THE EXTENSION AND FREEZING OF BOVINE SEMEN

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the College of Agriculture

University of Saskatchewan

by

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Written under the Supervision of

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April, 1956

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ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. W. E. Howell, Department of Animal Husbandry, for his guidance and advice during the development of this project.

The assistance extended by Mr. N. Shklov, Department of Mathematics, and by Mr. J. B. O'Neil, Poultry Department, is also gratefully acknowledged.

For her assistance during the experimental and statistical phases of the investigation gratitude is extended also to Miss Sandra Hay.

This project was made possible by a grant from the National Research Council of Canada, Ottawa.

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INTRODUCTION

Artificial insemination, the deposition of male reproductive cells (spermatozoa) in the female tract by mechanical means rather than by the direct service of a male, is not something new, but was known even in the fourteenth century among the Arabs. Very little was done in this field until the beginning of this century. At this time accessory sex gland secretions were found unnecessary to obtain fertilization, thereby introducing the possibility of semen extension. The application of artificial insemination increased steadily during the first half of this century.

At present approximately one-fifth of all the dairy cows in the United States and one-half of those in the British Isles are bred artificially. Until recently semen could be preserved for only a few days, which restricted the application of artificial insemination to areas of dense cattle populations. A technological advance in the technique of freezing semen to low temperatures gave new impetus to the field of animal breeding. The application of this method has enabled the extension of artificial insemination to outlying districts of lesser cattle numbers.

Research being conducted at present in artificial insemination concerns investigations of more accurate semen quality tests, optimal conditions for the handling of semen and improved storage techniques. The experiments reported

here were initiated to study the following:

(a) sample sizes necessary for the display of treatment responses as shown by live : dead cell counts,

(b) optimal levels of the components of a semen extender,

(c) controlled and uncontrolled methods of freezing bovine semen.

REASONS FOR THE STUDY

Much variation exists in the methods of handling semen from the time of collection to insemination. This applies particularily to the means of estimating semen quality and to the types and levels of extender components.

With the exception of actual use of semen in the field to derive conception rates, quality estimates are confined to laboratory determinations. Because these must be conducted at higher temperatures than are compatible with the retention of maximum sperm-cell livability, laboratory tests must be as simple, rapid and accurate as possible. Viability, as determined by proportions of differentially stained live and dead cells (Mayer et al., 1951) is fairly accurate as a measure of semen quality and it takes very little time to prepare the slides. In spite of the fairly wide use of this test there is little indication in the literature reviewed that sample size in the counting procedure has been derived from statistical inference. It was for this reason that the first experiment was designed to establish the sample size and counting procedure to obtain statistically adequate results.

Several levels of the widely used extender components, sodium citrate, glycerol and egg-yolk, have been suggested as optimum for sperm survival before and after freezing to -79°C. Because of this wide variance a second experiment was undertaken to determine the optima of these extender components for our conditions.

From 1949 to 1953 the freezing of bovine semen was achieved by a rather cumbersome method which entailed the reading of temperatures each minute throughout the freezing process (Polge and Lovelock, 1952). In 1953 a simpler method was suggested which is used commercially today (Polge, 1953). In this study a still simpler method of freezing is investigated which, if as efficient as the freezing methods mentioned above, would serve as a suitable substitute for them.

LITERATURE REVIEW

Determination of Semen Quality

With the exception of actual use of semen in the field to derive conception rates, quality tests are confined to laboratory determinations. Frequently used laboratory tests are methylene blue reduction, resazurin reduction, sperm concentration, percent motility, percent viability, respiration rate, glycolitic rate, livability, hyaluronidase content, color, fructolysis index, abnormality determinations, pH change, opacity measurements and pollution index. It has been pointed out by many workers (Branton et al., 1951; Bishop and Hancock, 1955; Buckner and Willett, 1950; Cupps et al., 1953; Erb et al., 1950; Johnston et al., 1952) that no single laboratory test is itself highly correlated with fertilizing power, but that a combination of tests is necessary for accurate estimations of semen quality. Various combinations have been used. Bratton et al., (1949), Beck and Salisbury (1943) and Mercier and Salisbury (1946) have indicated that a good sample must contain at least 900 x 10^6 spermatozoa per ml. and reduce methylene blue in nine minutes or less. Since then Branton et al. (1951, 1953) have reported that to be adequate for artificial breeding semen must possess at least 50% initial motility, 500 million spermatozoa per ml. (determined by hemocytometer, photoelectric colorimeter or opacity standards), and a methylene blue reduction time not exceeding nine minutes (using 300 million spermatozoa per ml.).

Branton also stated that semen must be extended on the basis of number of progressively motile spermatozoa rather than by volume (12 to 15 million motile cells per ml. of extended semen). Vandemark et al. (1945) recommended methylene blue reduction, spermatozoa concentration and initial motility estimates for routine prediction of semen quality. Harvey and Jackson (1945) made use of motility and abnormality estimates only. It was suggested by Reid et al. (1948) that pH change during controlled incubation is an accurate, simple and rapid test of semen quality. A correlation of 0.84±0.03 between incubation time (at 100°F. until motility ceased) and conception rate was found by Ludwick et al. (1948). Lasley (1951) reported the determination of percent motile cells by means of a hemocytometer to be a good measure of semen quality. Erb et al. (1952) disclosed that a modified resazurin reduction test offered a simple rapid method for estimating fertilizing capacity. Cupps et al. (1953) asserted that percent dead and percent abnormal sperm were more closely correlated to fertility than motility, fructolysis index or concentration. Bishop and Hancock (1955) have reported that concentration, percent living, and impedence change frequency are the most useful indices of the fertilizing capacity of bull semen. Several workers consider viability as a fairly accurate measure of semen quality (Lasley et al., 1942; Lasley, 1944; Mayer et al., 1947, 1951; Madden et al., 1947; Emik and Sidwell, 1947; Swanson and Bearden, 1951; Bonadonna, 1953;

and Bangham and Hancock, 1955).

Facilities would not permit the use of conception rate as a semen quality test in the present work. The laboratory tests used were percent motility, percent viability, and sperm cell concentration.

Establishment of a Counting Procedure:

Several variations have been suggested in the literature concerning the number of cells which should be counted. However, very little work has been conducted to determine sample size for statistically adequate results.

Salisbury et al. (1942) based their conclusions on a count of 550 spermatozoa per treatment. Lasley et al. (1942) combined the counts from several fields to a total of 500 cells per slide. Harvey and Jackson (1945) stated that a count of 400 to 600 spermatozoa gave sufficient accuracy in work with human semen. In routine practice they found a count of 200 to be within 3% of a count of 400 to 600. Variation among counts made by different observers were of about the same order. Hammond et al. (1947) used sixteen fields and counted a total of at least 100 cells. Shaffer and Almquist (1948) made four slides for each treatment and counted 100 cells per slide. In Good's (1948) translation of Russian work, it is indicated that at least 500 spermatozoa were counted to obtain a reliable estimate of pathological forms. Rowson (Polge, 1953) calculated the percentage of dead

spermatozoa by counting 50 fields per test which included a total of more than 500 spermatozoa. Dunn and Cruthers (1954) used two vials for each treatment and made two slides from each vial. Mixner and Saroff in the same year declared it sufficient to count approximately 300 spermatozoa on each slide (private communication, 1955).

In the literature reviewed, the number of spermatozoa counted per treatment varied from 100 to 600 on one to four slides, and included several fields per treatment or observation.

Determination of Optimal Levels of Extender Components:

Semen extenders have played a major role in the success of artificial insemination in dairy cattle. Basic in the use of extenders was the work of Phillips (1939), who first used egg-yolk in the extension of bull semen. He prepared an egg-yolk-phosphate extender consisting of 3 parts yolk: 2-3 parts of a phosphate buffer. An important advance was the introduction of an egg-yolk-sodium citrate extender by Salisbury et al. (1941). Hurst (1953) said, "A majority of the artificial breeding associations in the United States today use some form of the egg-yolk-sodium citrate diluter." The use of the word "some" in this quotation is an indication that at that time there was either little agreement concerning the optimal levels of the extender components, or that the optimal levels varied widely with changes in environmental conditions. Many variations of the original extender, as proposed by Salisbury et al. (1941) are still in use today. Examples of some of the variations used are as follows:

Egg yolk levels[1]

Salisbury et al., in 1941, recommended equal portions of egg-yolk and buffer. It was suggested by Meyer and Lasley (1944, 1945) that less than 50% egg-yolk could be used. They discovered an egg-yolk fraction that was insoluble in alcohol, acetone or ether, which in minute amounts was beneficial in protecting sperm against temperature shock. This factor acted as a buffer against drastic changes of temperature, osmotic pressure and pH. The egg-yolk fraction at an extension rate as high as 1 part: 250 parts of buffer gave better protection than whole egg-yolk. It was suggested that the protective action of the egg-yolk might be obtained at a lower concentration than 50%. Swanson (1948) reported that a yolk: citrate ratio of 1:3 gave results equal to the 1:1 ratio recommended by Salisbury et al. (1941). This was confirmed by Stewart et al. (1950). A 1:7 ratio gave inferior results (Swanson,1949) -- confirmed by Olds et al. (1951). Swanson continued this work and in 1949, stated that although 10% of egg-yolk was sufficient for protection against cold shock, motility was lowered at that level. The reduction in motility was not evident at the 20% egg-yolk level. Swanson's suggestion that extenders could contain less that 50% egg-yolk centered around time saved, lessened expense, and easier motility estimations (less yolk fats to interfere with the microscopic observation of individual spermatozoa). Hurst (1950) compared

[1] The percentage of egg-yolk indicated is the final concentration unless otherwise specified.

20% with 40% egg-yolk and obtained 61 and 66% conception, respectively (65 day non-return rates), after collecting data on 537 first services. In this experiment Hurst maintained a level of 0.1M or 2.94% sodium citrate. Salisbury et al. (1946) and Willett (1949, 1950) obtained satisfactory results with 1:100 egg-yolk=extender. Herrick (1950) suggested a pasteurized semen extender which was made up of 3 parts homogenized egg-yolk : 5 parts of sodium citrate solution. He was able also to lyophilize the product for storage. This was based on 0.1M or 2.94% sodium citrate. In the 1950 report of the National Association of Artificial Breeders (N.A.A.B.) it is indicated that one-half of their 48 member units were using less than 50% egg-yolk and several of these were using a concentration as low as 14 to 16%. Almquist (1951) compared egg-yolk : sodium citrate ratios of 1:1; 1:3; 1:5; and 1:7. He obtained no significant differences after collecting non-return data from 8,222 first service cows. He concluded that 12.5% egg-yolk gives results almost equal to 50% (freezing was not carried out for this experiment). Polge and Rowson (1952) used an extender which in its final combination contained 25% egg-yolk with the sodium citrate and glycerol constant at 2.94 and 10.0%, respectively. Dunn and Hafs (1953) reported that egg-yolk extenders in a final combination containing 10.0% glycerol and 25% egg-yolk were superior to other extenders containing 27.5 and 50% egg-yolk.

