AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented August 2, 1966

Title EFFECT OF RUMEN FLUID UPON THE PRESERVATION OF BOVINE SPERMATOZOA

Abstract approved [redacted]
(Major Professor)

Experiments were conducted to determine the effect of rumen fluid on live and motile spermatozoa of semen extended in yolk-citrate and stored at 4°C. Preliminary work showed that levels of rumen fluid below 10 percent were beneficial in keeping spermatozoan activity up to 14 days in storage. For more revealing information, 1664 observations for live and motility were made on 16 collections from 4 dairy bulls. Semen was extended in 0.5, 1.0 and 5.0 percent boiled or raw rumen fluid, treated or nontreated with 500 I.U. of penicillin and 500 micrograms of dihydrostreptomycin per ml. of extender. From microscopic evaluations to the ninth day, the results were seen to fall into three definite groups: control, antibiotic treated, and nontreated semen. Higher ratings were observed in the lower levels than in the 5 percent

level of rumen fluid ($p = .001$). Additional 768 observations to determine the effect of argon and oxygen on semen extended in different levels of rumen fluid showed highly significant differences in motility, but no differences in percent alive.

For the ninth day rating, rumen fluid at 1.0% or lower levels increased motility by 27 percent in comparison with control. Antibiotics decreased this figure to 11 percent. Oxygen decreased motility by 12 percent; however, at the 5 percent level, oxygen rated 25 percent higher than oxygen without rumen fluid. Argon showed an overall decline in motility of 8 percent.

It can be concluded that the activity of bovine spermatozoa can be increased with small amounts of rumen fluid. The detrimental effect of antibiotics on motility ($p < .0001$) and the nonharmful effect of oxygen on the life of the spermatozoa contradict present belief. The fact that boiling did not affect the beneficial effect of rumen fluid suggests sterile means for using rumen fluid in artificial insemination. Since the action of some substance produced by a rumen microorganism is suspected, the possibility of isolating this microorganism can not be overlooked.

EFFECT OF RUMEN FLUID UPON THE PRESERVATION OF BOVINE SPERMATOZOA

by

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A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1967

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Date thesis is presented August 2, 1966

Typed by Bernice Caceres

ACKNOWLEDGMENT

The author wishes to express his appreciation and thanks to Professor Floyd B. Wolberg for his guidance and support needed to carry out this study; to Dr. Ralph Bogart for his sincere advise in organizing and revising this material; to Kenneth Rowe for his suggestions in the statistical analyses; and to Merle Peters and George Hannay for their help in the collection of semen used in this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF APPENDIX TABLES

EFFECT OF RUMEN FLUID UPON THE PRESERVATION OF BOVINE SPERMATOZOA

INTRODUCTION

With increased use of artificial insemination in the livestock industry, means of preserving semen become more challenging. Although frozen semen has been and can be preserved for unknown periods of time, there is evidence (Salisbury, 1963) indicating that the fertilizing capacity of spermatozoa decreases within a few months after freezing. Besides looking for new ways of freezing semen, new substances may be found which may help in more effective storage of frozen semen.

The possibility also exists of finding substances which will improve semen extenders to use at room or common refrigeration temperatures. This could become an important factor when semen has to be transported considerable distances. It could also provide means for using unfrozen semen for longer periods of time than is recommended today. Finally, new substances could lead to the improvement of the fertilizing ability of the spermatozoa providing a new tool for faster genetic and economic progress.

In the present study, the use of rumen fluid for the storage of semen has been investigated. The study was carried out in three parts.

Part I was designed to determine the range of tolerance by the spermatozoa to rumen fluid. Boiled rumen fluid was compared with raw rumen fluid. Since antibiotics are commonly used in artificial insemination, antibiotic treated samples were compared with non-treated samples. Because of the high content of short chain fatty acids in rumen content, different percentages of these acids were also studied in this preliminary step.

Part II was designed to obtain more organized and revealing information concerning the range of rumen fluid which is most suitable to extend the life of the spermatozoa.

Part III was designed to study the effect of argon and oxygen gases upon semen extended in different amounts of rumen fluid. The idea behind this experiment was that argon should inhibit the metabolic rate of the organisms present in the medium. Oxygen, on the other hand, should activate the aerobic and to some extent kill the anaerobic microorganisms.

The composition of rumen fluid is not completely known. It is known, however, to contain large numbers of aerobic as well as anaerobic bacteria and protozoa. Limitations in time and resources did not permit the isolation of these organisms nor the inoculation of extenders with common rumen microorganisms. So far, nowhere in the literature has a similar study been reported.

REVIEW OF LITERATURE

CHARACTERISTICS OF RUMEN FLUID

Many functions in Ruminants are mediated by the microflora in the rumen. Some of these functions include (Doetch and Robinson, 1953) digestion of insoluble materials used as feed stuffs; synthesis of vitamins and other factors required for the well being of the animal; and synthesis of protein from non-protein nitrogen sources.

Using direct microscopic counts, Köhler (1940) reported maximum bacteria counts of 12.8×10^9 per gram of rumen content. By slightly different procedure, Stark and Loosli (1947) reported between 76 to 92 $\times 10^9$ bacteria per gram. Purser and Moir (1959) reported up to 3×10^5 protozoa per mililiter of rumen fluid at a pH of 5.3. The numbers increased up to 6.2×10^5 at a pH of 5.9. The presence of small numbers of yeast in the rumen has been also reported in more recent literature (Hungate, Bryant and Mah, 1964).

Gall and Huhtanen (1951) made about 5,000 isolations from the rumens of approximately 350 cattle and sheep and used these data to set up criteria for determining true rumen bacteria. Their criteria were: (a) Anaerobiosis; (b) presence in numbers of one million or more per gram of fresh rumen contents; (c) isolations of a similar type of bacterium at least ten times from at least two animals; (d) isolation from animals in at least two different locations;

and (e) "production by the organism of end products found in the rumen from substrates found in the rumen." Five lactic acid bacteria were described which fitted the preceeding criteria.

Annison and Lewis (1959) give descriptive tables of the microorganisms in the rumen.

At a particular time, the number or kinds of organisms present in the rumen depends on many factors (Kistner, 1965) such as pH, age of the animal, time relative to food ingestion and many others. Kistner (1965) points out that growth rates of different bacteria at a given time depends on their adaptation to the existent conditions in the rumen. Besides pH, temperature, Eh, osmatic pressure and concentrations of products of fermentation, the nature of these concentrations and nutrients available seem to have greater influence. As an example, Kistner mentions the branched-chain volatile fatty acids which are essential in the growth of Ruminococcus. These fatty acids are not present in the diet but are a result, at least in part, of deamination of corresponding amino acids. The contributions of nutrients by saliva illustrates another example.

The effect of some diets on the occurrence of cellulolytic bacteria in the rumen of cattle are presented in Table 1.

Elsden (1945) studied the individual volatile fatty acids (VFA) in the rumen of pasture-fed sheep. He found that acetic acid was present in larger amounts than other VFA. On a molar percentage

Table 1. Cellulolytic bacteria in cattle (condensed from Kistner, 1965).

Species, Reference, and Location	Diets	Numbers/ ml. or g.	Comments
A: *Ruminococcus* species			
1. Hungate (1957) Washington	Different combinations of chopped timothy hay, concentrates and salt, timothy hay constituting 2/3-8/9 of total digestible nutrients	Up to 10^7	Large variations in counts between animals and in same animal from month to month. Both *R. flavefaciens* and *R. albus* found in high sample dilutions.
2. Bryant *et al* (1958) Maryland	Alfalfa hay; alfalfa hay-grain; clover pasture; grain mixture	10^8	Both *R. flavefaciens* and *R. albus* found in some samples.
3. Bryant *et al* (1961) Maryland	22% alfalfa meal, 16% soybean oil meal, 61% barley, 1% salt	10^7-10^8	Bloat-provoking diet, before exposure to penicillin.
B: *Bacteroides succinogenes*			
1. Hungate (1957) Washington	Mainly timothy hay	Up to 10^5	Usually occurred in low numbers and in a small proportion of a group of 25 cows.

Table 1. Continued.

