# STUDIES OF THE SUITABILITY OF PREPARATIONS OF EWE AND COW MILK FOR STORING RAM SPERMATOZOA AT 37, 5, AND −79°C

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*Summary*

Ram semen was diluted in skim ewe or cow milk, and in preparations of the non-dialysable components of these resuspended in various synthetic diluents, and was then stored at 37, 5, or −79°C. There was little difference in the protective action of preparations from these two sources. The toxicity of fresh skim milk was reduced by heating at 92°C for 10 min, by the addition of 0·5 mg/ml of cysteine hydrochloride, or by the processes involved in dialysis and lyophilization of the milk solids. However, heat denatured the ewe milk more than the cow milk. The best method of preparing diluents from fresh skim milk was to add 0·5 mg/ml cysteine hydrochloride and then to heat. To remove the toxic factor in fresh cow milk, either heating or addition of cysteine was as effective as, or better than, a combination of both treatments. In the range 0·38–3·00% w/v, the optimum concentration of lyophilized, non-dialysable skim milk preparations added to synthetic diluents depended on their source (i.e. ewe or cow milk), the heat treatment before dialysis, and the measure of response.

## I. Introduction

The incorporation of a protein in diluents for ram or bull semen could buffer the solution against changes in pH, chelate any heavy metals present, and partially protect spermatozoa during dilution (Blackshaw 1953) and reduction in temperature (Choong and Wales 1962), and these are possible reasons for the success of milk as a diluent for bull semen (Melrose 1962).

Thacker and Almquist (1953) found that fresh cow milk is toxic to bull spermatozoa and Thacker, Flipse, and Almquist (1954) defined the toxic factor as being inactivated by heat at 92°C, non-dialysable, and present in the serum protein fraction of milk. Flipse, Patton, and Almquist (1954) confirmed opinions that lactenin, the antistreptoccal factor in milk (Jones and Simms 1930), is toxic to spermatozoa. Since lactenin is irreversibly inactivated by the addition of compounds with free sulphydryl groups (Wilson and Rosenblum 1952*a*, 1952*b*) and these groups are present in β-lactoglobulin when milk is heated above pasteurization temperatures (Larsen, Jenness, and Geddes 1949; Hutton and Patton 1952; Zweig and Block 1953), it was reasoned (Johnson, Flipse, and Almquist 1955) that heating milk "activated" sulphydryl groups of β-lactoglobulin which rendered lactenin non-toxic to spermatozoa. The latter authors reduced the toxicity of milk in this way and showed that bull spermatozoa can survive equally well in fresh milk containing

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1·0 mg/ml of cysteine hydrochloride or in milk previously heated at 92°C for 10 min. Concentrations of 0·5 and 2·0 mg/ml of cysteine were found to be "almost as effective" as 1·0 mg/ml, while 0·25 and 4·0 mg/ml were inferior to these levels.

Saacke, Almquist, and Flipse (1956) found that variations between batches of skim cow milk meant that different degrees of heat treatment were required for detoxification. Blackshaw (1960) found that, for ram spermatozoa, heating at 85°C for 5 min is sufficient to detoxify pasteurized milk.

Reactions of ram spermatozoa to the protein of cow milk could occur and the composition of ewe and cow milk is sufficiently different to warrant comparative studies of their value in semen diluents. Trials by Salamon and Robinson (1962) showed that the fertility of semen diluted with heated whole ewe milk was not as high as semen diluted with whole or skim cow milk. In this paper experiments are described which test fresh skim ewe milk or synthetic solutions containing lyophilized preparations of non-dialysable ewe and cow milk as diluents for storing ram spermatozoa at 37, 5, and −79°C.

## II. Materials and Methods

Semen was collected from Merino rams after electrical stimulation (Blackshaw 1954; Martin and Rees 1962) and only dense ejaculates with wave motion scores of 3·5–4·0 (range 0–4, Emmens 1947) were diluted for use in the experiments.