Flerchinger et al. (1953) still used 1 part egg-yolk to 1 part sodium citrate, whereas Graham and Marion (1953) claimed 10% egg-yolk to be the best. Hurst (1953) also used 20% egg-yolk. Thacker and Almquist (1953) experimented with 1:1 and 1:3 egg-yolk : sodium citrate and found the 1:1 ratio to be superior. Kinney and VanDemark (1954) found that extenders containing 16 and 24% egg-yolk gave better results than an extender containing 32% egg-yolk. They considered the optimal levels to be 1:1 semen : yolk-citrate with the addition of 7% glycerol. O'Dell and Almquist (1954) used 1:3 egg-yolk : sodium citrate in their work. Saroff and Mixner (1954) reported optimal results with 18% egg-yolk whereas Snyder et al. (1954) found 25% egg-yolk to give the best results. Saroff and Mixner (1955) studied the results of freezing semen in extenders containing 18.4, 23.0, and 27.6% egg-yolk as well as 15, 20, 25 and 30% in a second experiment. They obtained the best results with 20% egg-yolk (the glycerol level was 7%). Hafs and Elliott (1955) added egg-yolk to both the citrate solution and the glycerol citrate solution as compared to the usual practice of adding all of the egg-yolk to the citrate solution. They obtained superior results with the egg-yolk added to both portions. Cragle et al. (1955), for their work on levels of sodium citrate, glycerol and equilibration times, standardized the egg-yolk level at 24%.

Sodium citrate levels

The first sodium citrate extender which appeared in

published form was that prescribed by Salisbury et al. (1941) in which M/7.5 or 3.92% sodium citrate was used. Since then Mayer and Lasley (1944, 1945) found that 2.3% sodium citrate was superior to 1 or 4%. Salisbury and Knodt (1947) determined that 3.6% was optimum, with good results being obtained with 2.9% as well. Salisbury and Bratten (1948) finally decided upon the use of 2.9% sodium citrate. Swanson (1948) compared 1, 2, 3, 4, and 5% sodium citrate and obtained best results with 3%. Good results were had also with 2%. Foote and Bratten (1949) worked with 3.6% and Swanson in the same year compared concentrations from 1 to 5% and found from 2 to 3% to be optimum. VanDemark et al. (1950) employed 3.6% as did Foote and Bratten in the same year. Smith and Pelge (1950) used 3.92% sodium citrate in experiments designed to study freezing methods. Almquist (1951, 1954) used 2.9% sodium citrate for his work. Olds et al. (1951) made use of 3% sodium citrate. Dunn et al. (1950, 1953) found 2.9% to be optimal with whole egg after trying 1.9, 2.3, 2.9 and 3.6%. Flerchinger et al. (1953) claimed 3% to be optimal in egg-yolk and 2.8% in milk extenders. Pelge (1953) used 3.29% sodium citrate for freezing experiments. Cragle et al. (1955) and Cragle and Myers (1954) worked with 1.6, 2.3, 3.0, 3.6, and 4.4% sodium citrate. They found the optimum level to be 2.9%. Concentrations of 1.6% sodium citrate and 4.4% sodium citrate appeared to be detrimental. Dunn and Cruthers (1954) used 2.32% sodium citrate in an experiment

designed to determine minimal sample size for motility observations. Elliott et al. (1954) employed 2% sodium citrate in determining frozen semen results. Graham and Erickson (1954) used 2.7% sodium citrate in an egg-yolk extender. Kinney and VanDemark (1954) used 2.9% sodium citrate in a study to determine the effect of freezing upon egg-yolk, Mixner (1954) used 2.174% of sodium citrate in an extender comparison study. O'Dell and Almquist (1954) working with milk used 2% sodium citrate. Bratton et al. (1955) used 2.9% sodium citrate, as did Johnson et al. (1955).

Glycerol levels

It was discovered by Polge, Smith and Parkes (1949) that spermatozoa could be frozen with very little loss of motility if glycerol was included in the suspending medium. Although these workers are generally conceded to be the originators of the frozen semen technique, they were not the first to employ glycerol for this purpose. Rostand, in 1946, froze frog spermatozoa to -6°C. in a final concentration of about 20% glycerol (Bunge et al., 1954). At this temperature, however, the material would not be solid. He did not freeze spermatozoa to any very low temperatures (Polge and Parkes, 1952). However, even Rostand was not the first to freeze semen as Mantegazza, in 1866, froze human spermatozoa to -15°C. with some survival (Bunge et al., 1954) -- glycerol

was not used. It is stated that human spermatozoa are the most resistant to the absence of heat. Jahnel (1938), noted that a proportion of human spermatozoa survived freezing for comparatively long periods at -79°C. Polge et al. (1949) were the first to freeze fowl spermatozoa to -79°C., employing the use of glycerol. The level of glycerol used for this work was at a final concentration of 20% for fowl spermatozoa and 5% for human sperm. Smith and Polge (1950a) obtained maximum survival after freezing bull spermatozoa extended to contain 15 to 20% glycerol. At this level 80% of the bull spermatozoa survived freezing. Smith and Polge (1950b) had very good results using only 10 to 15% glycerol in final concentration with 5% glycerol giving almost equal results. Emmens and Blackshaw (1950) found that bull semen could be revived to the extent of swirling after slow cooling to -79°C., in the presence of 7.5 to 10% glycerol. Polge and Parkes (1952) reported 15 to 20% glycerol to be optimal, but Polge and Loveloek (1952) and Polge (1953) confirmed Emmens and Blackshaw's work (1950) that 7.5 to 10% glycerol gave good results. Degroot (1952) carried out an experiment to determine the optimal level of glycerol. Percentages of 0.75, 3.0, 7.5, 10.7, 12.0, 13.8 and 19.2 were compared; 7.5% gave the best results. Dunn et al. (1953) in studying the effects of antibiotics on spermatozoa used 10% glycerol in final concentration and Graham and Marion (1953) found 10% to be superior to 15 or 20% glycerol. Macpherson and

and Henderson (1953), and Macpherson (1954) used 10% glycerol in their work with milk extenders. Stower (1953) used 10% glycerol in freezing bovine spermatozoa. Cragle and Myers (1954) studied a range of 4 to 15% glycerol and found 7.6% to be optimum with good results from 7% as well. Miller and VanDemark (1954) tried 0 to 80% glycerol and reported 7% glycerol to be optimal. Since then 7% has been used by Bratton et al. (1955), Dunn and Cruthers (1954), Kinney and VanDemark (1954), Mixner (1954), Elliott et al. (1954), Erickson et al. (1954) and Williams (1954). Mixner and Saroff (1954) studied levels of glycerol from 2.5 to 12.5%. Results indicated that extenders containing 7.5% glycerol in final concentration gave a greater percent of motile spermatozoa after freezing than did extenders containing 5, 10, or 12.5% of glycerol. O'Dell and Almquist (1954), found levels of 10 and 13% glycerol to be better than 7%. Bunge and Sherman (1954) claim 10% glycerol to be best for human spermatozoa. Van Rensburg et al. (1954) obtained good results using 10% glycerol. Graham and Erickson (1954) obtained good conception rates using 10% glycerol in egg-yolk extenders. Snyder et al. (1954), used 8% glycerol in final concentration in comparing frozen and unfrozen semen. Saroff and Mixner (1955) carried out two tests, the first using 6, 8, and 10% glycerol, and the second using 5, 6, 7, 8, and 9% glycerol. They concluded that a final concentration of 7% glycerol gave the best results. From these reports one would expect that the optimal level of glycerol is most likely to range between 6.5 and 10%.

EXPERIMENT NO. I

ESTABLISHMENT OF A COUNTING PROCEDURE

Objectives:

(a) To establish the sample size and counting procedure to be used in conjunction with Mayer's et al. (1951) live: dead differential staining technique.

(b) To study the magnitude of technician differences in the counting results.

(c) To determine the effect on the viability of bovine spermatozoa of freezing by controlled and uncontrolled methods of temperature decline.

Materials and Methods:

(a) Experimental procedure -

On the day prior to semen collection several solutions were prepared and stored overnight at 5°C. These solutions were a 3% sodium citrate ($Na_3C_6H_5O_7.2H_2O$) solution, a gum tragacanth lubricant, a 15% glycerol-citrate (3%) solution, and a modified Mayer's stain (Mayer et al., 1951), which consisted of 3% sodium citrate instead of a M/8 phosphate buffer.

The semen used in the study was obtained from two Holstein-Friesian bulls by means of an artificial vagina (Green and Winters, 1948). Two ejaculates of 4 ml. and 2.5 ml. were obtained from one bull. From the other, the second ejaculate of 7 ml. was used. Immediately following collection

until arrival at the laboratory, the semen was maintained at body temperature. The samples were then cooled to room temperature (24°C.) in air while stained slides were being prepared for a viability estimate.

The staining solution and method of staining used was a modification of that recommended by Mayer et al. (1951). In this work a 3% sodium citrate solution was used instead of an M/8 phosphate buffer. The water used in the staining solution was twice distilled, the second distillation being over glass. A fresh staining solution was prepared for each experiment and stored in the refrigerator at 5°C. until required. Prior to its use, the staining solution was warmed to room temperature. The slides were prepared and dried within a few seconds after the staining procedure was started. They were then placed in slide holding trays which were kept in dessicators until counted. The counting was completed as soon as possible following the preparation of the slides. During the counting procedure the slides were kept dry with the hot air blast of a hair drier. Of the four slides made from each sample, the poorest preparation was discarded at the time of counting. The slides were labelled with a wax pencil for identification.

The three ejaculates were extended 1:10 with 50% egg-yolk,[1] in 3% sodium citrate, in 50 ml. erlenmeyer flasks. These flasks were set in a water bath at room temperature,[2]

[1] The method of egg-yolk preparation is given in the appendix.
[2] All equipment and solutions which were in contact with the semen were also at room temperature.

which was then placed in a refrigerator. The extended semen was cooled to 5°C. over a period of four hours. At this time an equal volume of 15% glycerol in 3% sodium citrate was added to the extended semen in six steps, at 5 minute intervals, in portions of 5, 12, 15, 20, 23 and 25% per step.

