.8
<i>P</i>			NS
Number of inseminations			
1	116	46	39.7
2	115	61	53.0
<i>P</i>			< 0.05
Overall	231	107	46.3

The results are presented in Table 5. Neither method of semen collection, nor decreasing the inseminate volume from 0.15 ml to 0.05 ml significantly affected the lambing results. The proportion of ewes lambing, however, was significantly affected by the number of inseminations. Performing a second insemination approximately 12 hr after the first improved the lambing rate from 39.7% to 53.0% ($P < 0.05$). A comparatively high overall lambing rate of 46.3% was obtained and the best treatment was two inseminations with 0.05 ml of semen

collected by artificial vagina (seventeen/thirty, 56.7%). There were no interactions.

An additional observation was made during the conduct of the experiment. In each of seven ewes, two pellets were thawed in the anterior vagina near the external cervical os. One ewe subsequently lambled.

EXPERIMENT 4

The effect of relaxin on depth of insemination and lambing following insemination with frozen ram spermatozoa was examined in Exp. 4.

Doses of 0, 100, 500 or 2500 guinea-pig units (g-p.u.) of relaxin were administered by intra-muscular injection approximately 12 hr before the first insemination. There were seventeen or eighteen ewes/treatment.

Ewes in oestrus were drafted from the flock at 08.00 hours and 18.00 hours daily and allocated at random to treatments. Morning- and evening-draft ewes were injected with relaxin at 09.00 hours and 21.00 hours respectively (i.e. 1 and 3 hr after drafting) and inseminated (cervical) twice at intervals of 12 and 24 hr after the injection.

The depth to which the inseminating pipette could be inserted into the cervical canal during insemination was influenced neither by the dose of relaxin (1.59, 1.77, 1.56 and 1.71 cm for the 0, 100, 500 and 2500 g-p.u. relaxin treatments respectively) nor the time of measurement after its administration (1.63 versus 1.68 cm for the first and second inseminations respectively). Depth of insemination was, however, closely related at both first and second inseminations ($\chi^2_4 = 13.79$; $P < 0.01$) and was significantly greater in ewes with higher numbers of cervical papillae (linear=linear; $\chi^2_1 = 4.20$; $P < 0.05$).

The proportions of ewes lambing in the 0, 100, 500 and 2500 g-p.u. relaxin treatments were 11/17 (64.7%), 9/18 (50.0%), 5/17 (29.4%) and 9/17 (52.9%) respectively. Although the proportion of ewes lambing in the relaxin treatments was lower than that for the control (44.2% versus 64.7%; $0.05 < P < 0.1$), owing to the low numbers of ewes, conclusions concerning the effect of relaxin on fertility cannot be drawn.

In view of the negative results on depth of insemination presented above, an additional observation was made during the conduct of the experiment. Three ewes were injected with 12,500 g-p.u. of relaxin. The mean depths of cervical penetration at the first and second insemination for these ewes were 1.33 and 1.67 cm respectively, and one of the ewes subsequently lambled.

EXPERIMENT 5

The effects of dose of oxytocin and time of its administration on lambing following insemination with frozen spermatozoa were examined in Exp. 5.

The experiment was of factorial design: 3×2 ; $n = 47$ to 50 , $N = 291$.

(1) Dose of oxytocin—0 versus 0.5 versus 5.0 i.u., i.m.

(2) Time of administration of oxytocin—immediately after first versus after second insemination

Ewes in oestrus were drafted from the experimental flock at 08.00 hours daily. All ewes received their first insemination immediately after drafting (approximately 1 to 25 hr after the onset of oestrus) and the second insemination approximately 10 hr later. The concentration of motile spermatozoa in the semen and inseminate volume used for all inseminations (cervical) were approximately 1.5×10^9 cells/ml and 0.1 ml respectively.

Semen used for this experiment was collected from twenty rams of unknown fertility and pellet-frozen under field conditions. The period of 10 to 12 hr for which the diluted semen was stored at 2 to 5°C before freezing was too long for optimal revival rates and subsequent viability of the thawed spermatozoa (Lightfoot & Salamon, 1969). Although there is no direct evidence, it is thought that these factors accounted for the comparatively low overall fertility (27.5% ewes lambing; Table 6) obtained in this experiment compared with that obtained with otherwise similar procedures in Exps 2, 3 and 4.