### (a) *Diluents for Storing Spermatozoa at 37 or 5°C*

(1) Reconstituted skim milk—an aqueous solution of 9% w/v dried skim milk solids and 17 mM fructose.

(2) Skim milk preparations—various preparations of fresh skim ewe or cow milk and skim pasteurized cow milk containing 17 mM fructose were used in experiments 1 and 2.

(3) Fructose synthetic—solutions of various concentrations (Tables 1 and 3) of the lyophilized, non-dialysable fraction of skim ewe or cow milk either heated or unheated (Martin, unpublished results) were prepared in 54 mM sodium chloride, 4 mM potassium chloride, 2·5 mM sodium dihydrogen phosphate, 2·5 mM potassium dihydrogen phosphate, and 202 mM fructose for use in experiments 1 and 2.

### (b) *Diluents for Deep-freezing Spermatozoa*

(i) *First-stage Diluent—Lactose Synthetic*

The ionic composition was the same as the fructose synthetic but the sugar component of this synthetic diluent was 185 mM lactose and 17 mM fructose (the concentrations of the lyophilized milk preparations are shown in Table 4).

(ii) *Second-stage Glycerol-containing Diluent*

In this, the fructose concentration was 140 mM and the diluents contained 1·77M glycerol, with the content of lactose and milk preparation the same as in the matching diluent (i).

In experiments 1, 2, and 3 cysteine hydrochloride was added to the solutions approximately 1–2 hr prior to their use as diluents. Cysteine hydrochloride was added either before or after heating of some of the preparations of skim milk used in experiment 4 and these preparations were stored for at least 12 hr at 5°C before use. Milk samples were heated in a water-bath so that the milk temperature reached 92°C for 10 min, and following this they were cooled rapidly to ambient temperature.

A diluent based on Krebs–Henseleit–Ringer (Jones 1965*a*) was used to incubate deep-frozen spermatozoa after thawing.

### (c) *Semen Processing*

In experiments 1 and 2 semen was diluted 40-fold and stored at 37°C or cooled over 2 hr to 5°C for storage. For deep-freezing (experiments 3 and 4) semen was initially diluted 20-fold with the first-stage diluent (30°C) and further diluted 1 : 1 at 5°C with the diluent containing glycerol. Diluted semen was cooled at a constant rate from 30 to 5°C in 2 hr, then "aged" at 5°C for 2 hr, "equilibrated" for 0·5 hr (Martin 1963), and frozen in 1-ml aliquots in an apparatus patterned on the device described by Polge and Lovelock (1952). After storage at −79°C for at least 1 day semen was thawed at 37°C, evaluated, concentrated by centrifugation, and resuspended for incubation at 37°C.

### (d) *Scoring Survival of Spermatozoa*

A system of coding and randomization of tubes which contained samples of semen of each treatment (Martin 1963) was used in all experiments so that, at the time of scoring, the treatment was not known and the "samples" were examined microscopically in a random order. As in previous studies (Jones 1965*a*) scores of progressive motility (range 0–4; Emmens 1947) and percentage of motile cells were made following microscopic examination of semen samples, and congo red–nigrosin smears were prepared for counting the percentage of unstained spermatozoa (Blackshaw 1955).

### (e) *Transformation and Analysis of Scores*

Scores of motility were doubled to remove fractions and the observations on proportions of stained and unstained spermatozoa and of motile and dead spermatozoa were transformed to angles (Claringbold, Biggers, and Emmens 1953) and these values were used as unit observations for the analyses of variance. The analyses were performed by the SILLIAC, an automatic electronic digital computer, using a programme described by Claringbold (1957) which generates sets of contrasts from the direct product of matrices of the orthogonal polynomial coefficients described by Fisher (1950) and tabulated by Fisher and Yates (1957). Other sets of orthogonal contrasts, each with a single degree of freedom, were used in a similar manner to partition components of a variable in the analyses of experiments 1 and 4, and as an example the matrix describing the contrasts used for experiment 1 is shown in Table 2. The significant effects in experiment 4 are described in the text.