The final extension ratio was 1:21 (semen:extender). The solution consisted of 4.5% semen, 22.7% egg-yolk, and 7.5% glycerol in 3% sodium citrate. All solutions were maintained at 5°C. during the glycerol addition period by adding ice cubes to the water bath. The pipettes were maintained at approximately 5°C. by flushing the cooled 3% sodium citrate. Following the addition of glycerol, the samples were returned to the refrigerator where equilibration continued for 18 hours.

Following equilibration the samples were gently agitated and 1 ml. was metered out into each of the 3 labelled[1] vials per treatment by means of a 2 ml. syringe and a 16 G. 1½ inch stainless steel needle. The glass ampoules were hermetically sealed by application of an oxymethane flame. During the entire charging and sealing procedure all equipment used was maintained at 5°C. and the vials were partially immersed in water, which was maintained just above freezing by the addition of ice cubes. In the event of breakage another vial, already labelled and cooled, was charged and sealed.

Two vials of each of the three ejaculates were frozen,

[1] The vials were permanently identified by marking with "Labink", which was applied with a straight pen, and fired to a red heat.

one by "Freezing Method No. 1" and one by "Freezing Method No. 2". Freezing method No. 1 was a modification of that outlined by Polge and Lovelock (1952). Several workers have stated that slow initial cooling to -10°C. followed by rapid freezing to -79°C. is most compatible with maximum retention of living cells (Bratton et al., 1954 and Elliott et al., 1954). The freezing in Method No. 1 was controlled to a decline in temperature as graphically represented in Figure 1. Freezing Method No. 2, which has given good results in this laboratory, involved the burial of sealed ampoules of glycerolated semen in a vacuum flask of crushed dry ice. A freezing process such as this was first outlined by Bruce (1953). The method was suggested to Bruce by Polge in private communication. Bruce obtained good recovery rates by placing ampoules in a metal can which was then placed in a dry ice storage box. Freezing was effected gradually by cold air. Sherman and Bunge (1953) reported that human spermatozoa, frozen by placing the vials in an insulated box containing dry ice were 67% viable after 3 months storage. The success and simplicity of this method warranted its comparison with that method described by Polge and Lovelock (1952) in which time:temperature relationships were controlled. The vials of frozen semen were stored at -79°C. for 24 hours in a mixture of dry ice and acetone before being thawed at 5°C. Three slides were prepared from each of the three vials frozen by the two methods giving a total of 18 slides.

Figure 1. Rate of temperature decline for Freezing Method Number 1. Experiment I.

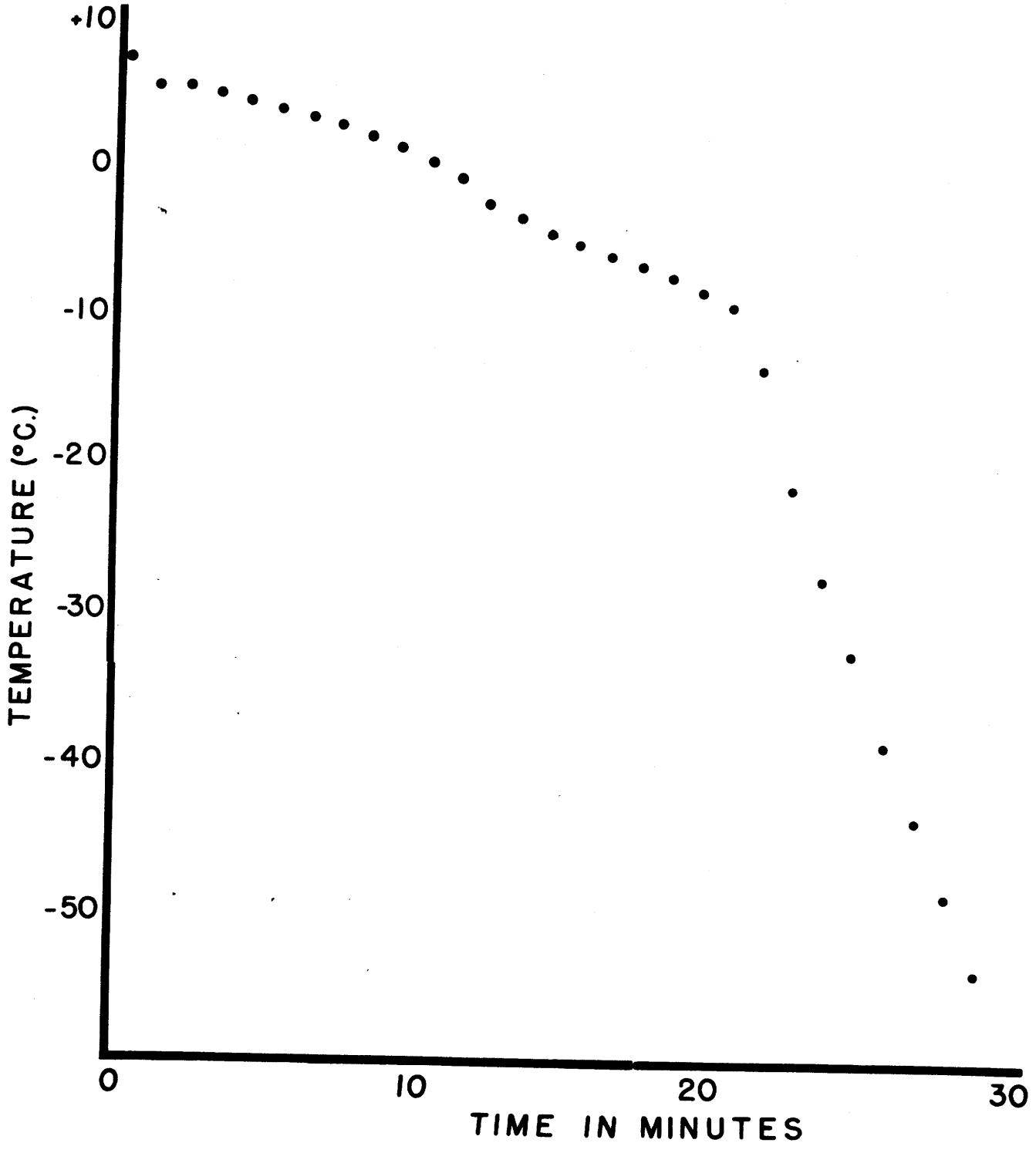


FIGURE 1

All glassware was cleaned by the standard methods outlined by Green and Winters (1948).

Immediately after use, the artificial vaginas were flushed with warm and then with hot water. No soap or detergent was used. Upon arrival at the laboratory the vaginas were disassembled and thoroughly flushed with hot water followed by a rinse in distilled water. Before hanging the liners and director cones to dry, they were rinsed with alcohol as a disinfectant and to hasten the drying process. Paper towels were then used to wrap the liners and cones in storage.

(b) Method of counting cells and statistical analysis

To obtain information as to differences between technicians, the counting in this study was independently performed by two technicians.

Two method of counting were compared. Count A involved two counts of 500 spermatozoa on each of two slides selected at random from the three slides prepared for each vial of each treatment. Count A was therefore completed by counting 2 slides x 2 counts x 500 spermatozoa x 2 technicians = 4,000 spermatozoa per treatment. Count 1 was made on the upper half of the slides by traversing horizontally until 500 spermatozoa were counted. Count 2 was made similarly on the lower half of the slide.

A second count, B, involved the three slides with two counts of 100 spermatozoa on each. By this method 1,200 spermatozoa were counted per treatment (3 slides x 2 counts

x 100 spermatozoa x 2 technicians). Count 1 was made on the right half of the slide by traversing vertically and Count 2 was made similarly on the left half of the slide. All cells in each field of observation were counted.

A problem which is encountered during the viability counting is the appearance of cells which are termed "half-stains". The anterior portion of the cell is clear and the posterior portion is stained. No account has been found which satisfactorily explains the cause of this staining phenomenon (Brochart,1953; Bishop and Hancock,1955). The "half-stains" in Experiment 1 were not counted, either as live or dead. The number encountered appeared to be fairly constant, presumably due to the routine manner in which the slides were prepared.

To analyze this investigation statistically, normality of distribution was assumed. An analysis of variance was carried out and the necessary differences were calculated, after Snedecor (1950), to test for significant differences between ejaculates, counting methods, freezing methods, technicians and their interactions.

Results and Discussion of Results

The mean treatment responses are contained in Table 1. Each average was obtained from 60 observations except those for the count comparison, in which the Count A and Count B averages were derived from 48 and 72 observations, respectively.

TABLE I

MEAN RESPONSES TO TREATMENTS (% LIVE SPERMATOZOA)

Freezing Method	Bull	Ejaculate Number	Count A		Count B		
			Technician A Average	Technician B Average	Technician A Average	Technician B Average	Average
	A	1	49.8	50.0	49.8	48.7	49.3
1	A	2	54.2	53.5	53.0	50.8	52.9
	B	2	56.6	56.2	56.6	55.3	56.2
	Average		53.6	53.2	53.1	51.6	52.9
	A	1	42.3	50.0	38.2	43.8	42.6
2	A	2	44.9	45.2	43.2	47.8	45.2
	B	2	43.4	47.2	42.5	49.0	45.5
	Average		43.5	46.1	41.3	46.8	44.4
Overall Average			48.6	49.6	47.2	49.2	48.6

Table 2 contains the results of the analysis of variance, differences between means and the necessary differences.

It will be observed that there was no significant difference between counting methods A and B (2000 cells vs. 600 cells per treatment).

Considering the comparison between technicians, the average of the counts performed by Technician A was 47.88% while that of Technician B was 49.42%. This difference of 1,54% was found to be significant ($P<0.05$) with a necessary difference of 1.37%.

The average viability after freezing by Method 1 was 52.9%, as compared to 44.4% for Method No. 2. By analysis of variance this difference of 8.41% was found to be highly significant ($P<0.01$). The necessary difference required was 1.37%.

The difference between the ejaculates used was also highly significant ($P<0.01$) with a necessary difference of 1.69%. The first order interaction of freezings x technicians and the third order interaction of ejaculates x freezings x technicians x counts both highly significant ($P<0.01$). Significance ($P<0.05$) was found in the interaction of ejaculates x freezings x counts.

