JANUARY, 1953 RESEARCH BULLETIN 519

UNIVERSITY OF MISSOURI COLLEGE OF AGRICULTURE

AGRICULTURAL EXPERIMENT STATION

J. H. LONGWELL, *Director*

Viability of Bull Spermatozoa as Influenced by Various Sugars and Electrolytes in the Storage Medium

Ralph F. Kampschmidt, Dennis T. Mayer, and H. A. Herman

Animal Husbandry, Agricultural Chemistry, and

Dairy Husbandry Departments, Cooperating

Publication authorized January 28, 1953

COLUMBIA, MISSOURI

TABLE OF CONTENTS

ABSTRACT

This study is a part of an investigation of the physical, chemical and physiological requirements of mammalian spermatozoa during a prolonged period of storage at low temperatures. Semen from 12 different bulls of the Jersey, Holstein and Guernsey breeds was used.

In a series of experiments in which osmotic balance was maintained by a proportionate increase of gulcose, it was observed that as the quantity of buffer salt, Na_2CO_3, was reduced there was a concomitant increase in the survival of bull spermatozoa during the storage period. However, if the buffer salt concentration is lowered below the minimum amount necessary to maintain the pH level during a storage period, the survival of these cells is detrimentally affected. Therefore, an optimum concentration of buffer salts is necessary for maximal storage results. Similarly, the results showed that an optimum quantity of a metabolizable sugar was necessary in the diluting medium. In order to meet the osmotic requirements, either glucose or some non-metabolizable sugar may be utilized in addition to the buffer salts and metabolizable sugar. It is understood that the diluting medium must include a source of certain storage factors found in egg yolk, boiled milk, or chick embryo.

Viability of Bull Spermatozoa as Influenced by Various Sugars and Electrolytes in the Storage Medium

RALPH F. KAMPSCHMIDT[1], DENNIS T. MAYER[2], AND H. A. HERMAN
Animal Husbandry, Agricultural Chemistry, and
Dairy Husbandry Departments, Cooperating

INTRODUCTION

The spermatozoa of the boar, ram, bull and stallion are able to produce energy for life processes by three distinct metabolic pathways: respiration, aerobic glycolysis, and anaerobic glycolysis. Spermatozoan metabolism, in the presence of sugar, has been shown to be predominantly glycolytic in character by Comstock (1939), MacLeod (1941), Lardy and Phillips (1941), Moore and Mayer (1941), Henle and Zittle (1942) and Mann (1948). Thus, spermatozoa are essentially dependent upon the energy derived from the breakdown of sugar to lactic acid. Details of spermatozoan metabolic activities are adequately described in the excellent review of Mann (1949), hence need not be repeated in this report.

MacLeod (1941) stated that glucose, fructose, mannose, and maltose were utilized by human spermatozoa. Moore and Mayer (1951) studied the configuration of sugars utilized in the metabolic activities of ram, boar and stallion spermatozoa. They found that glucose, fructose and mannose, monosaccharides with identical spacial configurations of carbon atoms 3 and 4, were glycolytically converted to lactic acid. Several workers besides MacLeod had reported that maltose was utilized by spermatozoa. Moore and Mayer found that maltose was not used as a source of energy, since the spermatozoa of none of the species studied produced enzymes which could split disaccharides or polysaccharides. These investigators reported that some samples of maltose contained glucose as an impurity and did yield lactic acid when incorporated into semen diluters. However, after purification procedures to remove the glucose, maltose was not utilizable by spermatozoa.

No differences in the rate of glycolytic activities were obtained by Moore and Mayer (1951) with different buffers in the diluting media.

[1]Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2]Supported (in part) by grants from the National Health Institute, Public Health Service, and Armour and Company. This bulletin reports on Department of Animal Husbandry project number 38 entitled, "Physiology of Reproduction."

Phosphate, citrate or bicarbonate buffers did not alter the rate of glycolysis. It was shown by Bogart and Mayer (1950) that buffers composed of highly ionizable metallic salts were harmful to stallion spermatozoa. Substitution of non-electrolytes such as glucose and sucrose gave very satisfactory results in the storage of these cells. Dubincik (1934) found the injurious effect of cations to be in the following order: Fe, Zn, Ca, NH_4, K. Among the anions, citrate then iodide and chloride are the most injurious, while sulphate and tartrate appear to have a favorable effect. The presence of a small amount of salts is necessary for normal irritability according to Milovanov (1933), although pure salt solutions may have an injurious effect on viability. He states that the most favorable media are those which contain a moderate amount of electrolytes, and that the amount of electrolytes varies with the type of semen and the nature of the electrolyte used.