Species, Reference, and Location	Diets	Numbers/ ml. or g.	Comments
B: Bacteroides succinogenes (Cont.)			
2. Hungate et al (1959) East Africa	Grass hay (zebu), otherwise unknown	10^6-10^9 (total cellulolytic count)	Camel included. B. succinogenes recognized by typical clearings in cellulose agar.
C: Butyrivibrio species			
1. Bryant and Small (1956) Maryland	Alfalfa hay; alfalfa hay-grain; alfalfa silage; soybean hay-grain; bluegrass pasture-grain; fresh green alfalfa; wheat straw; grain mixture	10^8	Only small proportion of isolates from non-selective medium showed visible loss of cellulose from liquid medium.
2. Hungate (1957) Washington	Mainly timothy hay	Up to 10^7	Large differences in counts between animals.
3. Gill and King (1958) Virginia	Hay-grain	10^7	Single isolate lost initial strong cellulolytic activity.

Table 1. Continued.

Species, Reference, and Location	Diets	Numbers/ ml. or g.	Comments
D: *Clostridium* species			
1. Hungate (1957) Washington	Mainly timothy hay	Up to 10^6	Ratio of sporeformers to total cellulolytic bacteria varied from animal to animal and month to month.
E: *Cillobacterium cellulosvens*			
1. Bryant *et al* (1958) Maryland	Clover pasture	10^8	Significance in rumen uncertain.

basis acetic acid ranged from 58 to 74 percent, propionic acid varied from 16 to 26 percent and butyric acid varied from 16 to 20 percent. Traces of valeric acid were also reported.

Emery, Smith and Huffman (1956) studied the VFA output and absorption by a set of twin Holstein cows and a Guernsey steer. An all alfalfa hay ration and a restricted hay-plus concentrate made up the two rations used. Two trials were carried out on each ration. The results showed rates of disappearance in percent per hour of 3.3 for acetic acid and 2.7 for butyric acid. For propionic acid, the corresponding figures were 4.6 in the concentrate ration and 3.2 in the hay ration. The rates of conversion of feed to VFA ranged from 28 to 84 percent.

Guided by reported data on VFA, Wolin (1960) assumed a ratio of 65: 20: 15 for acetate, propionate and butyrate, respectively. Using these figures, he proposed the equation:

$$57.5\ (C_6H_{12}O_6) \longrightarrow 60 \text{ acetate} + 20 \text{ propionate} +$$

$$15 \text{ butyrate} + 60CO_2 + 35CH_4 + 25H_2O$$

for the formation of gases from carbohydrates in the rumen. In balancing this equation, Wolin made the assumptions that $C_6H_{12}O_6$ is the only substrate, and CO_2 and CH_4 the only gases produced in the rumen other than VFA.

Mangan, Johns and Bailey (1959) suggested the possibility

of using penicillin against bloat producing bacteria in the rumen. Bryant et al (1960) studied the effect of penicillin against bloat, using six yearlings pastured on bloat-provoking Ladino clover. Three of these animals were known bloaters and three non-bloaters. Observations from the bloaters were taken 24 hours before and after 50 mg. of procaine penicillin were given orally to each animal. The study showed no differences for factors such as pH of rumen contents, anaerobic bacteria or cellulolytic bacteria. Facultative anaerobic streptococci, however, were significantly depressed in the penicillin treated group. Short chain streptococci formed from 60 to 95 percent of the organisms in the colonies studied in this experiment. It was concluded, therefore, that the effect of penicillin was mainly on streptococci.

EFFECT OF GASES ON SPERMATOZOAN ACTIVITY

Milovanov and Khabibulin (1933) reported that spermatozoa could be immobilized by lactic acid and its salts. A drop in pH accompanied the process, and reactivation was achieved by increasing temperature or by raising the pH. These workers proposed that lactic acid and carbon dioxide which are metabolic products of spermatozoa, along with temperature, were important factors in preserving semen at 5°C.

Shettles (1940) reported that carbon dioxide rapidly reduced

the respiration rate of human spermatozoa. Respiration was enhanced by the removal of this gas. He found that nitrous oxide and air at reduced pressure did not affect spermatozoan activity and that hydrogen and oxygen gases caused an increase in activity.

VanDemark, Salisbury and Branton (1949) placed extended bull semen in test tubes to which either air, oxygen or nitrogen was added. These samples were then placed under incubation for an hour at 46.5°C. or stored at 5°C. for ten days. Each increase in a relative amount of oxygen resulted in a relative decline in motility. During incubation and storage for four days, sugar disappearance was lower for semen under oxygen than for semen under the other treatments. However, after ten days of storage no significant differences were observed in sugar losses. The accumulation of lactic acid was larger for samples under air and lower for those under oxygen. The authors suggested that oxygen damage to extended semen may occur in routine handling and that shaking might increase the damage through oxygen exposure. Oxygen damage was largely eliminated by catalase in this experiment.

VanDemark and Couturier (1958) investigated the survival of bull spermatozoa using several dialysis fluids, various rates of fluid flow, several gaseous conditions and two temperatures. Each of eight samples of bull semen was split into 14 portions. Two of these were left unextended for control purpose. One half of the

remaining portions were extended in yolk-citrate with or without unheated pasteurized skim milk or with heated pasteurized skim milk. These six samples were kept in test tubes. The other samples were placed in the dialysis system using each of the extenders as dialysis fluids. One half of the portions was kept at 5°C. and the remainder at 26°C. For the 5°C. temperature, the results showed longer survival for the extended samples than in the dialysis systems. Opposite results were observed in the high temperature with yolk-citrate carrying the maximum survival. The lowest survival was observed in the controls. When nitrogen, oxygen or carbon dioxide was passed through the yolk-citrate dialysis, oxygen decreased survival while nitrogen increased it. The results with carbon dioxide were extremely variable. Oxygen depressed survival below the level observed when no gases were used.

Kliewer and Wolberg (1962) studied the effect of carbon dioxide, nitrogen and argon gases on motility, storage and fertilizing capacity of bull spermatozoa extended in yolk-citrate. All samples were treated with 1000 I. U. of penicillin and 1000 micrograms of dihydrostreptomycin per milliliter of extender. Gas pressure was applied at 20 p. s. i. to the extended semen during an hour and then all samples were stored at 4°C. after parafin sealing. Carbon dioxide and nitrogen kept the semen in useful condition up to seven days in storage. The conception rates for semen treated with these

two gases were 75.0 and 64.9 percent, respectively, for semen stored three to seven days. Semen treated with argon, on the other hand, showed 76.4 percent conceptions after storage for three to seven days and 68.6 percent conceptions after storage for eight to fourteen days. Although the control semen showed a conception rate of 81.5 percent after storage for one to four days, it was not used beyond four days due to the observed decline in motility.

More recent studies (Roussel et al, 1963; Steinbach and Foote, 1964) have been carried out to determine the effect of gases on post-freezing activity of bull spermatozoa. The results are somewhat inconclusive. Roussel et al (1963) reported a beneficial effect from nitrogen on post-freezing motility and a detrimental effect from argon after having used ampules flushed with either one of these two gases.

In a different experiment (Steinbach and Foote, 1964), argon showed a positive effect over nitrogen but the differences were not significant.

EXPERIMENTAL PROCEDURE

Part I

The purpose of this preliminary study was to determine if rumen fluid could be used to prolong the active life of bovine spermatozoa and, if so, to determine the amounts of rumen fluid which would produce the best results when combined with yolk-citrate extenders.

Fistulated animals, one Guernsey cow and one Hereford steer, were used as sources of rumen fluid. The fluid was brought to the laboratory and used part fresh and part after storage at 4°C. Four collections taken once a week from a two-year old Holstein bull were used as the semen supply. The semen was collected at the O. S. U. dairy barn using an artificial vagina filled with warm water to keep a temperature of 50°C. Another young bull was used as teasing animal. The semen was extended 1 to 20 with yolk-citrate extender. This extender was prepared using three parts of a 2. 9 percent sodium citrate solution and one part of egg yolk.

Antibiotics at the levels of 500 I. U. of penicillin (potasium crystalline G) and 500 micrograms of dihydrostreptomycin (sulfate solution) per mililiter of extended semen were added to half of the samples in each treatment. The treatments consisted of concentrations of 0, 1, 10, 20, 25, 50, 75, and 100 percent rumen fluid. The extended semen was evaluated for "alive" and "progressive motility"

at about a three-day interval between one and fourteen days of storage. All samples were stored under refrigeration at 4°C. Semen evaluation consisted of microscopic scores from zero to ten under 440 magnification for both alive and motility. By means of a warming plate, a temperature of about 34°C. was maintained below the slide during evaluation.