The levels of statistical significance of treatment main effects are shown in the relevant tables, and significant interactions are described in the text. The error variances for experiments 1–4 are shown at the base of Tables 2, 3, 4, and 6 respectively.

In tables of means $n$ refers to the number of observations used to calculate the mean.

## III. Results

### (a) *Experiment 1*

The toxicity, to spermatozoa stored at 37 or 5°C, of fresh skim ewe milk was studied in two experiments of similar design ($2^2$ factorial experiments replicated with ejaculates from four rams) with the levels of factors shown in Table 1. Two other diluents were used for comparison: heated pasteurized skim cow milk and a synthetic diluent containing fructose. Spermatozoa diluted in fresh ewe milk were immotile within a few hours of dilution so that scores relating to this treatment were not included in the analyses of variance (Table 2), which showed that the following effects were significant:

(1) For storage at 37°C, ewe milk prepared by heat and cysteine treatment was less toxic to spermatozoa than if prepared by either of these treatments alone.

(2) Spermatozoa survived better in heated ewe milk than in unheated ewe milk containing cysteine at 5°C.
(3) None of the ewe milk diluents was as satisfactory as heated pasteurized cow milk or fructose synthetic diluent at either 37 or 5°C.

TABLE 1

EXPERIMENT 1: VIABILITY OF RAM SPERMATOZOA AFTER INCUBATION FOR 2·5 HR AT 37°C OR FOR 3 DAYS AT 5°C IN VARIOUS PREPARATIONS OF FRESH SKIM EWE MILK, A FRUCTOSE SYNTHETIC DILUENT, AND HEATED FRESH PASTEURIZED COW MILK

Temperatures in parentheses are incubation temperatures

| Diluents ($n$ = 8) | Mean Scores of Survival | | | |
|---|---|---|---|---|
| | Motility (37°C) | % Motile (37°C) | Motility (5°C) | % Motile (5°C) |
| Ewe milk preparations | | | | |
| No heat, 0·0 mg/ml cysteine (1) | 0·00 | 0·0 | 0·00 | 0·0 |
| No heat, 0·5 mg/ml cysteine (2) | 1·44 | 23·8 | 2·25 | 28·8 |
| Heated, 0·0 mg/ml cysteine (3) | 1·06 | 16·3 | 2·25 | 42·5 |
| Heated, 0·5 mg/ml cysteine (4) | 2·63 | 46·3 | 2·38 | 42·5 |
| Fructose synthetic* (5) | 3·25 | 58·8 | 2·56 | 56·3 |
| Heated cow milk (6) | 3·06 | 55·0 | 2·69 | 60·0 |

* This diluent contained 1·6% w/v of lyophilized non-dialysable heated cow milk.

TABLE 2

ORTHOGONAL COEFFICIENTS USED TO PARTITION TREATMENT MAIN EFFECTS, AND STATISTICALLY SIGNIFICANT EFFECTS IN EXPERIMENT 1

Diluent numbers taken from Table 1. H, heated; C, cysteine-treated. FS, fructose synthetic diluent

| Orthogonal Coefficients for Diluents | | | | | Factor and Nature of Comparison | Significance of Effect on | | | |
|---|---|---|---|---|---|---|---|---|---|
| 2 | 3 | 4 | 5 | 6 | | Motility (37°C) | % Motile (37°C) | Motility (5°C) | % Motile (5°C) |
| −1 | 1 | 0 | 0 | 0 | Ewe milk: H $v.$ C | † | † | † | ** |
| −1 | −1 | 2 | 0 | 0 | Ewe milk: H or C $v.$ H+C | *** | *** | † | † |
| 0 | 0 | 0 | −1 | 1 | Cow milk $v.$ FS | † | † | † | † |
| 2 | 2 | 2 | −3 | −3 | FS+cow milk $v.$ all ewe milks | *** | *** | * | *** |
| | | | | | Error variance‡ | *1·08* | *38·92* | *0·55* | *29·03* |

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$. † Non-significant.
‡ 20 degrees of freedom.