Kampschmidt, *et al.* (1951) were concerned in their experiments with the favorable effects of increased glucose and the possible injurious effects of certain electrolytes. They found that the replacement of increasing proportions of the buffer salts, citrate or phosphate, with a metabolizable sugar led to an improvement in storage until the concentration of the salts was so low that the buffering capacity was exceeded. By using sodium bicarbonate, an exceptionally efficient buffer, the electrolytes could be substantially reduced without causing profound effects on the buffering capacity of the medium. A new medium was developed which contained a minimum of $NaHCO_3$, as the buffer, and an increased quantity of glucose, which served as an energy source and to meet osmotic requirements. This medium was superior to a number of others with higher electrolyte concentrations in prolonging the livability of bull spermatozoa during low temperature storage.

The present investigation was designed to determine the type and concentration of buffer ions and of metabolites which would be most beneficial in diluting media during the low temperature storage of bull spermatozoa.

MATERIALS AND METHODS

The success of artificial insemination of farm animals is dependent primarily upon the efficiency of semen storage and shipping processes. The efficiency of storage or shipping methods is usually judged by some criterion of spermatozoan viability or of fertilizing capacity. Thus, one measures either the efficiency with which viability is maintained or with which the inherent fertilizing capacity of a semen specimen is sustained during a storage or shipping period. Numerous investigators have reported results showing a high correlation between the storage capacity and the fertilizing capacity of semen specimens. Nevertheless,

the authors are of the opinion that laboratory results, in which spermatozoan survival during low temperature storage is used as an evaluation technique, should be substantiated by fertility studies under field conditions. Since it is not always feasible to evaluate storage results by both methods and since the latter method is both costly and time consuming, much work of an exploratory nature must be accomplished with survival data as the sole basis of evaluation. Survival of spermatozoan motility as judged by motility rating and survival of viability as judged by a differential staining technique were utilized in the present investigation. Substantiation of the results of this investigation by fertility studies under field conditions are now in progress.

Collection and Transport to the Laboratory

Samples were collected biweekly over a 2½ year period from bulls of the University of Missouri dairy herd. Twelve different bulls of proven fertility of the Holstein, Jersey and Guernsey breeds were used.

The semen was collected by use of the artificial vagina described by Herman and Ragsdale (1939) and Lambert and McKenzie (1940). In cold weather, precautions were taken to protect the semen from temperture shock by immersing the collection tube in a large tube of warm water. Immediately after collection the samples were placed in a thermos bottle containing water at 15-20° C. The time between collection and dilution varied from one-half hour to one hour.

Dilution

The ratio of semen to diluter in all experiments was 1:10. In all experiments where whole egg yolk was used, the ratio of egg yolk to buffer and/or sugar solutions was 1:4. The buffer salts and sugars were each dissolved separately in twice-distilled water in proportions to give a solution as nearly isosmotic with semen as could be conveniently calculated. In order to attain the correct osmotic conditions the following percentages of the various salts and sugars were used: 1.3% $NaHCO_3$, 5% glucose, 10% sucrose, 0.89% Na_2CO_3, .9% NaCl, 5% galactose, 2.2% Na_2SO_4, 0.62% Li_2CO_3, 1.1% K_2CO_3 and 0.94% $(NH_4)_2CO_3$. These isosmotic solutions were then pipetted to give the desired amounts of buffer and sugar solutions in the various mixtures used as spermatozoan storage media. Therefore, in every instance where mixtures of these solutions are mentioned, it will be in terms of volume ratios of these isosmotic solutions.

Rate of Cooling and Storage

Six ml. of the diluted semen were placed in a 12 by 35 mm. storage tube. To insure a slow rate of cooling, the tubes were arranged in a metal test tube rack and placed in 4 liters of water. The large vol-

ume of water surrounding the tubes slowed the rate of cooling. The refrigerator was maintained at a temperature between 4° and 7° C. The semen samples were removed from the refrigerator at 60, 120 and 240 hour storage intervals in order to determine the livability of the spermatozoa by the evaluation techniques described below.