An analysis was performed to determine whether or not there was a significant difference between the two counts within slides counted by Method B as regards the live:dead

TABLE II

ANALYSIS OF VARIANCE OF EJACULATES, FREEZINGS, TECHNICIANS AND COUNTS (% VIABILITY).
EXPERIMENT I

Source	Degrees of Freedom	Variance	Difference Between Means	Necessary Difference
Total	119			
Ejaculates	2	257.2**		1.69
Freezings	1	2133.6**	8.41	1.37
Technicians	1	86.7*	1.54	1.37
Counts	1	19.7		
Ejac. x Freez.	2	36.6		
Ejac. x Tech.	2	0.6		
Ejac. x Counts	2	7.8		
Freez. x Tech.	1	224.2**		1.94
Freez. x Counts	1	0.8		
Tech. x Counts	1	4.7		
Ejac. x Freez. x Tech	2	8.8		
Ejac. x Freez. x Counts	2	68.2*		3.37
Freez. x Tech. x Counts	1	32.6		
Ejac. x Freez. x Tech. x Counts	2	95.3**		4.76
Error	99	14.4		

ratio. The mean viabilities obtained for Count 1 and Count 2 were 47.3 and 49.1%, respectively. This difference of 1.8% was not significant as the necessary difference was calculated to be 3.37%.

Conclusions

The conclusions derived from the data obtained in Experiment I are as follows:

(a) A live:dead ratio obtained from a total count of 600 differentially stained bovine spermatozoa (100 cells on each end of 3 slides) gives a sufficiently accurate viability estimate of a semen sample for practical tests of treatment differences.

(b) Technician differences with respect to semen quality determination should be removed in the analysis of experimental results.

(c) The rate of temperature decline during freezing must be controlled within fairly narrow limits between 5°C. and -55°C. to obtain optimal survival of bovine spermatozoa frozen to -79°C.

EXPERIMENT II

DETERMINATION OF OPTIMAL LEVELS OF EXTENDER COMPONENTS

Objectives:

(a) To determine the optimal levels of the extender components sodium citrate, glycerol, and egg-yolk, for maximal sperm survival as estimated by live-dead differential staining and motility tests.

(b) To determine the effect on the survival of bovine spermatozoa of freezing to -79°C. by controlled temperature decline to -55°C., with no control, and by controlled temperature decline to -10°C.

(c) To compare motility and viability as quality tests for a semen sample.

Design and Analysis

A three dimensional central composite design was used to assign treatments in this experiment. This design as described by Box and Wilson (1951) and later by Box (1954) is adapted for the estimation of optimal operating conditions. Statisticians at the North Carolina State College were able to apply this design to experimental work to determine the best conditions for the freezing of bovine spermatozoa (Cragle et al., 1955). Using coded values for treatment combinations (Tables 3 and 4) the treatments necessary to determine the optimal levels of sodium citrate, glycerol, and egg-yolk may be graphically represented (Figure 2).

Treatments 1 through 8 form a 2^3 factorial experiment,

TABLE III

LEVELS OF EXTENDER COMPONENTS
IN
EXPERIMENT II

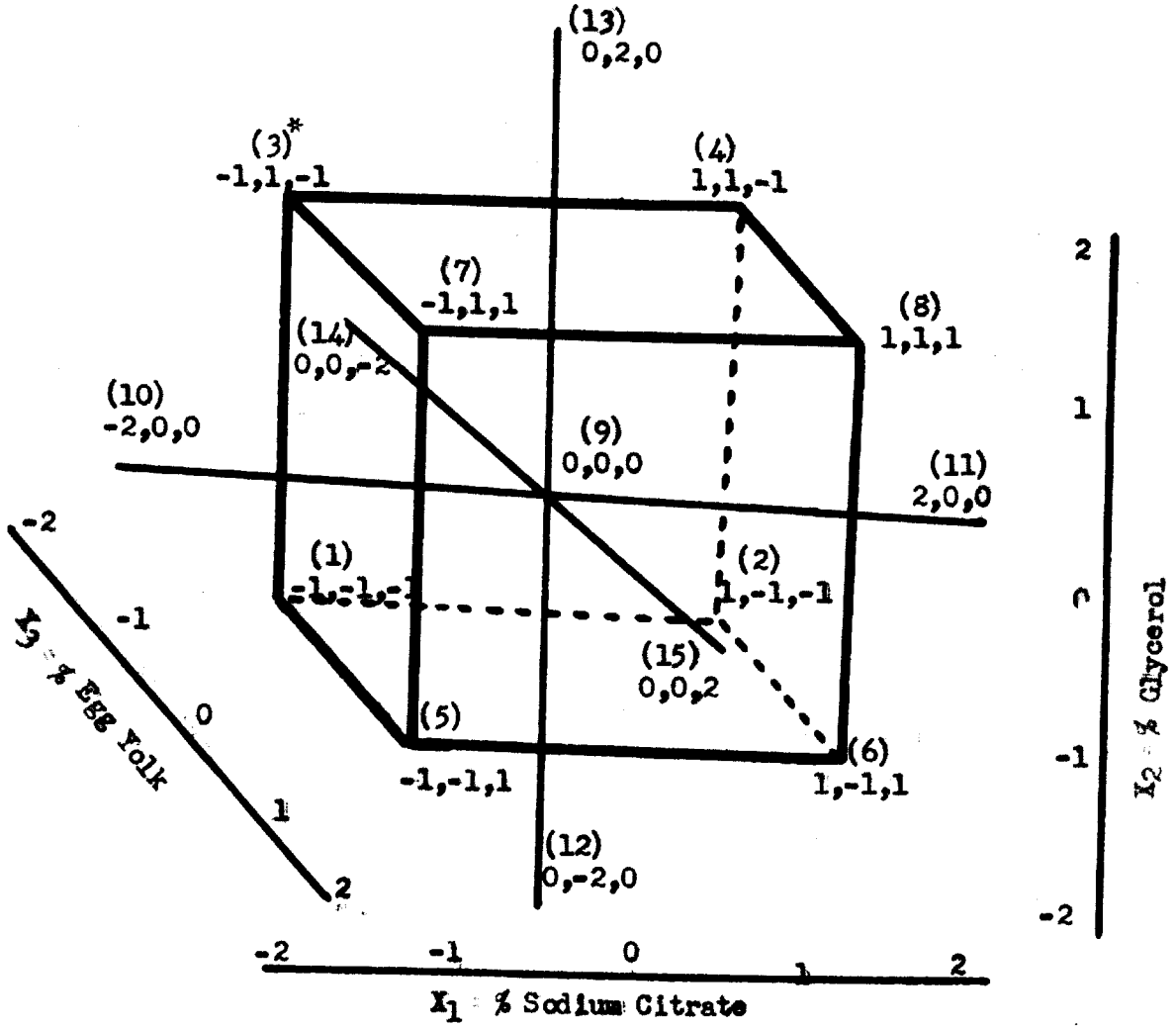
		Coded Values				
		-2	-1	0	+1	+2
Sodium Citrate	% (X_1)	2.4	3.2	4.0	4.8	5.6
Glycerol	% (X_2)	2.0	7.0	12.0	17.0	22.0
Egg Yolk	% (X_3)	10.0	20.0	30.0	40.0	50.0

TABLE IV

CODED VALUES OF THE TREATMENTS USED
IN
EXPERIMENT II

Treatment Number	Coded Values		
	X_1	X_2	X_3
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	0	0	0
10	-2	0	0
11	+2	0	0
12	0	-2	0
13	0	+2	0
14	0	0	-2
15	0	0	+2

Figure 2. The three-dimensional central composite design. ExperimentII (with treatment and coded values indicated).



*treatment number

FIGURE 2

and the additional treatments, 9 through 15, form a fractional 3^3 factorial experiment. The 2^3 factorial experiment allows good estimates to be made of the main effects and the two factor interactions. The fractional 3^3 factorial experiment affords a good method for the estimation of the quadratic effects. The linear, quadratic, and the linear x linear interaction effects were estimated by use of the multiple regression model which follows:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3$$

where Y = estimated response

B's = partial regression coefficients

This design is adapted to estimating a point of maximum survival of spermatozoa in terms of the levels of sodium citrate, glycerol and egg-yolk which correspond to that point.

The criteria used to estimate the effectiveness of the treatments were percent motility and percent viability. Five levels were chosen for each factor being studied (Table 3). The treatments are always as in Table 4 when using a three dimensional central composite design with 5 levels.

It is important to assign the coded value "0" to the level which one has reason to believe is near optimum. Treatment levels should be so designed that less recovery is obtained from "-2" and "2" levels than at the "0" level. One of the calculations for the determination of optimal levels is inserted in the appendix.

To analyze the responses obtained from the three freezing methods an analysis of variance and the necessary differences were calculated for both the motility and the viability tests.

For this experiment percent survival was calculated as follows:

$$\% \text{ survival of motile spermatozoa} = \frac{\% \text{ motile after storage}}{\% \text{ motile at collection}} \times 100$$

$$\% \text{ survival of viable spermatozoa} = \frac{\% \text{ viable after storage}}{\% \text{ viable at collection}} \times 100$$

Materials and Methods:

On the day prior to semen collection the solutions were prepared and stored overnight at 5°C. The first of these were the sodium citrate solutions. Sodium citrate ($Na_3C_6H_5O_7.2H_2O$) in amounts of 2.4, 3.2, 4.0, 4.8 and 5.6 gms. were placed into five 250 ml. measuring bottles. To each was added doubly distilled water until the 100 ml. mark was reached, with all of the sodium citrate dissolved. These bottles were then corked, labelled with wax pencil, and stored in the refrigerator. The glycerol-citrate solutions were then prepared. Various amounts of sodium citrate and glycerol were placed into fifteen labelled 250 ml. measuring bottles as shown in Table 5. Distilled water was added to each to the 100 ml. level. A Hayem's solution, which is used for extending and killing spermatozoa for the determination of concentration, was then prepared by dissolving 0.5 gms. of mercuric chloride, 5.0 gms. of sodium sulphate, and 1.0 gms. of sodium chloride in 200 ml. of distilled water. The gum tragacanth and Mayer's stain were

TABLE V

GLYCEROL SOLUTION COMPOSITION FOR EACH OF THE FIFTEEN TREATMENTS IN EXPERIMENT II

Treatment Number	Amount of Citrate gms.	Amount of Glycerol ml.
1	3.2	7.0
2	4.8	7.0
3	3.2	17.0
4	4.8	17.0
5	3.2	7.0
6	4.8	7.0
7	3.2	17.0
8	4.8	17.0
9	4.0	12.0
10	2.4	12.0
11	5.6	12.0
12	4.0	2.0
13	4.0	22.0
14	4.0	12.0
15	4.0	12.0

prepared as for Experiment I.