Using an erlenmeyer flask, part of the rumen fluid was heated to the boiling point. By removing the Bunsen burner as soon as boiling was reached, the expanding gases were prevented from leaving the flask. Used in equal proportions, the boiled fluid was compared to raw (unboiled) rumen fluid.

Knowing about the high content of short chain fatty acids in rumen fluid (Annison and Lewis, 1959), a short trial was carried out to compare these acids with rumen fluid relative to their effects on spermatozoan activity. Since the intentions still were to get preliminary ideas about rumen fluid, only two semen collections were used. Semen was processed as before, but this time only the one and ten percent concentrations of rumen fluid were tested. Potassium salts of acetic, propionic and butyric acids were added to the yolk-citrate extender at the levels of .05, 0.5, 1.0, and 5.0 percent. The extended semen samples were scored as before but only during the first, second and fourth days. After consideration of these preliminary data, part II of this study was initiated.

Part II

Sixteen semen samples, four from each of four bulls (two Holstein and two Jersey) were used in these experiments. The collections were made during the summer 1965 following the procedure explained for part I. A set of fistulated twin Hereford steers from the O. S. U. beef barn were used as sources of rumen fluid. These animals were alternated such that rumen fluid was collected each week from a different steer. The diets of these steers varied between good grass hay to alfalfa hay. A brownish color was observed in the collected rumen fluid when the animal was under grass hay and a greenish color was present when the animal was fed alfalfa hay. The pH of the fresh rumen fluid varied between 6. 8 to 6. 9. Only fresh fluid was used in this experiment.

The rumen fluid was taken from the animal by inserting one hand through the fistula, grasping a portion of the contents and squeezing the fluid into an erlenmeyer flask. Using hard paper towel, the mouth of the flask was protected from the entrance of other foreign substances. The fluid was still warm when brough to the laboratory.

When required, the fluid was filtered using a piece of clean cloth, then divided into two portions. One of these portions was boiled as in part I and cooled by inserting the flask into cool water.

Raw and boiled rumen fluid were then added to previously made yolk-citrate extender at the levels of one-half, one, and five percent. Each one of these new extenders was further divided in two parts and one of these parts treated with 500 I. U. of penicillin and 500 micrograms of dihydrostreptomycin as in part I. A portion of yolk-citrate was left without rumen fluid and without antibiotics to be used as a control. The 13 extenders were left at room temperature in the laboratory in graduated 100-mililiter cylinders while the semen was being collected. Total collection and transport time of the semen to the laboratory ranged from 30 to 45 minutes.

A microscopic evaluation of the fresh semen from each bull was made immediately after collection. The evaluation consisted of a 0-10 score for minimum and maximum motility and concentration. The rating was made under 100 magnification. Due to unpredicted bull behavior or semen losses, it took about eight weeks to obtain the required four collections from each bull. When two collections were needed to obtain sufficient semen, they were mixed and considered as one collection. The characteristics of the fresh semen used in this experiment are shown in Table 2.

Each collection was divided into 13 parts, one for each extender. The semen was extended at 1 to 20. The 13 portions were subdivided into four parts to be evaluated at days 0, 3, 6, and 9, respectively. Each of these four samples were placed in 5 ml. test

Table 2. Characteristics of the raw semen used in part II of this study. (No. in parenthesis represents the age of each bull in months, left no. in score stands for concentration, right no. stands for motility.)

Date of Collection	Holstein Bulls				Jersey Bulls			
	Comet (48)		Prescott (53)		Pioneer (62)		Milad (38)	
	ml	score	ml	score	ml	score	ml	score
6-17-65	8.0	9-10						
6-25-65	11.6*	9-8.5					11.1*	7-8.5
7-2-65	4.5	9-10	7.2	5-10	8.7*	8.5-10		
7-8-65	6.0	7-10					5.8	7-10
7-13-65			7.7	8-10			7.6*	9-7.5
7-20-65			7.2	6-10	4.2	9-10	6.0	10-8
7-29-65			5.6	8-9	6.5*	8-10		
8-4-65					10.6*	8.5-9.5		
Averages	7.5	8.5-9.6	6.9	6.8-9.8	7.5	8.5-9.9	7.6	8.2-8.5

*Two collections

tubes (vials). Three milliliters of extended semen were placed in each vial. All vials were corked. The first set of samples was scored immediately after being extended and the remaining 39 samples were stored at 4°C. Since only one person worked in the processing, it required between two and three hours from collection till the semen was ready for storage. The longest processing time occurred when semen from all bulls was made available. Overall handling of the semen during processing or during evaluation was done at random within treatments.

Statistical analysis of the results was made using a split-plot design (Snedecor, 1956; Cochran and Cox, 1957) with treatments as main plots and periods as subplots. Separate analyses were made for alive motile and net effect (motile + alive x 1/2).

Part III

This experiment was designed to determine the effect of argon and oxygen gases upon life and motility of bull semen extended with different amounts of rumen fluid. Eight collections of semen from five dairy bulls were used. Rumen fluid was obtained in the same manner and from the same animals as in part II. Raw fluid was added to the yolk-citrate extender at the levels of 0, 0.1, 1.0, and 5.0 percent. Antibiotics and boiling were not used.

Preparing of extenders, collecting and extending of semen

were made as described in part II. The vials of extended semen were divided into three portions. One portion was placed in a chamber for argon gas, the other in a chamber for oxygen gas and the third portion was kept for control. Argon and oxygen gas pressure at the rate of 22 p. s. i. (pounds per square inch) was applied to the open vials during an hour after which the vials were corked and sealed with parafin for storage at 4^{o}C. Both gases were applied in aluminum chambers of a cylindrical type, with a cover which could be tightly sealed. Gas supply came from cylinders where both argon and oxygen are stored at about 2,000 p. s. i. Rubber tubing connected the inlet-outlet valves between cylinders and chambers. The control samples were kept at room temperature while the other samples were in the gas chambers. These control samples were also sealed with parafin.

Evaluation of the samples for "dead-alive" sperm and progressive motility was made during the second day of storage (day 1) and days 5, 9, and 14. As in the previous parts of this study, different samples were used each time the semen was evaluated.

Motility scores were given by the same technique used previously in this study. Alive percentages were determined using a modified Blom's differential stain (Swanson and Beardon, 1951). The stain was prepared by dissolving 1 gram of eosin and 5 grams of nigrosin in 100 mililiters of 3 percent sodium chloride. To prepare

the slide, a drop of stain was placed on a clean slide, a small drop of extended semen added, mixed thoroughly, pressed against another slide, separated, and dried on a warm plate (150°C.). Two-hundred sperms were counted on each slide under oil immersion at 970 magnification. Those sperms which did not take the stain were considered alive. An Adams laboratory counter was used to keep score of dead, alive, and total during the microscopic count.

The results were analyzed statistically by the split-plot procedure as in part II with eight repetitions (collections), twelve treatments and four periods. Different analyses were made for alive and motility.

RESULTS AND DISCUSSION

Part I

Tolerance of Spermatozoa to Rumen Fluid

Data showing the tolerance of spermatozoa to rumen fluid are shown in Table 3. Four collections of semen were used; but since the data were collected by a trial and error technique, the figures given represent averages from two to four observations. The beneficial effect of rumen fluid for extending the life of the spermatozoa decreased proportionately with the amount used. Maximum spermatozoan activity was observed when the semen was extended in 100 percent rumen fluid. However, due perhaps to acidity and other unknown factors, rumen fluid alone did not support the life of the spermatozoa beyond a few hours. Comparing the scores from one to fourteen days, the one percent level gave the best results, while the ten percent was approximately equivalent to the control.

Rumen Fluid in Comparison to Fatty Acids

The potassium salts of acetic, propionic and butyric acids were studied at the levels of .05, 0.5, 1.0, and 5.0 percent and compared with 1 and 10 percent rumen fluid. The results showed that all three acids activated the spermatozoa. These results were

Table 3. Scores for alive and motile spermatozoa under different concentrations of rumen fluid and stored up to 14 days. (Left score stands for alive, right score stands for motility.)