### (b) *Experiment 2*

Samples of the unheated and heated ewe milk preparations used in the previous experiment were dialysed and the non-dialysable fraction lyophilized for use in experiments 2 and 3. Experiment 2 was a $2 \times 3^2$ factorial design with replicates from

four ejaculates (Table 3), to which, for a "control" comparison, a diluent containing 1·6% w/v of lyophilized non-dialysable heated skim cow milk was added.

The lyophilized non-dialysable ewe milk preparation did not dissolve completely in the synthetic diluents used in experiments 2 and 3, even when stored for 18 hr at 5°C. Individual *F*-tests showed that spermatozoa survived as well in the best diluent containing a preparation of ewe milk as in the control diluent. The following treatment effects were significant in the analyses of variance.

TABLE 3

EXPERIMENT 2: VIABILITY OF RAM SPERMATOZOA AFTER INCUBATION FOR 4 HR AT 37°C OR 5 DAYS AT 5°C IN FRUCTOSE SYNTHETIC DILUENTS CONTAINING VARYING CONCENTRATIONS OF CYSTEINE AND PREPARATIONS OF LYOPHILIZED NON-DIALYSABLE EWE AND COW MILK

Incubation temperatures given in parentheses

| Factor and Level | Mean Scores of Survival | | | |
|---|---|---|---|---|
| | Motility (37°C) | % Motile (37°C) | Motility (5°C) | % Motile (5°C) |
| Ewe milk preparation ($n$ = 36) | | | | |
| Not heated | 2·11 | 35·3 | 1·94 | 34·0 |
| Heated | 1·93 | 28·7 | 2·04 | 34·3 |
| Level of ewe milk preparation ($n$ = 24) | | | | |
| 0·5% w/v | 1·73 | 23·8 | 1·56 | 25·6 |
| 1·0% w/v | 2·29 | 37·1 | 2·10 | 34·2 |
| 2·0% w/v | 2·04 | 34·0 | 2·31 | 42·7 |
| $P$ (linear) | † | ** | *** | *** |
| $P$ (quadratic) | * | ** | † | † |
| Level of cysteine ($n$ = 24) | | | | |
| 0·0 mg/ml | 3·08 | 52·1 | 2·31 | 42·9 |
| 0·5 mg/ml | 1·92 | 27·8 | 2·40 | 42·5 |
| 1·0 mg/ml | 1·06 | 16·1 | 1·27 | 17·1 |
| $P$ (linear) | *** | *** | *** | *** |
| $P$ (quadratic) | † | † | *** | *** |
| Control diluent‡ ($n$ = 4) | 3·50 | 65·0 | 2·88 | 65·0 |
| Error variances§ | *1·69* | *88·12* | *0·94* | *72·47* |

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$. † Non-significant.

‡ 1·6% w/v lyophilized heated cow milk preparation.

§ 40 degrees of freedom.

(i) *After Incubation at 37°C*

Spermatozoa survived storage better in the solutions containing 1·0 rather than 0·5 or 2·0% of the lyophilized ewe milk preparation. The interaction of milk preparation levels and cysteine content of the diluent was significant ($P < 0·05$) in the analysis of motility scores and it was found that motility was very poor in the diluent with 0·5% milk solids and 1·0 mg/ml cysteine.

An increase in cysteine from 0·0 to 0·5 to 1·0 mg/ml lowered the survival of spermatozoa, although the viability of some ejaculates was not reduced as much as other ejaculates, i.e. interaction of level of cysteine × ejaculates was significant ($P < 0{\cdot}001$) for both measures of response.

(ii) *Spermatozoa Stored at 5°C*

An increase in concentration of the lyophilized milk preparation from 0·5 to 2·0% caused a corresponding increase in the survival of spermatozoa during storage in a fructose synthetic diluent.