The semen used in this experiment was obtained from one Shorthorn and two Holstein-Friesian bulls by means of an artificial vagina (Green and Winters,1948). Two ejaculates from each of the three bulls were pooled to give sufficient volume to conduct the experiment. Immediately following collection, until arrival at the laboratory, the semen was maintained at body temperature. The sample was then cooled to room temperature (24°C.) in air while the initial motility estimate was being made and the stained slides were being prepared for the viability estimate.

Estimations of motility were conducted at 37°C. by the use of a slide warmer. A drop of semen was placed on a microscope slide and covered with a slip. Low power was used except when the rate of motility was very high, in which case both high and low power were used in an effort to obtain a higher degree of accuracy. Two slides were made from each vial. If a discrepancy was noted between these, a third or a fourth slide was prepared. The total area under the coverslip was examined for each estimation. All motility estimates were determined by one technician.

$$\text{Response or \% survival} = \frac{\text{motility estimate after a given storage time}}{\text{motility estimate immediately after collection}} \times 100$$

Sperm cell concentration was determined by use of a haemocytometer counting chamber. A haemocytometer counting plate is divided into halves possessing a total of 400 squares

within a square millimeter (25 large squares of 16 each) on each half. Using high power on the microscope, one-fifth of these squares were counted, that is, 5 of the 25 larger squares on each side of the plate. The squares to be counted were selected at random. By completing 6 repetitions using a fresh semen sample each time, an average count of 40 spermatozoa was obtained. Therefore the concentration of spermatozoa in the pooled sample was:

40 (counts) x 5 (squares) x 10 (1/10 mm. thickness) x 1000 (to change units to ml.) x 200 (dilution) = 400 million spermatozoa per ml.

Extension was carried out immediately following the initial quality tests. It was necessary to complete the haemocytometer test before extension was undertaken in order to determine what extension was necessary to result in a sufficiently high sperm concentration for insemination purposes (to obtain applicable results).

Olds et al. (1953) inseminated 9,558 cows with 4 to 48 million spermatozoa per insemination and obtained no significant differences in conception results. It was found, however, that there was a tendency for the higher concentration, larger dosages, and deeper depositions to produce the highest fertility with an average increase of 1.2% in non-returns for each 10 million additional sperm deposited.

In this experiment the initial concentration was found to be approximately 400 million spermatozoa per ml. A final

extension of 1:21 (semen to extender) would result in a concentration of about 20 million spermatozoa per ml. Providing that the vitality of this semen approximated that used in Olds' experiment, this final concentration would be sufficient. This meant that the semen would have to be extended 1:10 with yolk-citrate and the volume be doubled with glycerol-citrate.

Fifteen 50 ml. erlenmeyer flasks were fastened in a shallow tray with elastics to keep them upright. Into each of the flasks was measured the components as shown in Table 6. The egg-yolk was found to be very hard to measure accurately until the following method was devised:

(1) Into a 10 ml. graduate pour the required amount of sodium citrate.

(2) Add liquid egg-yolk until the 10 ml. level is reached.

(3) Then, without mixing, quickly pour the contents into the appropriate erlenmeyer flask.

By this method the yolk did not stick to the sides of the graduate. The contents of each flask were thoroughly mixed and one ml. of the unextended, pooled, semen was pipetted into each flask. All erlenmeyers were then corked and gently rotated. The tray, containing the flasks, was then filled with tap water at room temperature (24°C.) and placed in the refrigerator to cool to 5°C.

The extended semen was cooled to 5°C. over a period of 4 hours. Motility and viability were again checked, and an equal volume of glycerol-sodium citrate solution was added at

TABLE VI

COMPOSITION OF THE YOLK-CITRATE EXTENDER

EXPERIMENT II

Treatment Number	Sodium Citrate		Egg Yolk
	Amount ml.	Concentration %	ml.
1	8	3.2	2
2	8	4.8	2
3	8	3.2	2
4	8	4.8	2
5	6	3.2	4
6	6	4.8	4
7	6	3.2	4
8	6	4.8	4
9	7	4.0	3
10	7	2.4	3
11	7	5.6	3
12	7	4.0	3
13	7	4.0	3
14	9	4.0	1
15	5	4.0	5

six equal intervals over a period of 35 minutes with 5, 12, 15, 20, 23 and 25% of the total added per interval according to the experimental design. This method of glycerol addition, a modification of the method used by Elliott et al. (1954), has given good results at this laboratory in the past. Throughout this time the samples and solutions were maintained at 5°C. by immersion in ice water. The samples were then returned to the refrigerator for a glycerol-equilibration period of 18 hours after which motility and viability were again determined. Nine one cc. portions of extended semen from each treatment were pipetted into labelled 1c.c. glass ampoules and hermetically sealed. All samples, solutions and glassware were maintained at 5°C. during these operations. Freezing was then carried out by one of three methods.

Freezing Methods Nos. 1 and 2 were as in Experiment 1. Freezing Method No. 3 was introduced in this experiment. This freezing method was similar to Freezing Method No. 1 except that the temperature was controlled by the gradual addition of dry ice rather than by the gradual cooling effect of the freezing baths used in the former freezing method. When -10°C. was reached the bath was filled with dry ice to cool the semen to -79°C. as rapidly as possible, whereas in Method No. 1 the rate of 5°C. per minute was more or less adhered to until -55°C. was reached (Polge and Lovelock,1952).

The advantages of this method over the first is that

many more vials can be frozen at the same time and after -10°C. is reached no attention need be paid to the freezing process until the vials are to be stored.

This freezing method was first employed by Stower (1953). He cooled the semen, by adding solid CO_2 to a vacuum flask containing alcohol, at a rate sufficient to cause a decline in temperature approximating 1°C. per minute from 5°C. to -55°C. When -55°C. was reached the flask was filled with solid CO_2 and the temperature made to drop more rapidly. The whole cooling operation took 65 to 75 minutes. Macpherson and Henderson (1953), using milk as an extender, cooled semen from 5°C. to -10°C. in 45 minutes and from -10° to -15°C. in 5 minutes. Dry ice was then added in quantity.

The rate of temperature decline for Freezing Methods Nos. 1 and 3 for Experiment 11 are graphically represented in Figure 3. Three vials of each treatment were frozen by each of the three methods.

After freezing, the samples were stored for 5 days at -79°C. in a vacuum bottle containing dry ice. The samples were then thawed by application of body heat. One vial was used for motility estimates and viability staining. The other two vials were prepared in the event that breakage occurred.

Results and Discussion of Results:

The initial quality of the semen collected was as follows: 55% initial motility, 66.3% initial viability, and a concentration of 400 million cells per ml.

Figure 3. Rate of temperature decline for Freezing Method Numbers 1 (circle) and 2 (triangle). Experiment II.

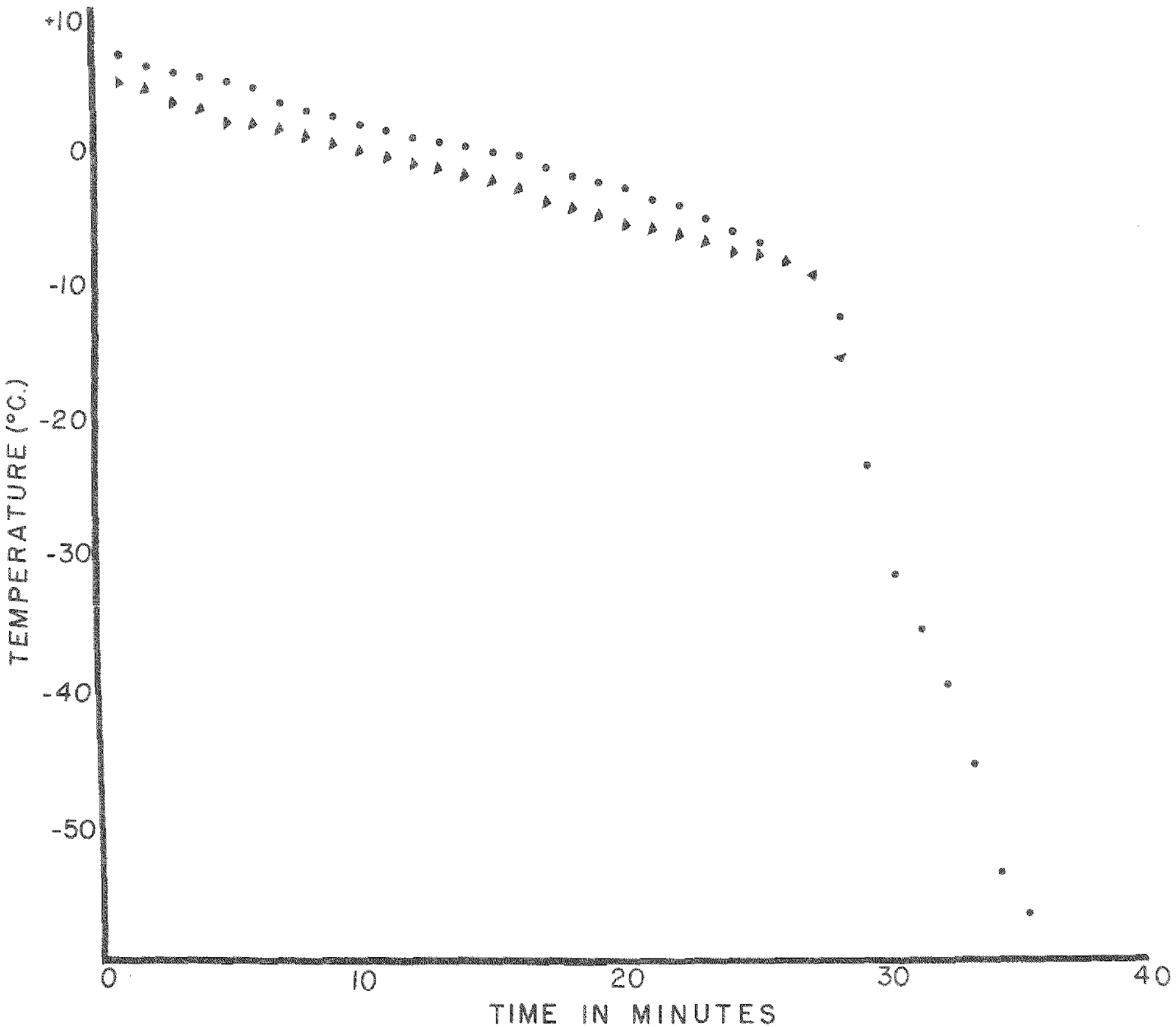


FIGURE 3

The results for the determination of optimum levels are presented in Table 7 and in the Appendix Table. An example calculation for the determination of the optimum levels from the treatment responses is given in the appendix.