TABLE 6
LAMBING FOLLOWING THE ADMINISTRATION OF OXYTOCIN AT
EITHER THE FIRST OR SECOND INSEMINATION WITH FROZEN RAM
SPERMATOZOA

Treatment	No. of ewes		% of ewes lambing
	Inseminated	Lambing	
Dose of oxytocin (i.u., i.m.)			
0	99	24	24.2
0.5	96	36	37.5
5.0	96	20	20.8
<i>P</i> , 0 versus 0.5 and 5.0			NS
0.5 versus 5.0			<0.01
Time of administration of oxytocin			
After first insemination	144	37	25.7
After second insemination†	147	43	29.3
<i>P</i>			NS
Overall	291	80	27.5

† The second insemination was performed approximately 10 hr after the first insemination.

Injection of 5.0 i.u. oxytocin after insemination resulted in a lower proportion of ewes lambing (20.8%) than occurred in the 0.5 i.u. oxytocin (37.5%) or control (24.2%) treatments. The difference between the former two treatments was statistically significant ($P < 0.01$). The lambing rate was not affected by time of administering oxytocin.

DISCUSSION

It would appear from the results of experiments reported here that the methods employed in freezing and thawing ram spermatozoa by the pellet technique are critical for the attainment of satisfactory lambing results. The use of sub-optimal methods of dilution, equilibration and/or thawing in the preparation of semen for Exps 1 and 5 was associated with comparatively poor post-thawing sperm

activity and low fertility, although it is likely that other factors were also involved (e.g. one insemination only, low concentration of spermatozoa—Exp. 1; rams of unknown fertility—Exp. 5).

The importance of achieving a high concentration of motile spermatozoa in the inseminate to increase the number of spermatozoa entering the cervix was shown by Lightfoot & Salamon (1970a). In the experiments reported here, the concentration of spermatozoa in the inseminate was found to be a major factor influencing the proportion of ewes lambing. The low fertility observed in Exp. 1, in which semen containing only 0.4×10^9 motile spermatozoa/ml was used, is partly attributable to this factor.

In contrast to the importance of sperm concentration, the inseminate volume appeared to have little effect on the fertility, which is in agreement with the literature for fresh semen (e.g. Robinson, 1956, 1958; Sinclair, 1957; Kuznecov & Kuprijanova, 1961; Jones, Martin & Lapwood, 1969). Reducing the inseminate volume from 0.3 to 0.1 ml in Exp. 2 (0.5×10^9 motile spermatozoa/ml) or from 0.15 to 0.05 ml in Exp. 3 (1.6×10^9 motile spermatozoa/ml) did not significantly affect the lambing rate. It seems likely, at least with ewes in which semen can be deposited into the cervical os, that use of a concentrated inseminate of even less than 0.05 ml may be practical.

The increase in the lambing rate that was achieved by double insemination with frozen semen was greater than that normally reported for inseminations with fresh ram semen (e.g. Sinclair, 1957; Salamon & Robinson, 1962; Dunlop & Tallis, 1964). This is compatible with the evidence (Salamon & Lightfoot, 1967; Mattner, Entwistle & Martin, 1969; Lightfoot & Salamon, 1970a) that infertility following insemination with deep frozen ram semen is associated with a failure of sperm entry and viability within the ewe's genital tract. The magnitude of the response to double insemination with frozen semen, however, seemed to vary according to the characteristics of the inseminate and the timing of insemination in relation to the stage of oestrus of the ewe. In the former case, the increase in fertility was greater with dilute than concentrated semen (Exp. 2, Table 2), and in the latter case, more advantage was gained from performing a second insemination when the first had been given early, rather than towards the middle of oestrus (Table 3). It should be noted, however, that the results reported here conflict with those of other workers who have failed to obtain a fertility response of similar magnitude to double insemination with frozen ram semen (First, Sevinge & Henneman, 1961; Lopatko, 1963; Salamon, 1967).

More experimentation is required before the value of the cervical traction method of insemination can be adequately assessed. In Exp. 1, the results were in favour of this method, but the overall fertility was very low. Normal cervical insemination was superior to the cervical traction method, both in Exp. 2 and in an experiment reported elsewhere (Lightfoot & Salamon, 1970b). Ten En Bon (1965) used frozen semen and reported that 36.0% ewes (201) versus 46.4% ewes (28) lambed following the normal and a 'deep cervical' method of insemination respectively. However, it appears that only those ewes in which a depth of 2 to 5 cm insemination could be achieved were included in the latter treatment.