Overall, a concentration of 1·0 mg of cysteine per millilitre of diluent greatly decreased survival of spermatozoa. However, an interaction of heat treatment of the milk preparation and cysteine levels (significant for both measures of response; $P < 0{\cdot}001$) is illustrated in Figure 1. If no cysteine was present, diluents containing unheated milk preparations were better for storing spermatozoa than diluents containing heated milk, but preparations of heated milk were substantially improved by the addition of 0·5 mg/ml of cysteine to the diluent.

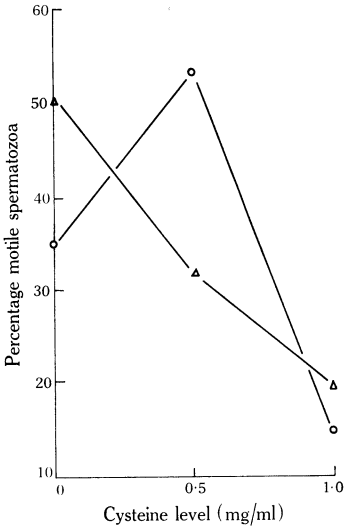


Fig. 1.—Effects of adding cysteine to diluents containing lyophilized preparations of non-dialysable fractions of skim ewe milk on the survival of ram spermatozoa at 5°C.
△ Unheated preparation.
○ Heated preparation.

(c) *Experiment 3*

Spermatozoa diluted in a lactose synthetic diluent were deep-frozen in this experiment (a 2×3×4 factorial design replicated with ejaculates from four rams), for which the factors and levels are shown in Table 4. Additionally, a sample of spermatozoa from each ejaculate was frozen in a reconstituted skim milk diluent. Individual $F$-tests showed that the mean scores of motility and percentage motile spermatozoa for semen samples frozen in this control diluent did not differ significantly from the mean scores for samples frozen in the best of the synthetic diluents. A higher proportion of unstained spermatozoa were observed for samples frozen in

either of the two diluents containing 3·00% of the unheated ewe milk preparation than in the control diluent ($P < 0·001$).

Some of the factors tested interacted significantly with ejaculates in the analyses of variance, so that the relevant treatment × ejaculate interaction was used as the estimate of error for the $F$-test when examining the significance of main effects. After using this test in the appropriate cases, the effects discussed below were significant.

TABLE 4

EXPERIMENT 3: MEAN CHARACTERISTICS IMMEDIATELY UPON THAWING AND AFTER INCUBATION FOR 1 HR AT 37°C OF RAM SPERMATOZOA DEEP-FROZEN IN RECONSTITUTED SKIM COW MILK AND LACTOSE SYNTHETIC DILUENTS CONTAINING VARIOUS PREPARATIONS OF LYOPHILIZED NON-DIALYSABLE EWE AND COW MILK

| Factor and Level | Mean Scores of Survival | | | | |
|---|---|---|---|---|---|
| | Motility: | | % Motile: | | % Unstained: |
| | Thawed | Incubated | Thawed | Incubated | Thawed |
| I. Lyophilized prepn. ($n$ = 32) | | | | | |
| Ewe milk, not heated (1) | 2·17 | 2·17 | 26·6 | 23·8 | 40·7 |
| Ewe milk, heated (2) | 1·33 | 1·28 | 10·2 | 8·4 | 18·2 |
| Cow milk, heated (3) | 1·94 | 1·77 | 23·4 | 15·0 | 29·2 |
| $P$ (Ia: 2 versus 3) | *** | ** | *** | *** | *** |
| $P$ (Ib: 1 versus mean of 2 and 3) | *** | *** | *** | *** | *** |
| II. Level of lyophilized prepn. ($n$ = 24) | | | | | |
| 0·38% w/v | 1·63 | 1·75 | 17·1 | 12·5 | 24·3 |
| 0·75% w/v | 1·71 | 1·58 | 17·7 | 14·0 | 27·3 |
| 1·50% w/v | 1·90 | 1·73 | 22·3 | 18·8 | 33·3 |
| 3·00% w/v | 2·02 | 1·90 | 23·1 | 17·7 | 32·5 |
| $P$ (linear) | * | † | * | ** | *** |
| III. Level of cysteine ($n$ = 48) | | | | | |
| 0·0 mg/ml | 1·69 | 1·97 | 15·9 | 16·3 | 22·3 |
| 0·5 mg/ml | 1·94 | 1·59 | 24·2 | 15·2 | 36·5 |
| $P$ | * | * | *** | † | *** |
| Control diluent‡ ($n$ = 4) | 2·75 | 3·00 | 35·0 | 27·5 | 27·0 |
| Error variance§ | *1·43* | *1·51* | *47·07* | *28·09* | *26·77* |