Day	Percent Rumen Fluid								
	0	1	10	20	25	50	75	100	Ave.
0	7-8	7-9	7-9	7-9	7-10	7-10	7-10	8-10	7.1-9.4
3	6-7	6-8	6-8	6-9	7-8	6-8	6-1	0-0	5.4-6.1
5	5-6	7-8	7-7	6-7	7-8	6-5	4-2	0-0	5.2-5.4
7	5-5	6-5	5-5	5-4	6-8	3-4	8-1	0-0	4.8-4.0
9	6-5	6-9	5-6	5-6	7-6	2-3	1-1	0-0	4.1-4.6
11	5-3	5-5	6-5	3-2	5-4	1-1	0-0	0-0	3.1-2.5
14	4-4	5-5	9-4	2-1	3-1	1-1	0-0	0-0	2.4-2.0
Ave.	5.3-5.4	6.0-7.0	5.9-6.6	4.9-5.4	6.0-6.4	3.7-4.6	3.7-2.1	1.1-1.4	

somewhat consistent with the literature (Mann, 1954). Since all three acids behaved in a similar way, the results were combined and shown in Table 4. The five percent level of fatty acids showed a detrimental effect on extended semen stored more than two days. For the same length of time, rumen fluid at one or ten percent maintained the semen in good condition. It was concluded from these preliminary results that the effect of rumen fluid might be due in part to its VFA content, but that there was something else contributing to the beneficial effect of rumen fluid. The action of some substance produced by some kind of rumen microorganism was suspected.

Effect of Antibiotics

It was noticed in this preliminary work that when 500 I.U. of penicillin and 500 micrograms of dehydrostreptomycin per ml. of extender were used, the spermatozoa did not survive as well as when no antibiotics were used. The effect of antibiotics has been further investigated in part II of this study and, therefore, not much is said at this point.

Part II

A total of 832 observations for alive and 832 for motility were made to determine the effect of three levels of boiled or raw rumen fluid, with or without antibiotics, on semen stored up to nine days

Table 4. Rating of semen stored up to four days under different levels of fatty acids and rumen fluid. (Left number in score stands for alive, right number stands for motility.)

Treatment	% Used	Rating			Ave.
		Day 0	Day 2	Day 4	
Volatile fatty acids	.05	8-10	6-9	5-7	6-9
	0.5	8-10	6-9	5-7	6-9
	1.0	7-10	6-8	5-5	6-8
	5.0	6-10	5-6	3-3	5-6
Average		7-10	6-8	4-5	6-8
Rumen fluid	1.0	7-9	7-9	7-9	7-9
	10.0	7-10	7-9	7-7	7-9
Average		7-10	7-9	7-8	7-9
Control		6-8	6-8	4-5	5-7

at 4°C. The averages of four collections from each of four bulls are shown in Table 5. The analyses of variance made on the original data are shown in Tables A to C of the Appendix. These analyses showed highly significant differences for bulls and periods (linearity) for both motile and alive spermatozoa ($p < .0001$).

The effect of rumen fluid (all levels combined) is shown in Table 6. Although the analyses were made on the actual scores (0-10) the following tables are expressed in percentages for easy interpretation.

Table 5. Averages for alive and motility scores of the 13 treatments in part II of this study. (Each figure comes from 16 observations.)

Treatment*	Alive					Motility				
	Day 0	Day 3	Day 6	Day 9	Ave.	Day 0	Day 3	Day 6	Day 9	Ave.
RT 1/2%	7.0	5.6	4.8	4.1	5.38	9.8	8.4	6.2	4.8	7.30
1%	6.9	5.2	4.8	3.9	5.20	9.9	8.2	6.1	4.1	7.08
5%	6.4	5.1	4.3	3.6	4.85	9.8	7.8	5.7	3.8	6.78
RU 1/2%	6.9	5.9	4.6	3.8	5.30	9.9	8.7	7.6	6.2	8.10
1%	7.0	5.7	4.6	3.9	5.30	9.8	8.8	7.7	7.3	8.40
5%	6.8	5.2	3.8	3.1	4.72	9.9	9.1	7.8	6.2	8.25
BT 1/2%	6.8	5.4	4.4	4.2	5.20	9.7	8.1	6.8	5.2	7.45
1%	7.2	5.6	4.6	4.2	5.40	9.9	7.8	6.2	4.7	7.15
5%	6.3	5.4	4.5	3.5	4.92	9.9	8.1	5.4	3.8	6.80
BU 1/2%	6.6	5.8	4.7	3.9	5.25	10	9.1	7.6	6.7	8.35
1%	6.8	5.7	4.8	3.8	5.28	9.9	9.1	8.1	6.9	8.50
5%	7.0	5.3	3.9	3.1	4.82	9.9	8.9	7.6	6.6	8.25
Control	7.2	5.2	4.0	3.2	4.90	9.9	8.4	5.5	4.1	6.98

*RT = Raw treated (antibiotics)
RU = Raw untreated
BT = Boiled treated
BU = Boiled untreated

The Overall Effect of Rumen Fluid

A review of the literature failed to show any work or give any ideas which would help to interpret the positive results shown in Tables 6 and 7. The recognition of fructose as being the sugar of semen and glycolysis as the process which supplies most of the energy for motility is well established (Mann, 1945, 1954). The utilization of other sources of energy besides fructose by the spermatozoa of different species is known also (Mann, 1954). Among these substances the utilization of organic acids has been investigated to a small extent (Terner, 1960; Scott, White and Annison, 1962). Evidence for the oxidation of acetic acid by bull spermatozoa was first given by Lardy and Phillips (1944). Oxidation of acetate in preference to glucose by ram and bull spermatozoa has been reported (Scott, White and Annison; 1962), although opposite results are reported for human spermatozoa (Terner, 1960).

Work in this laboratory (Wolberg and Lobo, 1964) shows that short chain volatile fatty acids used at levels below one percent improve motility in bull semen up to nine days in storage. Somewhat similar results for bull spermatozoa plus the stimulation of O_2 uptake by acetate have also been reported (Flipse, 1960; Scott et al, 1961). Evidence for the presence of some of these organic acids in bovine semen and in particular acetic and formic acids has been reported

Table 6. Effect of rumen fluid on alive spermatozoa. (Data represents combined averages for 0.5, 1 and 5 percent rumen fluid.)

Treatment	% Alive				
	Day 0	Day 3	Day 6	Day 9	Ave.
Raw treated	69.8	53.5	46.5	38.3	52.0
Raw untreated	68.8	56.0	43.5	36.2	51.1
Boiled treated	67.9	54.4	45.0	39.8	51.8
Boiled untreated	67.9	55.8	44.4	36.0	51.0
Control	71.9	51.9	40.0	32.5	49.1

Table 7. Effect of rumen fluid on progressive motility. (Data represents combined averages for 0.5, 1 and 5 percent rumen fluid.)

Treatment	% Motile				
	Day 0	Day 3	Day 6	Day 9	Ave.
Raw treated	98.3	81.5	60.0	42.1	70.5
Raw untreated	98.8	88.3	76.9	65.8	82.4
Boiled treated	98.3	79.8	61.2	45.6	71.2
Boiled untreated	99.4	90.0	77.5	67.1	83.5
Control	98.8	83.8	55.0	41.2	69.7

(Flipse and Porter, 1955; Scott, White and Annison, 1961; Ramsey et al, 1963). Based on these reports, it is possible that the results of the present study may be in part due to the fatty acid content in rumen fluid. A somewhat unknown factor is suspected, however.

The Effect of Different Levels of Rumen Fluid

The effect of different levels of rumen fluid upon the life and motility of the spermatozoa is shown in Tables 8 and 9. These tables show that the five percent level of rumen fluid had some detrimental effect on the life ($p < .0001$) and to a less degree on motility of the spermatozoa. There were no differences between the 0.5 and 1 percent levels of rumen fluid in this experiment. There was a highly significant difference between control and combined treatments as far as motility ($p = .01$) but due to the detrimental effect of the five percent level of rumen fluid, this difference was not significant for alive spermatozoa. Excluding the five percent level of rumen fluid, both differences are highly significant. It was, therefore, concluded that the effect of rumen fluid in this experiment gave its maximum benefit at levels 0.5 and 1 percent.