Considering motility, it would seem that the optimal levels of sodium citrate, glycerol and egg-yolk would approximate 3.4, 12.4 and 33%, respectively, whether stored for only 23 hours after collection or frozen by Method No. 1. In final concentration these levels would be 6.2% glycerol and 15% egg-yolk in 3.4% sodium citrate at the extension rate used for this experiment (i.e. 1 part semen : 21 parts extender). These results are in accordance with the results of a large number of authors. For example, 3.4% sodium citrate is in agreement with Salisbury and Knodt (1947), Foote and Bratton (1949), VanDemark et al. (1950) and Polge (1953); 6.3% glycerol is close to the results of all of those workers which recommended 7%, such as Miller and VanDemark (1954), Bratton et al. (1955), Dunn and Cruthers (1954), Kinney and VanDemark (1954), Mixner (1954), Elliott et al. (1954), Erickson et al. (1954) and Williams (1954); 15% egg-yolk is in agreement with Mayer and Lasley (1944), Salisbury et al. (1941), Stewart et al. (1950), Almquist (1951), Dunn and Hafs (1953), Hurst (1953), Kinney and VanDemark (1954) and Saroff and Mixner (1954). Any deviation from these levels and those of the authors quoted above, could be accounted for in the variation due to the different extension

TABLE VII

TREATMENTS AND PERCENT SURVIVAL AVERAGES
EXPERIMENT II

Treat. No.	Sodium Citrate[a] %	Glycerol[a] %	Yolk[a] %	% Survival — Viability[c] 5 hrs.	23 hrs.	Freezing No.1	Freezing No.2	Freezing No.3	Motility[d] 5 hrs.	23 hrs.	Freezing No.1	Freezing No.2	Freezing No.3
1	3.2(-1)[b]	7(-1)	20(-1)	102.3[e]	103.8	64.1	67.9	81.4	90.9	81.8	54.5	45.5	45.5
2	4.8(+1)	7(-1)	20(-1)	105.1	101.4	59.6	73.2	71.6	90.9	45.5	5.5	1.8	3.6
3	3.2(-1)	17(+1)	20(-1)	101.1	104.4	59.3	60.6	59.6	100.0	90.9	45.5	54.5	45.5
4	4.8(+1)	17(+1)	20(-1)	99.1	97.0	57.0	56.1	71.6	90.9	50.9	3.6	1.8	3.6
5	3.2(-1)	7(-1)	40(+1)	102.3	105.6	56.6	61.2	58.5	81.8	90.9	36.4	12.7	9.1
6	4.8(+1)	7(-1)	40(+1)	99.2	104.4	48.7	58.5	46.0	85.5	36.4	3.6	5.5	0
7	3.2(-1)	17(+1)	40(+1)	102.6	102.6	40.0	55.1	48.0	90.9	90.9	45.5	54.5	54.5
8	4.8(+1)	17(+1)	40(+1)	101.1	105.4	59.3	67.6	60.8	96.4	54.5	9.1	7.3	9.9
9	4.0(0)	12(0)	30(0)	100.0	98.8	51.6	67.9	47.0	81.8	100.0	72.7	54.5	54.5
10	2.6(-2)	12(0)	30(0)	100.3	102.6	83.7	60.3	62.9	94.5	100.0	1.8	0	0
11	5.6(+2)	12(0)	30(0)	107.5	100.0	47.2	26.8	32.7	63.6	7.3	45.5	36.4	54.5
12	4.0(0)	2(-2)	30(0)	96.8	99.8	53.1	55.5	56.9	90.9	90.9	0	3.6	0
13	4.0(0)	22(+2)	30(0)	96.5	87.5	31.4	47.2	39.7	81.8	81.8	54.5	63.6	58.2
14	4.0(0)	12(0)	10(-2)	91.0	96.2	54.6	56.3	41.9	76.4	45.5	36.4	27.3	36.4
15	4.0(0)	12(0)	50(+2)	101.5	103.6	55.4	49.3	50.1	90.9	81.8	63.6	54.5	27.3
OPTIMAL LEVELS	%Sodium Citrate (X_1)				4.68	5.42	4.31	4.14		3.43	3.44	4.69	3.07
	% Glycerol (X_2)				9.73	16.81	13.41	3.36		11.03	13.89	Out of	
	% Egg Yolk (X_3)				36.75	19.16	22.77	26.32		34.07	32.08	range	used.

a Concentration of sodium citrate, glycerol and egg yolk used.
b Coded values in parenthesis.
c Percent survival based on the average of six counts(two counts on each of three slides) of one vial per treatment.
d Percent survival based on two or more observations per vial.
e Percent survivals of one hundred or over were obtained during the five and ~~eighteen~~ 23 hour periods if the sample estimate was higher than the average initial estimate made upon the semen sample before it was treated in any manner.

rates used; especially in regard to the percent of sodium citrate and glycerol.

Concerning viability, the optimum levels of extender components vary widely depending upon the proposed length of storage or the method of freezing employed. No report has been found in the literature reviewed which is based solely upon viability estimates to which these results could be compared. This investigation indicates, that to attain maximum viability, different levels of sodium citrate, glycerol and egg-yolk are required than to attain maximum motility. Also, more variation is observed in the optimal levels for viability between methods of processing than is observed for motility in the same respect. However, it must be borne in mind that viability and motility are two completely different quality tests in that the former measures the ratio of live:dead cells whereas the latter measures the ratio of motile:non-motile cells.

It was noticed that the motility estimates have a definite pattern related for the most part to the quantity of sodium citrate used. The motility estimates were high when the level of sodium citrate was low and vice versa. This same relationship seemed to hold regardless of freezing method. It may be that the sodium salts interfere with spermatozoan motility, especially during the freezing process. The computation of a correlation coefficient indicated a

negative association between viability and motility estimates, although, not significantly so at the 5% level (Table 8).

It would be difficult to determine which is closer to the proper value of any sample in question without running a field test; however, it is generally considered that spermatozoan motility is necessary for fertilization. It would seem logical to believe, therefore, that in those cases in which no, or very little, motility is observed, that regardless of the number of unstained spermatozoa counted, the sample is still of little practical value (Cupps et al., 1953, would be at variance with this). In such a case the viability count would either be in error or the cells would be alive but motionless (Lasley,1951).

It would appear from these observations that further work is required to investigate these differences. In the interim, motility should be considered as the more accurate test.

Following an examination of the results, it appears that the only plausible explanation concerning the optimal levels of these three components is that the optimal level of each depends upon the other two and upon the method of processing. If a quantitative or qualitative substitution were made for one of the components it would seem logical from the foregoing that the optimal levels of the remaining extender components would vary. For example, if more or less egg-yolk were used

TABLE VIII

ANALYSIS OF VARIANCE FOR QUALITY TESTS USED EXPERIMENT II

Source	Degrees of Freedom	Variance	Difference Between Means	Necessary Difference
Total	524			
Quality Tests	1	40086.5**	26.9	4.48
Error	523	706.1		

r = -0.223 (r at 5% level from tables = -0.482)

TABLE IX

ANALYSIS OF VARIANCE FOR FREEZING METHODS USED EXPERIMENT II

	Viability		Motility	
Source	Degrees of Freedom	Variance	Degrees of Freedom	Variance
Total	269		44	
Freezings	2	202.1	2	103.3
Error	267	201.0	42	603.3

or if whole egg was used to replace egg-yolk, the optimal levels of sodium citrate and glycerol, determined here, would not likely apply. If this is true, then optimal levels determined under the conditions of this experiment would be applicable only under these conditions and would serve merely as a guide for other circumstances.

Triplicate samples of the fifteen treatments were frozen by each of the three freezing methods outlined in "Methods and Materials". An analysis of variance indicated that there were no significant differences among these methods considering either the motility or the viability responses (Table 9). Hence, under these conditions the simpler and more economical method of freezing semen (Method No. 2) gives as adequate a response as does either of the more cumbersome, standard procedures, and would serve as a practical substitute for them (Table 10).

A significant difference was observed between Freezing Methods No. 1 and 2 in Experiment I; however, no significant difference was observed between the same two methods in Experiment II. It should be noted that the conditions of the two experiments were not directly comparable in that the vials were frozen for 5 days in Experiment II, as compared to 24 hours in Experiment I and, also, that 45 vials were placed in a layer in Experiment II, whereas only 6 were frozen for Experiment I. Dunn et al. (1954) found that the effect of freezing on semen samples stored at -75°C. is not

TABLE X

FREEZING RESPONSES OBTAINED EXPERIMENT II

	Source	Average	n	Standard Error	Co-efficient of Variability
Viability	Freezing Method No. 1	54.8%	90	0.82	1.47
	Freezing Method No. 2	57.6%	90	0.88	1.57
	Freezing Method No. 3	55.3%	90	0.89	1.59
Motility[a]	Freezing Method No. 1	28.2%	15	6.3	21.7
	Freezing Method No. 2	31.9%	15	6.6	22.8
	Freezing Method No. 3	26.8%	15	6.1	21.0

[a] The motility estimates were low as these figures represent the average of the treatment responses. These treatments were designed over such a range as to give low responses in some cases.

completed until 3 days after freezing. In view of this, it may be that the length of storage in the frozen state would partially explain the difference noted between experiments. It is postulated that to freeze spermatozoa successfully a standard temperature decline must be followed within fairly narrow limits regardless of the freezing method used. The number of vials used in the first experiment may have altered the rate of temperature decline sufficiently to obtain a lowered survival due to too rapid freezing. It is suggested that for uncontrolled freezings, the procedure used for Freezing Method No. 2 in Experiment II be closely adhered to for favorable results until further investigations are carried out.

Conclusions:

Based on data from this experiment concerning optimal levels of extender components, two quality tests and three freezing methods the conclusions are as follows:

(a) Optimal levels of sodium citrate, glycerol, and egg-yolk based on motility estimates before and after freezing by Method 1 (controlled temperature decline to -55°C.) were 3.42, 11.03, and 34.07% and 3.44, 13.99, and 32.08% respectively. The optimal levels after freezing by Methods 2 and 3 were out of the range used and were therefore incalculable.

(b) Optimal levels of the three extender components studied varied considerably when estimates of semen quality were based on viability (live:dead differential staining). These

optimal levels vary depending upon the length of storage in the unfrozen state and upon the method of freezing.

(c) There appears to be very little agreement between the results obtained from the two quality tests employed; that is, motility and viability. The motility test is considered the more reliable estimate of semen quality.

(d) A freezing method is proposed which consists simply of surrounding the semen ampoules with solid CO_2. This method resulted in responses equally as good as those obtained from either of the methods in general use. Because this result is at variance with that obtained in Experiment I further work is necessary to study these methods of freezing.