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$. † Non-significant.

‡ Reconstituted skim cow milk. § 57 degrees of freedom.

(1) All scores of response show that the best lyophilized milk used was prepared from fresh unheated ewe milk and that preparations from heated cow milk were better than preparations from heated ewe milk. Significant interactions of milk preparations and ejaculates arose because of variation in response to heated cow and ewe milk preparations from ejaculate to ejaculate.

The interaction between level and type of milk preparation was significant for all measures of response. Figure 2 illustrates this interaction for scores of percentage motile spermatozoa after incubation at 37°C following thawing. The preparation from unheated ewe milk reached an optimum at 1·5% w/v, whereas a similar preparation from heated ewe milk showed no such response.

(2) As an average effect, the addition of cysteine to the diluents caused an increase in motility scores upon thawing. The interaction of cysteine levels and ejaculates for scores of percentage motile ($P < 0{\cdot}01$) and unstained ($P < 0{\cdot}001$) spermatozoa upon thawing, and motility ($P < 0{\cdot}01$) and percentage motile after incubation ($P < 0{\cdot}05$), showed that the effect of cysteine was greater on some ejaculates than others.

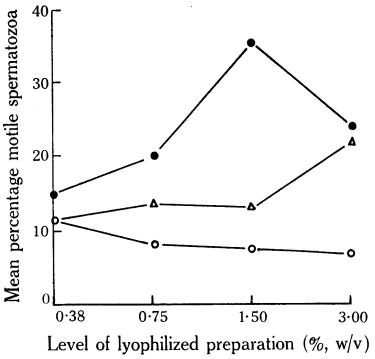


Fig. 2.—Effects of levels of various preparations of lyophilized non-dialysable skim milk on the percentage of motile ram spermatozoa after deep-freezing and incubation for 1 hr at 37°C after thawing.
● Unheated ewe milk.
○ Heated ewe milk.
△ Heated cow milk.

(3) Cysteine did not have the same effect on the three milk preparations used in the diluents (Table 5). The Ia × III component of the interaction for scores of motility after incubation shows that the addition of cysteine was slightly beneficial to spermatozoa in diluents containing heated ewe milk preparations, but detrimental in diluents containing preparations of heated cow milk. For scores of motility upon thawing and motility and percentage motile spermatozoa after incubation, the Ib × III interaction shows that when these scores are averaged for the two diluents containing heated milk preparations, the effect of adding cysteine was small, whilst its presence in diluents containing unheated ewe milk caused a marked reduction in these scores of survival.

### (*d*) *Experiment 4*

The treatments in this 2 × 4 factorial experiment, with six replicates, are shown in Table 6. Samples of spermatozoa diluted in fresh skim milk were also prepared, but since 95–100% were immotile after chilling to 5°C, they were not frozen. Additionally, samples from each ejaculate were frozen in a reconstituted skim milk diluent ("control" diluent). For preparation of the diluents in this experiment, fresh milk was collected from a cow and five ewes, all of which were in their second or third week of lactation. Upon heating after skimming, the ewe milk coagulated, but the heated cow milk did not. A second collection of ewe milk from ewes in their 10th and 14th week of lactation did not coagulate on heating, so this and a fresh sample of cow milk was used in the experiment. *F*-tests, which compared average survival rates in the "control" diluent with the best average survival rates observed in the fresh milk diluents, showed that reconstituted skim milk was the superior diluent, as measured