Evaluation of Samples During Storage

All semen samples were warmed to 37° C. prior to making the estimations of motility, thus insuring a constant temperature for each rating made. The motility was scored in thirds of a unit from 0 to 5, zero representing no motility and five the maximum.

The percentage of the spermatozoa surviving the storage period was obtained with the staining technique developed by Lasley, Easley and McKenzie (1942) and as modified by Easley, Mayer and Bogart (1942) and Mayer, Squiers, Bogart and Oloufa (1951). The stain used was composed of 2 per cent fast green and 0.8 per cent erythrosin in 100 ml. of isosmotic phosphate buffer. The slides were made immediately after dilution and at 60, 120 and 240 hour storage intervals. The percentage of the spermatozoa surviving a storage period was obtained by dividing the percentage of unstained cells at the end of a given storage period by the percentage of the cells which were unstained immediately after dilution and then multiplying by 100.

The percentage survival after cold shock was calculated in the manner described above, except that the spermatozoa were subjected to a temperature of 0° C. for 10 minutes instead of a storage period at 4-7° C. This technique was described and discussed by Lasley and Bogart (1943) and Lasley and Mayer (1944).

The pH of the samples was determined with the aid of a Beckman glass electrode pH meter.

RESULTS

The Effects of Increased Concentration of Glucose Solutions with Corresponding Decreases in Electrolytes in the Storage Medium Upon Spermatozoan Viability

Kampschmidt, *et al.* (1951) reported that increased concentrations of glucose with a proportional reduction in the citrate or phosphate salts leads to an increased survival of spermatozoa in an egg yolk medium. In addition, they reported that the electrolyte concentration of the medium could be further reduced by substituting $NaHCO_3$ as the buffer salt. Since sodium carbonate would also provide sufficient buffering capacity in much lower concentrations, its effects with various proportions of glucose solution were tested.

The sodium carbonate solution was prepared by dissolving 890 mg. of Na_2CO_3 in 100 ml. of double-distilled water. Different proportions of

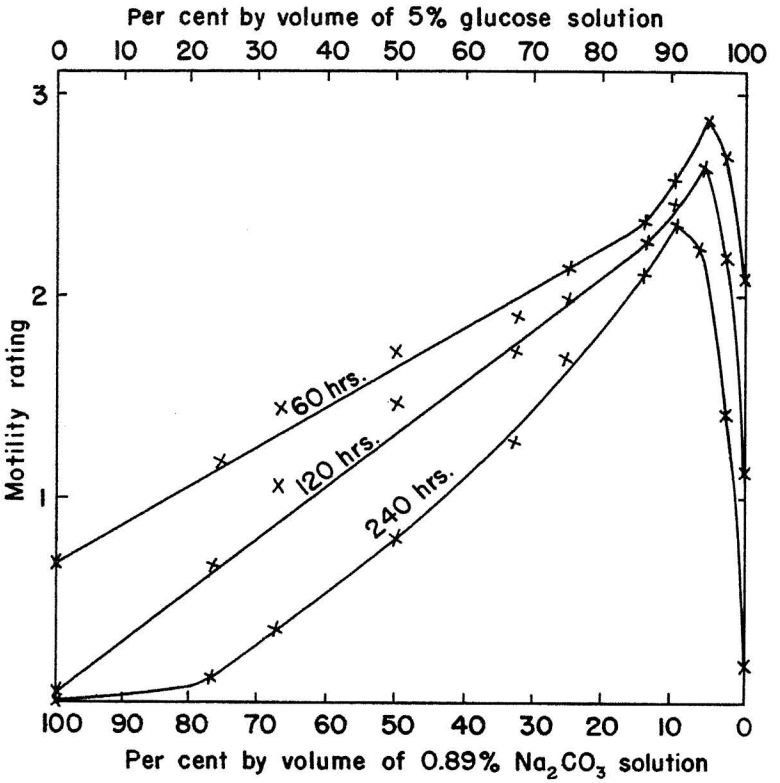
this Na_2CO_3 solution were used and in each case the solution was adjusted to a constant final volume with isosmotic glucose solution. To the carbonate-glucose solution one-fourth of a volume of egg yolk was added. Below the percentages, by volume, of isotonic Na_2CO_3 solution used in these diluters are presented; the remainder of the volume being isotonic glucose solution. The pH levels to which each of the final mixtures was adjusted with CO_2 gas are also shown.