SUMMARY

An experiment was conducted to establish the sample size and counting procedure to be used in conjunction with a live: dead differential staining technique to obtain statistically adequate results. The semen used in the study was obtained from two bulls by means of an artificial vagina. The ejaculates were extended 1:21 with a glycerol-yolk-citrate extender. Samples were frozen in 1 ml. ampoules and stored for 24 hours at -79°C. Following thawing at 5°C., two methods of counting were compared. Count A involved two counts each of 500 spermatozoa on each of two slides prepared for each vial. Count B involved three slides with two counts of 100 spermatozoa on each. The counting was independently performed by each of two technicians.

From an analysis of variance with 119 degrees of freedom there were no significant differences in the results obtained concerning the two counting procedures; however, a significant difference was found between the counting of technicians.

A second experiment was designed to investigate the effect on semen quality of an extender comprised of various levels of the commonly used components sodium citrate, egg-yolk, and glycerol. A three-dimensional central composite experimental design was used to determine the optimal levels which would allow maximum survival of spermatozoa frozen to

and stored at low temperatures. The pooled semen sample collected from 3 bulls had an initial concentration of 400 million spermatozoa per ml. An extension rate of 1:21 (semen:extender) was used. The optimal levels were calculated, based on two qualtiy tests and three methods of freezing.

It was noticed that the motility estimates had a definite pattern related to the concentration of sodium citrate. The motility estimates were high when the level of sodium citrate was 3.2% or less and were low when the level of sodium citrate was 4.8% or more. The same relationship held regardless of storage time, but increased in effect as storage progressed.

Very little correlation was apparent between the two quality tests, viability and motility. Further work is required to investigate this difference. In the interim motility should be considered as the more accurate test since the fertilizing capacity of non-motile, living spermatozoa is questionable.

The results from three freezing methods would indicate that a standard temperature decline must be followed within fairly narrow limits for maximum survival.

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APPENDIX A

Preparation of the Liquid Egg-Yolk

The egg-yolk was obtained as follows:

1) The shell was cracked and carefully split into halves, retaining the whole yolk and allowing the albumen and chalazae to escape.

2) The yolk, still contained within the vitelline membrane, was poured from one-half of the egg shell into the other until separation from the albumen was complete.

3) A paper towel was twice folded to form a funnel into which the yolk was placed (as the yolk slid towards the bottom of the funnel all remaining albumen was left attached to the towel).

4) A needle, inserted through the bottom of the funnel, punctured the vitelline membrane allowing the liquid yolk to flow into a glass graduate. The vitelline membrane adhered to the funnel.

A clean paper towel was used for each egg. In the event that the vitelline membrane was broken before completely separated from the albumen, or, that the yolk became contaminated in any way, the material was discarded.

APPENDIX B_1

VIABILITY RESPONSES OBTAINED FROM FIFTEEN TREATMENTS AT FIVE AND TWENTY-THREE HOURS AND THE THREE FREEZING METHODS

Slide 1		Slide 2		Slide 3		Slide 1		Slide 2		Slide 3	
Ct.1	Ct.2	Ct.1	Ct.2	Ct.1	Ct.2	Ct.1	Ct.2	Ct.1	Ct.2	Ct.1	Ct.2
FIVE HOURS						TWENTY-THREE HOURS					
102.6	111.6	93.5	101.0	98.0	107.1	111.6	108.6	96.5	107.1	90.5	108.6
117.6	101.0	84.5	110.1	99.5	117.6	107.1	89.1	111.6	102.6	104.1	93.5
98.0	107.1	98.0	93.5	92.0	117.6	95.0	105.6	104.1	102.6	113.1	105.6
95.0	108.6	84.5	86.0	110.1	110.1	111.6	99.5	107.1	108.6	96.5	58.8
111.6	95.0	92.0	113.1	101.0	101.0	104.1	96.5	104.1	110.1	111.6	107.1
102.6	105.6	105.6	114.6	79.9	87.5	104.1	108.6	95.0	92.0	102.6	105.6
95.0	111.6	120.7	96.5	84.5	107.1	98.0	108.6	113.1	99.5	102.6	108.6
104.1	102.6	87.5	108.6	101.0	102.6	101.1	107.1	104.1	102.6	111.6	105.6
92.0	119.6	101.0	101.0	93.5	101.0	101.1	93.5	92.0	99.5	105.6	101.1
114.6	107.1	96.5	107.1	81.4	95.0	113.1	102.6	99.5	95.0	105.6	99.5
96.5	119.1	105.6	116.1	111.6	96.5	102.6	108.6	105.6	92.0	101.1	90.5
93.5	98.0	98.0	104.1	96.5	90.5	99.5	101.1	99.5	110.1	87.5	101.1
102.6	96.5	84.5	95.0	102.6	98.0	87.5	87.5	87.5	87.5	87.5	87.5
81.4	96.5	82.9	93.5	108.6	82.9	101.1	89.1	95.0	98.0	96.5	98.0
96.5	101.0	99.5	98.0	110.1	104.1	104.1	107.1	101.1	108.6	86.0	114.6

APPENDIX B_2

VIABILITY RESPONSES OBTAINED FROM FIFTEEN TREATMENTS AT FIVE AND TWENTY-THREE HOURS AND THE THREE FREEZING METHODS

Slide 1		Slide 2		Slide 3	
Ct.1	Ct.2	Ct.1	Ct.2	Ct.1	Ct.2
FREEZING METHOD NO. 1					
69.4	64.9	67.9	72.4	49.8	60.3
70.9	66.4	43.7	61.8	58.8	55.8
72.4	55.8	73.9	55.8	40.7	57.3
61.8	61.8	57.3	46.8	58.8	55.8
58.8	51.3	49.8	63.4	51.3	64.9
46.8	52.8	64.9	28.7	42.2	57.3
54.3	31.7	28.7	42.2	43.7	39.2
57.3	60.3	54.3	73.9	60.3	49.8
51.3	46.8	43.7	45.2	67.9	54.3
95.0	89.1	78.4	82.0	86.0	70.9
49.8	42.2	69.4	43.7	33.2	45.2
42.2	49.8	48.3	43.7	51.3	83.0
43.7	22.6	28.7	30.2	31.3	31.3
51.3	60.3	45.2	51.3	55.8	63.4
58.8	34.7	60.3	52.8	67.9	57.3

Slide 1		Slide 2		Slide 3	
Ct.1	Ct.2	Ct.1	Ct.2	Ct.1	Ct.2
FREEZING METHOD NO. 2					
73.9	52.8	89.0	64.8	63.3	63.3
70.9	87.5	76.9	70.9	73.9	58.8
49.8	76.9	63.3	63.3	48.3	61.8
73.9	24.1	58.8	70.9	57.3	51.3
63.3	57.3	63.3	69.4	58.8	55.8
51.3	57.3	52.8	67.9	58.8	63.3
49.8	60.3	51.3	54.3	57.3	57.3
76.9	70.9	67.9	64.8	67.9	57.3
78.4	67.9	72.4	58.8	63.3	66.4
55.8	63.3	54.3	66.4	57.3	64.8
24.1	25.6	31.7	25.6	30.2	24.1
45.2	55.8	60.3	52.8	61.8	57.3
37.7	43.7	49.8	36.2	45.2	70.9
54.3	54.3	55.8	51.3	64.8	57.3
57.3	45.2	46.7	37.7	49.8	58.8

APPENDIX B_3

VIABILITY RESPONSES OBTAINED FROM FIFTEEN TREATMENTS AT FIVE AND TWENTY-THREE HOURS AND THE FREEZING METHODS

Slide 1		Slide 2		Slide 3	
Ct.1	Ct.2	Ct.1	Ct.2	Ct.1	Ct.2
FREEZING METHOD NO. 3					
84.5	76.9	76.9	87.5	83.0	79.9
66.4	84.5	67.9	75.4	63.4	72.4
46.8	51.3	64.9	55.8	64.9	73.9
75.4	67.9	69.4	67.9	78.4	70.9
66.4	75.4	60.3	51.3	45.3	52.8
33.2	46.8	51.3	40.7	51.3	52.8
55.8	45.3	45.3	43.7	57.3	40.7
58.8	57.3	61.8	73.9	54.3	58.8
45.3	36.2	45.3	51.3	54.3	49.8
64.9	61.8	58.8	64.9	69.4	57.3
24.1	43.7	37.7	33.2	18.1	39.2
55.8	63.4	48.3	64.9	58.8	49.8
31.7	42.2	36.2	49.8	43.7	34.7
46.8	30.2	33.2	46.8	48.3	46.8
54.3	45.3	52.8	46.8	42.2	58.8

APPENDIX C

EXAMPLE CALCULATION OF OPTIMUM LEVELS FROM FIFTEEN RESPONSES

MOTILITY TWENTY-THREE HOURS

Treatment No.	Coded values X_1	X_2	X_3	Response Motility 23 hrs.
1	-1	-1	-1	81.8
2	1	-1	-1	45.5
3	-1	1	-1	90.9
4	1	1	-1	50.9
5	-1	-1	1	90.9
6	1	-1	1	36.4
7	-1	1	1	90.9
8	1	1	1	54.5
9	0	0	0	100.0
10	-2	0	0	100.0
11	2	0	0	7.3
12	0	-2	0	90.9
13	0	2	0	81.8
14	0	0	-2	45.5
15	0	0	2	81.8

APPENDIX C

EXAMPLE CALCULATION OF OPTIMUM LEVELS FROM FIFTEEN RESPONSES

X_0 = sum of 15 treatments = +1049.1

X_1 = -1(81.8) +1(45.5) -1(90.9) +1(50.9) -1(90.9)+1(36.4)-1(90.9)+1(54.5)
+0(100.0)-2(100.0)+2(7.3)+0(90.9) ...0(81.8) = -352.6

X_2 = -1(81.8)-1(45.5)+1(90.9)+1(50.9)-1(90.9)-1(36.4)+1(90.9)+1(54.5)
+0(100.0) ...0(7.3)-2(90.9)+2(81.8)+0(45.5) ...0(81.8) = -36.5

X_3 = -1(81.8)-1(45.5)-1(90.9)-1(50.9)+1(90.9)+1(36.4)+1(90.9)+1(54.5)
+0(100.0) ...0(81.8)-2(45.5)+2(81.8) = +76.2

X_{11}= 1(81.8)+1(45.5)+1(90.9)+1(50.9)+1(90.9)+1(36.4)+1(90.9)+1(54.5)
+0(100.0)+4(100.0)+4(7.3)+0(90.9) ...0(81.8) = +971.0