TABLE 5

EXPERIMENT 3: EFFECT OF CYSTEINE ON THE VIABILITY OF RAM SPERMATOZOA DEEP-FROZEN IN LACTOSE SYNTHETIC DILUENTS CONTAINING PREPARATIONS OF LYOPHILIZED NON-DIALYSABLE EWE OR COW MILK ($n = 16$)

A, upon thawing; B, after 1 hr at 37°C

| Treatment | Mean Scores of: Motility (A) | Motility (B) | % Motile (B) |
|---|---|---|---|
| Ewe milk prepn., not heated | | | |
| 0·0 mg/ml cysteine | 2·25 | 2·53 | 27·2 |
| 0·5 mg/ml cysteine | 2·09 | 1·81 | 20·3 |
| Ewe milk prepn., heated | | | |
| 0·0 mg/ml cysteine | 1·13 | 1·09 | 7·2 |
| 0·5 mg/ml cysteine | 1·53 | 1·47 | 9·7 |
| Cow milk prepn., heated | | | |
| 0·0 mg/ml cysteine | 1·69 | 2·03 | 14·4 |
| 0·5 mg/ml cysteine | 2·19 | 1·50 | 15·6 |
| $P$ (Ia × III)‡ | † | ** | † |
| $P$ (Ib × III)‡ | * | * | ** |

* $P < 0·05$. ** $P < 0·01$. † Non-significant.

‡ Contrasts defined in Table 4.

TABLE 6

EXPERIMENT 4: CHARACTERISTICS IMMEDIATELY UPON THAWING AND AFTER INCUBATION FOR 2 HR AT 37°C OF RAM SPERMATOZOA DEEP-FROZEN IN VARIOUS PREPARATIONS OF FRESH SKIM EWE AND COW MILK OR RECONSTITUTED SKIM COW MILK

| Treatments† | Mean Scores of Survival: Motility: Thawed | Motility: Incubated | % Motile: Thawed | % Motile: Incubated | % Unstained: Thawed |
|---|---|---|---|---|---|
| Ewe milk preparations | | | | | |
| Not heated cysteine added‡ | 2·58 | 2·33 | 34·1 | 23·3 | 51·5 |
| Heated, no cysteine | 2·25 | 1·58 | 28·3 | 10·8 | 55·5 |
| Heated, cysteine added before heating | 2·67 | 2·50 | 41·7 | 28·3 | 59·5 |
| Heated, cysteine added after heating | 2·33 | 2·50 | 27·5 | 20·8 | 50·5 |
| Mean | *2·46* | *2·23* | *32·9* | *20·8* | *54·3* |
| Cow milk preparations ($n = 6$) | | | | | |
| Not heated, cysteine added | 2·67 | 2·25 | 38·3 | 22·5 | 45·0 |
| Heated, no cysteine | 2·42 | 2·17 | 35·0 | 23·3 | 44·3 |
| Heated, cysteine added before heating | 2·83 | 2·50 | 42·5 | 22·5 | 50·2 |
| Heated, cysteine added after heating | 2·83 | 1·08 | 42·5 | 9·2 | 51·8 |
| Mean | *2·69* | *2·00* | *39·6* | *19·4* | *47·8* |
| Control diluents§ ($n = 6$) | 3·50 | 3·00 | 60·0 | 44·2 | 59·8 |
| Error variance‖ | *0·96* | *1·86* | *62·79* | *50·91* | *38·64* |

† 95–100% of spermatozoa diluted in untreated ewe or cow milk were immotile after chilling to 5°C and were not frozen. ‡ 0·5 mg/ml cysteine added. § Reconstituted skim cow milk. ‖ 15 degrees of freedom.

by scores of motility upon thawing and percentage motile spermatozoa after incubation ($P < 0{\cdot}05$).