Effect of Boiling and Antibiotics

Although the preliminary study seemed to indicate different effects when rumen fluid was boiled, it was shown in this experiment

Table 8. Overall effect of different levels of rumen fluid on alive spermatozoa. (Averages of all treatments.)

% Rumen Fluid	% Alive				
	Day 0	Day 3	Day 6	Day 9	Ave.
0.5	68.3	56.6	46.2	40.2	52.8
1.0	69.7	55.3	47.0	39.5	52.8
5.0	66.4	53.0	41.2	33.1	48.4
Control	71.9	51.9	40.0	32.5	49.1

Table 9. Overall effect of different levels of rumen fluid on progressive motility. (Average of all treatments.)

% Rumen Fluid	% Motile				
	Day 0	Day 3	Day 6	Day 9	Ave.
0.5	98.4	85.6	70.5	57.3	78.8
1.0	98.9	84.7	70.2	57.3	77.8
5.0	98.8	84.4	66.1	50.8	75.0
Control	98.8	83.8	55.0	41.2	69.7

that it made no difference whether the rumen fluid was boiled or used raw. The results are shown in Tables 10 and 11. These results seem to indicate that whatever substance is beneficial to the spermatozoa, it is not destroyed by boiling.

A definite detrimental effect on motility is shown in Table 11, when 500 I. U. of penicillin and 500 micrograms of dihydrostreptomycin were used per ml. of extender. Whether the negative results were due to the effect of antibiotics on the extender used or on the spermatozoa directly is not known.

The effect of penicillin upon microorganism in the rumen has been studied to a small degree (Mangan, Johns and Bailey, 1959; Bryant et al, 1960; Bryant, Robinson and Lindahl, 1961). These studies have been concerned with the control of bloat producing bacteria. Bryant et al (1960) reports significant effects from penicillin against facultative streptococci in the rumen. As far as toxicity of antibiotics to the spermatozoa, Sykes and Mixner (1951) found no toxic effects at levels eight times larger than the levels used in the present experiment. The fact that antibiotics were detrimental to motility whether used with boiled or with raw rumen fluid suggests that the harmful effect may have been in part directly affecting the spermatozoa rather than on the microflora of the extender used.

Table 10. Effect of raw versus boiled rumen fluid, and antibiotics versus no antibiotics, on alive spermatozoa. (Averages from all observations.)

Treatment	% Alive				
	Day 0	Day 3	Day 6	Day 9	Ave.
Raw	69.8	54.8	45.0	37.2	51.6
Boiled	67.9	55.1	44.7	37.9	51.4
Treated	68.8	54.0	45.4	39.0	51.8
Untreated	68.4	55.9	44.0	36.1	51.1

Table 11. Effect of raw versus boiled rumen fluid, and antibiotics versus no antibiotics on progressive motility. (Averages from all observations.)

Treatment	% Motile				
	Day 0	Day 3	Day 6	Day 9	Ave.
Raw	98.5	84.9	68.4	54.0	76.4
Boiled	98.8	84.9	69.2	56.4	77.3
Treated	98.3	80.6	60.6	43.8	70.8
Untreated	91.1	89.2	77.2	66.4	83.0

The Net-Effect

In as much as a linear relationship exists between alive and motile spermatozoa (Tables A, B, C in Appendix), a "net-effect" may be calculated by getting an average from motility and alive scores. At times and for convenience this may show the overall results more clearly. The results expressed as net-effect for the combined treatments in this experiment are shown in Table 12. It can be seen that the control semen rated consistently lower than semen under the other treatments for both alive ($p = .05$) and motile ($p = .001$) spermatozoa. As expected, there are highly significant differences between nontreated and antibiotic treated semen ($p < .0001$) and the low levels compared with the five percent level ($p = .001$) of rumen fluid.

A summary of the results in Table 12 is shown in Figure 1 (5 percent level excluded). This figure shows the separation of the results into three definite groups: control, antibiotics, and no antibiotics. Again these results indicated the possibility of the isolation of some kind of microorganism or its product that may be beneficial in preserving extended semen for longer periods of time than is possible with only yolk-citrate extender. This work suggests also the fact that bovine spermatozoa may support bacterial infection or live jointly with bacteria beyond present thinking.

Table 12. Net-effect of rumen fluid on semen stored up to 9 days. (Each figure represents the average from 32 observations.)

Treatment	% Rumen Fluid	Rating Day 0	Day 3	Day 6	Day 9	Ave.
Raw treated	0.5	83.4	71.9	55.6	44.4	63.8
	1.0	83.1	66.9	55.6	41.9	61.9
	5.0	81.6	64.7	50.6	39.7	59.2
Raw untreated	0.5	83.1	72.8	61.9	54.1	68.0
	1.0	82.5	73.4	62.2	56.9	68.7
	5.0	81.6	73.7	59.1	48.1	65.6
Boiled treated	0.5	81.3	66.9	57.2	47.5	63.2
	1.0	84.7	67.2	55.0	44.7	62.9
	5.0	82.2	68.4	52.2	38.8	60.4
Boiled untreated	0.5	82.2	73.8	61.6	52.8	67.6
	1.0	82.8	73.8	64.7	53.8	68.8
	5.0	84.1	71.6	58.8	50.0	66.1
Control		83.4	68.8	47.8	37.2	59.3

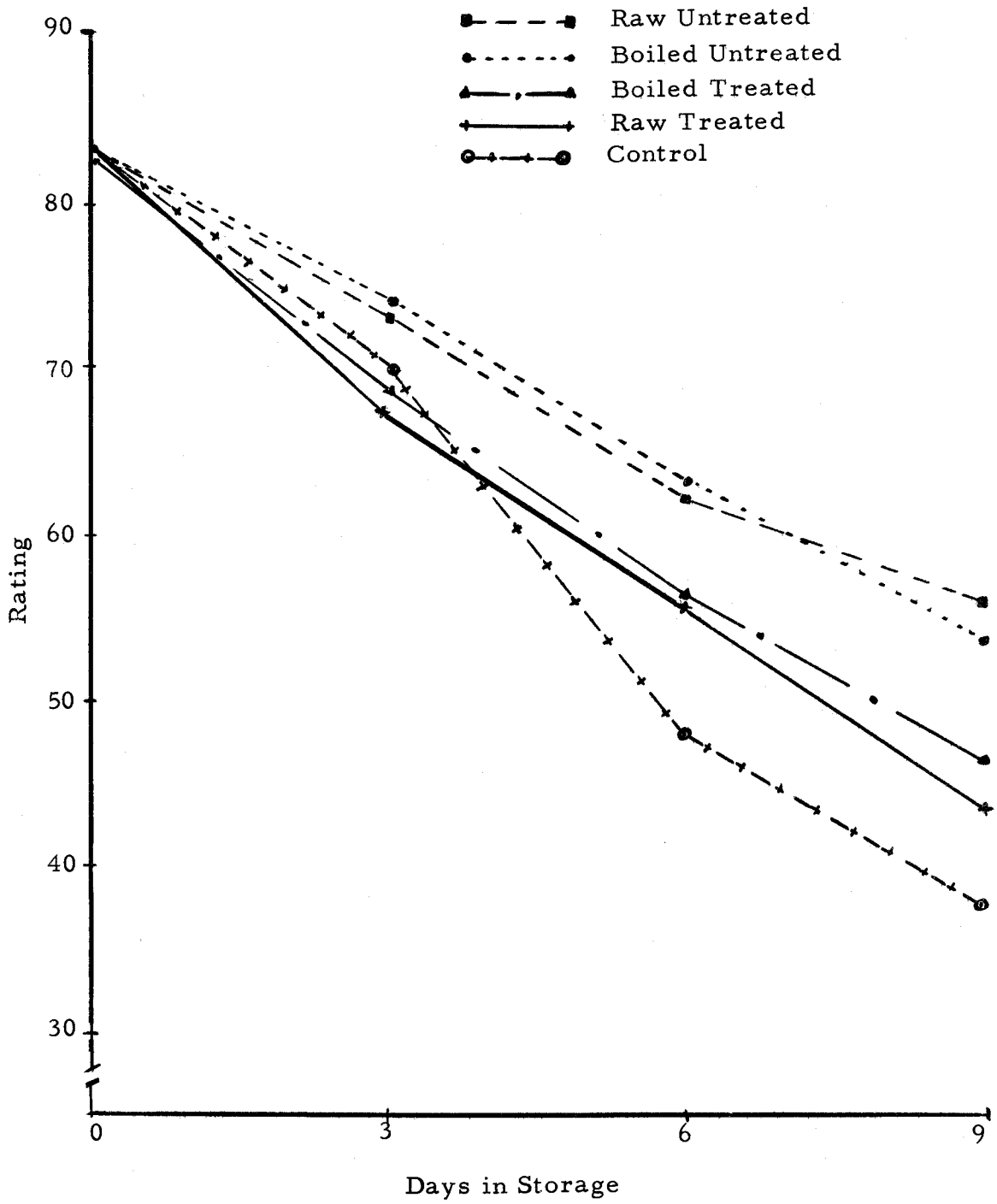

Figure 1. Net-effect of rumen fluid on semen stored up to nine days at 4°C.