The relationship between depth of insemination and ewe fertility, as indicated in Exp. 2 and reported by Šarapa (1967), is difficult to interpret as insemination depth is closely related to the number of cervical papillae (Exps 2 and 4) which is, in turn, associated with both ewe fertility (Exp. 2) and increasing parity in ewes (Dun, 1955). It is possible that, when using frozen semen, increasing numbers and complexity of the cervical papillae assist in retaining a greater proportion of the inseminate in contact with mucus at the external cervical os, resulting in better penetration of spermatozoa into the cervix. This hypothesis is supported by the observation (Exp. 2) that, in ewes with little mucus and in which only shallow insemination can be achieved, fertility is much reduced.

The hormonal requirements for cervical relaxation in non-pregnant farm animals are not clear. Cervical relaxation has been reported in both swine and cattle with doses of relaxin varying from 500 to 15,000 g-p.u., usually preceded by oestrogen treatment (Graham & Dracy, 1953; Zarrow, Sikes & Neher, 1954; Zarrow, Neher, Sikes, Brennan & Bullard, 1956; Eggee & Dracy, 1966). However, Smith & Nalbandov (1958) found that relaxin injections (750 g-p.u.) did not cause cervical relaxation in sows when given concurrently with oestrogen. Although the range of doses used in the present study with oestrous ewes (100 to 12,500 g-p.u.) seems to be adequate in relation to those used by other workers, there was no visible effect of the hormone on the external os of the cervix, nor was a deeper insemination achieved.

The results of the present experiments do not support the use of oxytocin as an agent to improve fertility in the ewe, contrary to evidence concerning its use in cows (Hays, VanDemark & Ormiston, 1953, 1958; Milovanov & Sergeev, 1961; Milovanov, Sokolovskaja, Korotkov, Gorohov, Masenko, Rymarj & Rahman, 1963; Stepanov, 1964; Masenko, 1966) and sows (Stratman, Self & Smith, 1958; Milovanov & Sergeev, 1961; Sergeev, 1963; Ikoev & Ermakov, 1965). The high doses (5.0 to 10.0 i.u.) used in Exps 1 and 5 depressed ewe fertility, thereby confirming earlier results (Lightfoot & Salamon, 1970a). The lambing rate was slightly higher than controls following the administration of a low dose of oxytocin (0.5 i.u.; Exp. 5) but the effect was not significant, in agreement with experiments conducted with fresh semen by Jones (1968) and Jones *et al.* (1969). Derjažencev (1966), however, reported increased fertility in a group of ewes inseminated with fresh semen to which a posterior pituitary preparation had been added.

Apart from more recent reports, the literature concerning fertility of deep frozen ram semen has been reviewed by Emmens (1961), Emmens & Robinson (1962) and Sadleir (1966). These reviews point out that critical assessment of many of the reports is difficult as the authors frequently omit essential details concerning the conduct of the experiments. The highest fertility—61 to 66%—has been reported by Mackepladze, Gugušvili, Bregadze & Haratišvili (1960) and by Lopatko (1963)—41 to 67%—but the latter results have not been confirmed (Branny, Pilch & Wierzbowski, 1966; Salamon, 1967; Lopyrin, 1969). Lopyrin reported that the high fertility results claimed by the Soviet workers have subsequently been tested under supervision of a commission and “none of the authors could confirm their own results and obtain a fertility rate higher

than 14% after one insemination. Evidently, this was due to the replacement of fertile teaser rams by vasectomized rams. Thus, the possibility of occasional fertilization of ewes by natural mating has been excluded". Aamdal & Andersen (1968) recently reported 62% pregnancy in twenty-six ewes inseminated with semen frozen in straws. Reasonably good results have also been claimed by Kalév & Vénkov (1961; 55% non-returns), Vlachos & Tsakalof (1965; 23 to 56%) and Ten En Bon (1965; 36 to 46%) but the authors have not mentioned what type of teaser rams were used for detection of ewes in oestrus.

Previous publications concerned with ram semen frozen by the pellet method, mentioned earlier, have reported generally low fertility. The comparatively high fertility reported by Lightfoot & Salamon (1970b) has been confirmed in the experiments reported here and a number of factors that may affect the fertility of pellet-frozen ram spermatozoa have been identified. Further development may lie in a critical assessment of the minimum inseminate volume that may be used without reduced fertility to achieve the most economical use of frozen semen.

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