The analyses of variance showed that: (1) Spermatozoa survived freezing and thawing as well in heated milk preparations as in unheated preparations containing cysteine. (2) A greater proportion of spermatozoa were motile after incubation for 2 hr when they were frozen in a ewe or cow milk diluent prepared by adding cysteine before rather than after heating ($P < 0{\cdot}05$). A similar effect was found in the data of scores of motility after incubation of samples frozen in cow milk preparations ($P < 0{\cdot}05$). Observations made after incubation of the samples showed an interaction of the factors milk source and method of preparation ($P < 0{\cdot}05$). Here, spermatozoa frozen in the heated ewe milk preparation without added cysteine, or in heated cow milk with cysteine added after heating, survived incubation at body temperature very poorly.

## IV. Discussion

Johnson, Flipse, and Almquist (1955) and Saacke, Almquist, and Flipse (1956) studied the effects of heat and cysteine treatment on the toxicity of cow milk to bull spermatozoa, and Blackshaw (1960) confirmed the effects of heating on milk to be used as a diluent for ram spermatozoa. These reports and a separate study of the toxicity of fresh cow milk indicated that this was a regular characteristic of cow milk, and only heated preparations were used in experiments 1, 2, and 3. There appears to be no advantage in using ewe milk as a diluent for ram semen.

Johnson, Flipse, and Almquist (1955) concluded that the toxicity to spermatozoa of fresh milk is due to the presence of free sulphydryl groups in the protein fraction (suggested to be all present in a crude fraction named lactenin) which can be blocked by chemical bonding with either "activated" $\beta$-lactoglobulin during heating or by exogenous cysteine. This may, however, be an oversimplification of the process, since "activation" was not defined and a quantitative determination of amino acids in $\beta$-lactoglobulin by Brand *et al.* (1945) suggests that there are free sulphydryl groups present in this protein, whilst casein contains $0{\cdot}36\%$ cysteine and $3{\cdot}5\%$ methionine (Block and Bolling 1950). Also, other end groups such as amide, carboxyl, phenol, amine, pyrrolidine, indole, and imidazole may contribute to toxicity, and their destruction during heating may provide a reason for the superiority of the milk diluent prepared by cysteine and heat treatment in experiment 1.

Neither heat nor cysteine treatments alone appear sufficient to detoxify milk completely. Biological variation in the concentration of lactenin in different batches of milk (Jones and Simms 1930; Wilson and Rosenblum 1952*b*) means that the amount of cysteine required to detoxify each batch of milk is not likely to be constant. The presence of free cysteine may account for poor survival of spermatozoa in diluents containing $1{\cdot}0$ mg/ml of cysteine and the interaction of levels of lyophilized ewe milk and cysteine levels shows that each of these substances is toxic to spermatozoa at the high end of the range tested. The benefit from the addition of cysteine before, rather than after, heating milk (experiment 4) could be explained as a conversion

of unbounded cysteine to cystine by heat (Fruton and Simmons 1954); or, if insufficient cysteine were added, then the endogenous toxic groups would be destroyed by heat.

If the protective action of milk protein during freezing is dependent upon minimal denaturation, then a chemical treatment would be preferable to a heat treatment, which would be expected to cause at least some disruption of the tertiary structure. Probably the best method of preparing non-dialysable skim milk for use as a diluent for spermatozoa, if only sulphydryl groups are responsible for milk toxicity, is to add amounts of cysteine to fresh milk in excess of the quantity required to detoxify and then remove any free cysteine by dialysis. The advantage of using this technique was confirmed by Jones (1965*b*). The mechanism of the detoxification reaction during heating could be by reorientation of the tertiary structure of protein allowing disulphide bond formation among the sulphydryl groups. If such modification of tertiary structure can decrease protein toxicity, then milk chemically treated with, for example, acids, alkalis, salts, etc., or with specific enzymes such as trypsin, chymotrypsin, carboxypeptidase, and aminopeptidase is worth considering for the preparation of diluents for semen.

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