Part III

A total of 768 observations were made to study the effect of argon and oxygen gases upon bull semen extended with different amounts of rumen fluid and stored up to 14 days at 4°C.

Percentages of live spermatozoa were determined by an eosin stain technique and motility by the method described in parts I and II of this study. The averages from eight replications (eight semen samples) are given in Table 13 for alive spermatozoa and Table 14 for motility. Analyses of variance (Tables D and E in Appendix) were made on the percentages of alive and on the 0-10 scores for motility. Highly significant differences were found in both alive and motility for replications and periods (both linear and quadratic) and for main treatments and gases in motility alone. A significant ($p = .05$) gas by periods interaction for alive spermatozoa shows the different behavior of the gases through the different periods of evaluation. No significant differences were observed for gases or levels of rumen fluid in the percentages of alive spermatozoa.

In regard to levels of rumen fluid, the results were somewhat similar to the results obtained in the previous experiment (Tables 15 and 16). The differences in motility ($p = .01$) are due to the higher ratings of 0.1 and 1.0 percent levels of rumen fluid. The 5 percent level rated about equal with the control. These figures show that

Table 13. Effect of oxygen and argon upon alive spermatozoa in semen extended with different amounts of rumen fluid. (Data represents the averages from eight replications.)

Treatment	% Rumen Liquid	% Alive				
		Day 1	Day 5	Day 9	Day 14	Ave.
Oxygen	0	68.8	69.5	60.5	49.7	62.1
	0.1	68.2	66.2	60.7	45.0	60.0
	1.0	73.9	63.7	58.8	46.3	60.7
	5.0	69.5	60.7	61.6	49.2	60.2
	Ave.	70.1	65.0	60.4	47.6	
Argon	0	65.5	68.2	65.5	46.4	61.3
	0.1	65.7	70.0	58.6	48.1	60.6
	1.0	61.8	69.1	65.1	43.8	60.0
	5.0	66.7	66.3	63.1	44.8	60.2
	Ave.	64.9	68.4	63.0	45.8	
Control	0	72.0	69.9	58.8	51.1	63.1
	0.1	65.2	72.2	52.6	56.0	61.5
	1.0	66.6	65.6	55.0	53.6	60.2
	5.0	69.2	64.6	64.1	52.2	63.3
	Ave.	68.2	68.1	57.6	54.2	

Table 14. Effect of oxygen and argon upon progressive motility of semen extended with different amounts of rumen fluid. (Data represents the averages from eight replications.)

Treatment	% Rumen Liquid	% Motility				
		Day 1	Day 5	Day 9	Day 14	Ave.
Oxygen	0	72.5	41.2	27.5	25.0	41.5
	0.1	82.5	53.8	42.5	28.8	51.9
	1.0	81.2	61.2	45.0	40.0	56.8
	5.0	80.0	56.2	52.5	36.2	56.2
	Ave.	79.0	53.1	41.9	32.5	
Argon	0	95.0	63.8	46.2	42.5	61.9
	0.1	90.0	67.5	61.2	46.2	66.2
	1.0	91.2	61.2	47.5	32.5	58.1
	5.0	90.0	61.2	43.8	28.8	56.0
	Ave.	91.5	63.4	49.7	37.5	
Control	0	87.2	62.5	52.5	38.8	60.3
	0.1	88.8	70.0	61.2	46.2	66.5
	1.0	95.0	71.2	68.8	43.8	69.7
	5.0	90.0	45.0	50.0	36.2	55.3
	Ave.	90.3	62.2	58.1	41.2	

Table 15. Effect of different levels of rumen fluid on alive spermatozoa in oxygen and argon treated semen. (Data represents averages from 24 observations.)

% Rumen Fluid	% Alive				
	Day 1	Day 5	Day 9	Day 14	Ave.
0	68.8	69.2	61.5	48.7	62.1
0.1	66.4	68.8	57.3	49.7	60.6
1.0	67.4	65.7	59.6	47.1	60.0
5.0	68.5	63.9	63.5	49.7	61.4
Ave.	67.8	66.9	60.5	48.8	61.0

Table 16. Effect of different levels of rumen fluid on progressive motility of oxygen and argon treated semen. (Data represents averages from 24 observations.)

% Rumen Fluid	% Motile				
	Day 1	Day 5	Day 9	Day 14	Ave.
0	86.0	55.8	42.1	35.4	54.8
0.1	87.1	59.2	55.0	40.4	60.4
1.0	89.2	64.6	53.8	39.2	61.7
5.0	86.7	54.2	48.8	33.8	55.9
Ave.	87.2	58.4	49.7	37.2	58.2

rumen fluid was beneficial even at the level of 0. 1 percent. No difference was observed between the 0, 1 and 1. 0 percent levels of rumen fluid used in this experiment. The averages shown in Tables 13 and 14 have been plotted in Figure 2 for alive and Figure 3 for motility. In regard to alive spermatozoa, the results in Figure 2 are somewhat contradictory to reports in the literature (VanDemark, Salisbury and Branton, 1949; VanDemark and Couturier, 1958). Serious oxygen damages on spermatozoan survival has been reported by these workers.

Using the same technique as was employed in the present experiment, Kliewer and Wolberg (1962) reported that argon increased the life and motility of bull spermatozoa and kept it in useful condition up to 14 days when stored at 4°C. In the present experiment, however, the results are more similar to those of more recent reports (Roussel et al, 1963; Steinbach and Foote, 1964) where argon failed to improve survival or motility of bull spermatozoa. As is shown in Figure 2, argon was better or equal to oxygen and control during the central period but failed at the extremes. These results agree, however, with the original hypothesis that if argon was to inhibit the organisms in rumen fluid, negative results should be obtained. The fact that oxygen rated on the average five percent higher during the second day of storage suggests that at least for a while oxygen could have been beneficial rather than detrimental to the life