X_{22}= 1(81.8)+1(45.5)+1(90.9)+1(50.9)+1(90.9)+1(36.4)+1(90.9)+1(54.5)
+0(100.0) ...0(7.3)+4(90.9)+4(81.8)+0(45.5) ...0(81.8) = +1232.6

X_{33}= 1(81.8)+1(45.5)+1(90.9)+1(50.9)+1(90.9)+1(36.4)+1(90.9)+1(54.5)
+0(100.0) ...0(81.8)+4(45.5)+4(81.8) = +1051.0

X_{12}= 1(81.8)-1(45.5)-1(90.9)+1(50.9)+1(90.9)-1(36.4)-1(90.9)+1(54.5)
+0(100.0) ...0(81.8) = +14.4

X_{13}= 1(81.8)-1(45.5)+1(90.9)-1(50.9)-1(90.9)+1(36.4)-1(90.9)+1(54.5)
+0(100.0) ...0(81.8) = -14.6

X_{23}= 1(81.8)+1(45.5)-1(90.9)-1(50.9)-1(90.9)-1(36.4)+1(90.9)+1(54.5)
+0(100.0) ...0(81.8) = +3.6

APPENDIX C

EXAMPLE CALCULATION OF OPTIMUM LEVELS FROM FIFTEEN RESPONSES

$$B_0 = \tfrac{1}{9}(81.8)+\tfrac{1}{9}(45.5)+\tfrac{1}{9}(90.9)+\tfrac{1}{9}(50.9)+\tfrac{1}{9}(90.9)+\tfrac{1}{9}(36.4)+\tfrac{1}{9}(90.9)+\tfrac{1}{9}(54.5)$$
$$+\tfrac{1}{9}(100.0)-\tfrac{1}{9}(100.0)-\tfrac{1}{9}(7.3)-\tfrac{1}{9}(90.9)-\tfrac{1}{9}(81.8)-\tfrac{1}{9}(45.5)-\tfrac{1}{9}(81.8) = +92.72221$$

$$B_1 = -\tfrac{1}{16}(81.8)+\tfrac{1}{16}(45.5)-\tfrac{1}{16}(90.9)+\tfrac{1}{16}(50.9)-\tfrac{1}{16}(90.9)+\tfrac{1}{16}(36.4)-\tfrac{1}{16}(90.9)$$
$$+\tfrac{1}{16}(54.5)+0(100.0)-\tfrac{2}{16}(100.0)+\tfrac{2}{16}(7.3)+0(90.9)\ \ldots 0(81.8) = -22.037500$$

$$B_2 = -\tfrac{1}{16}(81.8)-\tfrac{1}{16}(45.5)+\tfrac{1}{16}(90.9)+\tfrac{1}{16}(50.9)-\tfrac{1}{16}(90.9)-\tfrac{1}{16}(36.4)+\tfrac{1}{16}(90.9)+\tfrac{1}{16}(54.5)$$
$$+0(100.0)\ \ldots 0(7.3)-\tfrac{2}{16}(90.9)+\tfrac{2}{16}(81.8)+0(45.5)\ \ldots 0(81.8) = +0.900000$$

$$B_3 = -\tfrac{1}{16}(81.8)-\tfrac{1}{16}(45.5)-\tfrac{1}{16}(90.9)-\tfrac{1}{16}(50.9)+\tfrac{1}{16}(90.9)+\tfrac{1}{16}(36.4)+\tfrac{1}{16}(90.9)+\tfrac{1}{16}(54.5)$$
$$+0(100.0)\ \ldots 0(81.8)-\tfrac{2}{16}(45.5)+\tfrac{2}{16}(81.8) = +4.762500$$

$$B_{11} = -\tfrac{1}{72}(81.8)-\tfrac{1}{72}(45.5)-\tfrac{1}{72}(90.9)-\tfrac{1}{72}(50.9)-\tfrac{1}{72}(90.9)-\tfrac{1}{72}(36.4)-\tfrac{1}{72}(90.9)-\tfrac{1}{72}(54.5)$$
$$-\tfrac{16}{72}(100.0)+\tfrac{10}{72}(100.0)+\tfrac{10}{72}(7.3)+\tfrac{1}{72}(90.9)+\tfrac{1}{72}(81.8)+\tfrac{1}{72}(45.5)+\tfrac{1}{72}(81.8) = -10.677778$$

$$B_{22} = -\tfrac{1}{72}(81.8)-\tfrac{1}{72}(45.5)-\tfrac{1}{72}(90.9)-\tfrac{1}{72}(50.9)-\tfrac{1}{72}(90.9)-\tfrac{1}{72}(36.4)-\tfrac{1}{72}(90.9)-\tfrac{1}{72}(54.5)$$
$$-\tfrac{16}{72}(100.0)+\tfrac{1}{72}(100.0)+\tfrac{1}{72}(7.3)+\tfrac{10}{72}(90.9)+\tfrac{10}{72}(81.8)+\tfrac{1}{72}(45.5)+\tfrac{1}{72}(81.8) = -2.727778$$

$$B_{33} = -\tfrac{1}{72}(81.8)-\tfrac{1}{72}(45.5)-\tfrac{1}{72}(90.9)-\tfrac{1}{72}(50.9)-\tfrac{1}{72}(90.9)-\tfrac{1}{72}(36.4)-\tfrac{1}{72}(90.9)-\tfrac{1}{72}(54.5)$$
$$-\tfrac{16}{72}(100.0)+\tfrac{1}{72}(100.0)+\tfrac{1}{72}(7.3)+\tfrac{1}{72}(90.9)+\tfrac{1}{72}(81.8)+\tfrac{10}{72}(45.5)+\tfrac{10}{72}(81.8) = -8.402778$$

$$B_{12} = \tfrac{1}{8}(81.8)-\tfrac{1}{8}(45.5)-\tfrac{1}{8}(90.9)+\tfrac{1}{8}(50.9)+\tfrac{1}{8}(90.9)-\tfrac{1}{8}(36.4)-\tfrac{1}{8}(90.9)+\tfrac{1}{8}(54.5)$$
$$+0(100.0)\ \ldots 0(81.8) = +1.900000$$

$$B_{13} = \tfrac{1}{8}(81.8)-\tfrac{1}{8}(45.5)+\tfrac{1}{8}(90.9)-\tfrac{1}{8}(50.9)-\tfrac{1}{8}(90.9)+\tfrac{1}{8}(36.4)-\tfrac{1}{8}(90.9)+\tfrac{1}{8}(54.5)$$
$$+0(100.0)\ \ldots 0(81.8) = -1.825000$$

$$B_{23} = \tfrac{1}{8}(81.8)+\tfrac{1}{8}(45.5)-\tfrac{1}{8}(90.9)-\tfrac{1}{8}(50.9)-\tfrac{1}{8}(90.9)-\tfrac{1}{8}(36.4)+\tfrac{1}{8}(90.9)+\tfrac{1}{8}(54.5)$$
$$+0(100.0)\ \ldots 0(81.8) = +0.450000$$

APPENDIX C

EXAMPLE CALCULATION OF OPTIMUM LEVELS FROM FIFTEEN RESPONSES

	X_1Y		B
X_0	+1049.1	B_0	+92.722210
X_1	- 352.6	B_1	-22.037500
X_2	- 36.5	B_2	+ 0.900000
X_3	+ 76.2	B_3	+ 4.762500
X_{11}	+ 971.0	B_{11}	-10.677778
X_{22}	+1232.6	B_{22}	- 2.727778
X_{33}	+1051.0	B_{33}	- 8.402778
X_{12}	+ 14.4	B_{12}	+ 1.900000
X_{13}	- 14.6	B_{13}	- 1.825000
X_{23}	+ 3.6	B_{23}	+ 0.450000

$$Y = +92.722210 - 22.037500X_1 + 0.900000X_2 + 4.762500X_3 - 10.677778X_1^2 - 2.727778X_2^2 - 8.402778X_3^2 + 1.900000X_1X_2 - 1.825000X_1X_3 + 0.450000X_2X_3$$

$$\frac{\partial}{\partial X_1} -22.037500 - 21.355556X_1 + 1.900000X_2 - 1.825000X_3 = 0$$

$$\frac{\partial}{\partial X_2} + 0.900000 - 5.455556X_2 + 1.900000X_1 + 0.450000X_3 = 0$$

$$\frac{\partial}{\partial X_3} + 4.762500 - 16.805556X_3 - 1.825000X_1 + 0.450000X_2 = 0$$

APPENDIX C

EXAMPLE CALCULATION OF OPTIMUM LEVELS FROM FIFTEEN RESPONSES

Equation 1 — $-21.355556X_1 + 1.9000000X_2 - 1.825000X_3 = 22.037500$

Equation 2 — $+ 1.900000X_1 - 5.455556X_2 + 0.450000X_3 = - 0.900000$

Equation 3 — $- 1.825000X_1 + 0.450000X_2 - 16.805556X_3 = - 4.762500$

Doolittle Solution

		X_1	X_2	X_3	X_0	Check
I	a	-21.355556	1.900000	- 1.825000	22.037500	+ 0.756944
	b		- 5.455556	+ 0.450000	- 0.900000	- 4.005556
	c			-16.805556	- 4.762500	-22.9430560
II	a	-21.355556	1.900000	- 1.825000	22.037500	+ 0.756944
	b	1	- 0.088970	+ 0.085458	- 1.031933	- 0.035445
IIIa			- 5.286513	+ 0.287630	+ 1.060673	- 3.938211
	b		1	- 0.054408	- 0.200638	+ 0.744954
IV	a			-16.633946	- 6.588068	-23.222014
	b			1	+ 0.396062	+ 1.396062

$X_3^\circ = + 0.396062$

$X_2^\circ = - 0.2006375 - (-0.054408X + 0.396062) = - 0.1790886$

$X_1^\circ = - 1.031933 - (+1.900000X - 0.1790886) - (+0.085458X + 0.396062) = -0.657818$

Therefore the stationary point is

$X_1^\circ = - 0.657818 = 3.474\%$ Sodium Citrate

$X_2^\circ = - 0.1790886 = 11.11\%$ Glycerol

$X_3^\circ = + 0.396062 = 33.961\%$ Yolk

APPENDIX C

EXAMPLE CALCULATION OF OPTIMUM LEVELS FROM FIFTEEN RESPONSES

$$Y_o = 92.722210 + 14.496664 - .161180 + 1.886245 - 4.620541 - .874878$$
$$- 1.318102 + .223835 + .475479 - .319187$$
$$Y_o = 103.148919$$