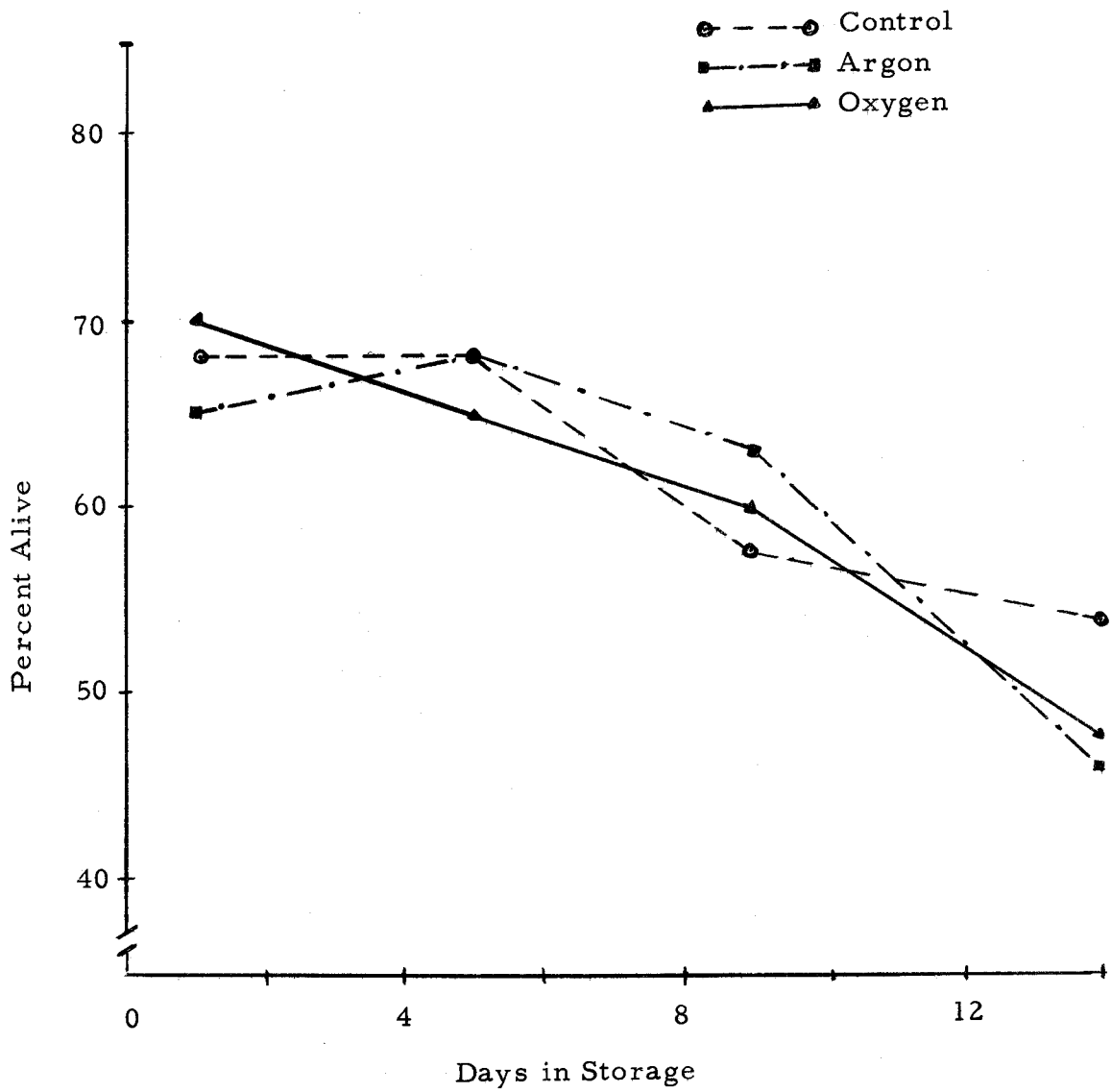

Figure 2. Effect of argon and oxygen gases on the life of spermatozoa of semen stored up to 14 days at 4°C.

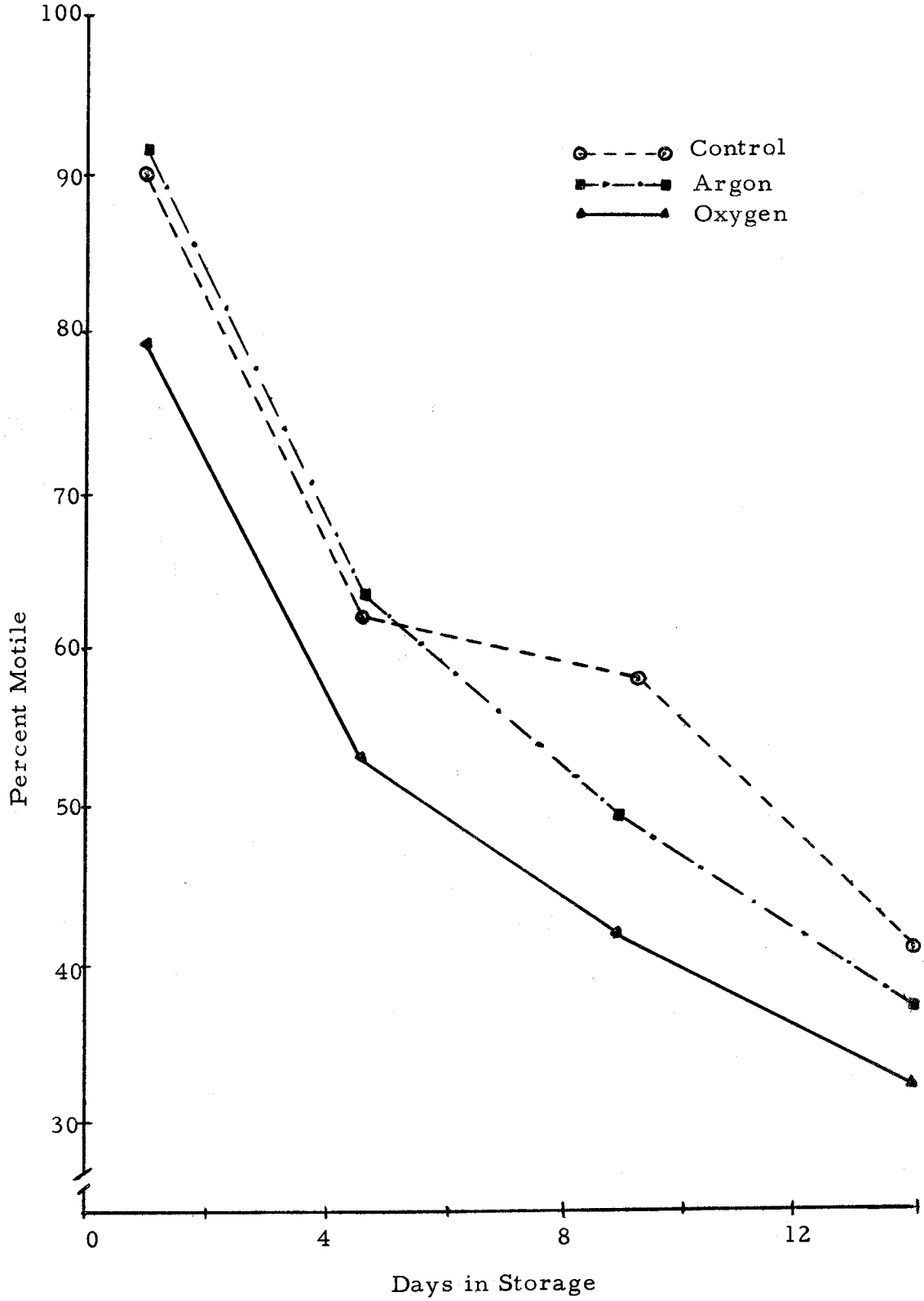

Figure 3. Effect of oxygen and argon gases on motility of spermatozoa in semen stored up to 14 days at 4°C.

of the spermatozoa. Had the replications been more uniform, these differences in treatments might have been significant. Although the differences in replications were large for all treatments, they were higher in those treatments involving oxygen. Two Holstein bulls, which in the previous experiment were quite similar in semen characteristics, under the oxygen treatments of this experiment, they differed markedly. Regardless of the level of rumen fluid, one bull showed perfect scores for motility many times even at the 9th day of rating. The other bull at times showed "0" scores even during the rating on the fifth day. These differences sometimes were also observed within the same semen sample. The author has no explanation for such drastic differences.

Except for one report (Shettles, 1940), the differences ($p < .0001$) shown in Figure 3 are consistent with the literature in respect to oxygen (VanDemark, Salisbury and Branton, 1949; VanDemark and Couturier, 1958). Argon showed a slight superiority over the control during the first two ratings, but failed at the end. These results for argon again fit the hypothesis that this gas may be inhibiting some microorganisms and, therefore, producing negative results on spermatozoan activity. As far as motility is concerned, argon showed higher ratings at the low levels of rumen fluid, oxygen at the higher levels and the control at the intermediate ones.

A comparison between Figures 2 and 3 for alive and motile

spermatozoa shows a somewhat contrasting shape. The death rate (Figure 2) progressed slowly at first, moving faster toward the 14th day. Motility, on the other hand, decreased fast during the first five days of storage, followed by a slow decline toward the 14th day. Both graphs showed highly significant quadratics ($p < .0001$). Figures 2 and 3 also show the odd behavior of the control semen, in that its decline in both alive and motile spermatozoa followed an unpredicted pattern (p for cubic in motile = .025). The superiority of the control semen specially at the 14th day rating accounts for part of this odd behavior.

The results of this experiment have shown once more that small amounts of rumen fluid are beneficial to stored semen extended in yolk-citrate. The contrasting results for argon, oxygen and control in relation to levels of rumen fluid suggest that the effect of rumen fluid is due to some kind of microorganism. Oxygen was detrimental to motility but not to survival of the spermatozoa.

SUMMARY AND CONCLUSIONS

Experiments were conducted to determine the effect of rumen fluid on the life and motility of bovine spermatozoa stored at $4^{\circ}C$.

Preliminary work showed that amounts below 10 percent of rumen fluid in the extender were beneficial in comparison to yolk-citrate alone. To obtain revealing information, 1664 observations were made on 16 collections of semen from four dairy bulls. The semen was extended in levels of 0.5, 1.0 and 5.0 percent of boiled or raw rumen fluid, treated or nontreated with 500 I.U. of penicillin and 500 micrograms of dihydrostreptomycin per ml. of extender.

Microscopic evaluations between the first and ninth day of storage showed significant differences ($p = .05$) for alive and highly significant differences ($p < .0001$) for motility for the various treatments. Highly significant differences ($p < .0001$) were observed for alive spermatozoa between the five percent and the other two levels of rumen fluid in favor of the lower levels. Control rated consistently lower than the 0.5 and 1.0 percent levels of rumen fluid and about equal to the 5 percent level. Antibiotics did not affect the life of the spermatozoa but were highly detrimental to motility ($p < .0001$).

When a net-effect was calculated from alive and motility, the results fitted into three groups: control, antibiotic treated, and non-treated semen, in that ascending order. Boiled rated slightly higher

than the raw rumen fluid but the differences were nonsignificant.

An additional 768 observations were made on eight collections of semen to determine the effect of argon and oxygen gases on semen extended with 0, 0.1, 1.0 and 5.0 percent levels of raw rumen fluid. Gas pressure at 22 p. s. i. was applied to the extended semen and microscopic evaluation made for percent alive and motility between 1 and 14 days of storage.

Oxygen decreased motility but did not affect the life of the spermatozoa. Nongased semen rated higher for both alive and motile toward the 14th day of storage. Argon and nongased semen rated about equal in motility at the 0.1 percent level of rumen fluid. The beneficial effect of argon decreased with increase in concentration of rumen fluid. Oxygen, on the other hand, rated higher in the high levels of rumen fluid.

The ninth-day ratings showed that rumen fluid at 1.0 percent or lower levels increased motility by 27 percent in comparison with control. Antibiotics decreased this figure to 11 percent. Oxygen decreased motility by 12 percent. However, oxygen at the 5 percent level of rumen fluid rated 25 percent higher than oxygen with no rumen fluid. Argon on the overall decreased motility by 8 percent.

The activity of bovine spermatozoa in stored semen can be increased by small amounts of rumen fluid. The fact that raw rumen fluid did not harm the spermatozoa illustrates the ability of the

spermatozoa to survive in the presence of large numbers of micro-organisms. The detrimental effect of antibiotics on motility and non-harmful effect of oxygen on alive spermatozoa contradicts present belief.

Since boiling did not affect the beneficial effect of rumen fluid, the use of rumen fluid seems feasible in artificial insemination. However, the possibility of isolating a microorganism from rumen fluid, beneficial in preserving semen, can not be overlooked.

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APPENDIX

Appendix Table A. Analysis of variance for spermatozoa alive (Part II).

Source of Variance	Degrees of Freedom	Mean Square	F Ratio	Probability
TOTAL	207			
Bulls	3	61.0000	35.4301	<.0001
Main plots	12	3.5017	2.0339	.05
Control vs. treatments	1	3.0683	1.7821	.25
Dilution 1 vs. 2	1	.0078	.0045	
Dilutions 1 and 2 vs. 3	1	35.1927	20.4000	<.0001
Boiled vs. raw	1	.0326	.0189	
Antibiotics	1	.3763	.2186	
Dilutions x boiled	2	.8398	.4878	
Dilutions x antibiotics	2	.1834	.1065	
Boiled x antibiotics	1	.1575	.0915	
Dilution x boiled x antibiotics	2	.5692	.3306	
Error a	36	1.7217		
Periods	3			
Linear	1	1118.4252	142.0872	<.0001
Quadratic	1	22.5589	2.8659	.1
Remainder	1	.0877	.0111	
Days x treatments	36	1.1104	.1411	
Error b	117	7.8714		

Appendix Table B. Analysis of variance for motile spermatozoa (Part II).

Source of Variance	Degrees of Freedom	Mean Square	F Ratio	Probability
TOTAL	207			
Bulls	3	124.0000	32.9323	<.0001
Main plots	12	27.7042	7.3578	<.0001
Control vs. treatments	1	30.6300	8.1348	.01
Dilution 1 vs. 2	1	.0176	.0047	
Dilutions 1 and 2 vs. 3	1	12.5588	3.3354	.1
Boiled vs. raw	1	1.7826	.4734	
Antibiotics	1	274.3242	72.8559	<.0001
Dilutions x boiled	2	.0698	.0185	
Dilutions x antibiotics	2	6.2428	1.6580	.25
Boiled x antibiotics	1	.0117	.0031	
Dilution x boiled x antibiotics	2	.2485	.0660	
Error a	36	3.7653		
Periods	3			
Linear	1	2362.0000	150.0740	<.0001
Quadratic	1	.0001	.0000	
Remainder	1	4.4458	.2825	
Days x treatments	36	5.6819	.3610	
Error b	117	15.7389		

Appendix Table C. Analysis of variance for net-effect (Part II).

Source of Variance	Degrees of Freedom	Mean Square	F Ratio	Probability
TOTAL	207			
Bulls	3	78.4767	41.4387	<.0001
Main plots	12	8.3368	4.4022	.001
Control vs. treatments	1	10.0333	5.2980	.05
Dilution 1 vs. 2	1	.0005	.0003	
Dilutions 1 and 2 vs. 3	1	21.4468	11.3247	.001
Boiled vs. raw	1	.6019	.3178	
Antibiotics	1	64.4613	34.0381	<.0001
Dilutions x boiled	2	.1288	.0680	
Dilutions x antibiotics	2	1.5058	.7951	
Boiled x antibiotics	1	.1081	.0571	
Dilution x boiled x antibiotics	2	.0602	.0318	
Error a	36	1.8938		
Periods	3			
Linear	1	1688.7380	275.3886	<.0001
Quadratic	1	4.6950	.7656	
Remainder	1	1.4069	.2294	
Days x treatments	36	1.5872	.2588	
Error b	117	6.1322		

Appendix Table D. Analysis of variance for spermatozoa alive (Part III).

Source of Variance	Degrees of Freedom	Mean Square	F Ratio	Probability
TOTAL	383			
Replicas	7	9210.6414	121.1256	<.0001
Treatments	11	48.1354	.6359	
Dilutions	3	73.9367	.9766	
Gases	2	77.4850	1.0234	.25-.50
D x G	6	25.4183	.3357	
Error a	77	75.7103		
Periods	3			
Linear	1	19319.8900	136.7934	<.0001
Quadratic	1	2832.4000	20.0546	<.0001
Remainder	1	23.9300	.1694	
Treatment x periods	33	92.1424	.6524	
D x P	9	102.5844	.7263	
G x P	6	338.5667	2.3972	.05
D x G x P	18	4.7450	.0336	
Error b	252	141.2341		

Appendix Table E. Analysis of variance for motile spermatozoa (Part III).

Source of Variance	Degrees of Freedom	Mean Square	F Ratio	Probability
TOTAL	283			
Replicas	7	289.2571	97.0564	<.0001
Treatments	11	17.8982	6.0055	<.0001
Dilutions	3	12.9600	4.3200	.01
Gases	2	45.3350	22.6675	<.0001
D x G	6	11.2217	1.8703	.10-.25
Error a	77	2.9803		
Periods	3			
Linear	1	1217.6300	369.7182	<.0001
Quadratic	1	51.7700	15.7193	<.0001
Remainder	1	21.4600	6.5161	.025
Treatment x periods	33	2.7589	.8286	
D x P	9	1.5749	.4785	
G x P	6	2.2977	.6977	
D x G x P	18	3.5603	1.0810	
Error b	252	3.2934		