Percentage of isotonic Na_2CO_3 solution	pH to which the carbonate-glucose solution was adjusted
100	7.1
75	7.2
66.6	7.25
50	7.3
33.3	7.35
25	7.4
14.3	7.5
10	7.6
5	7.8
2	8.0

This initial gradient in pH was used, in an endeavor to maintain a value nearer the optimum for spermatozoan storage throughout the storage period.

Results obtained with these storage media are illustrated in Graph 1. Fifteen within-sample comparisons with these mixtures are shown. There was a gradual increase in the maintenance of motility as higher percentages of glucose solution and lower percentages of carbonate solution were used. However, when the glucose solution was increased to such an extent that less than 2% of the total volume was Na_2CO_3 solution, the buffering capacity of the medium was exceeded and a rapid drop in pH occurred during a prolonged storage period. The low pH level developed during storage with very low concentrations of buffer in the medium appeared to be the major factor concerned in the rapid decrease in motility rating. In the previous experiments of Kampschmidt, *et al.* (1951) with citrate and phosphate salts, increased acidity was shown to be the cause of lowered motility. High proportions of Na_2CO_3 solution in the medium resulted in a rise in pH during storage. This was especially true with the poorer semen samples. An increase in the proportion of glucose solution in relation to buffer solution has been shown to result in an improvement in the storage of spermatozoa. This occurs regardless of the type of buffer salt used. The improvement in storage of spermatozoa under these conditions might be attributed either to: (1) an increased glucose concentration, which would make greater quantities of this metabolite available, or

(2) to the harmful effects of an increased electrolyte concentration in the medium on the spermatozoa.

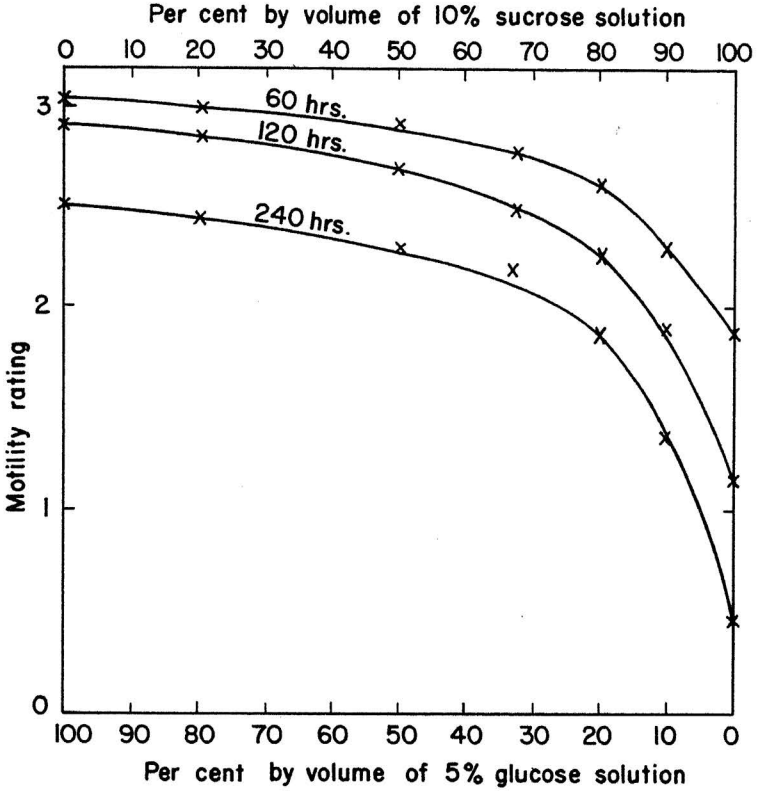


Graph 1.— The effect of increased concentrations of Na_2CO_3 solution in a Na_2CO_3-glucose-egg yolk medium upon the motility rating of bovine spermatozoa following prolonged storage.

Replacement of Part of the Glucose Solution by an Isotonic Solution of a Non-Metabolizable Sugar

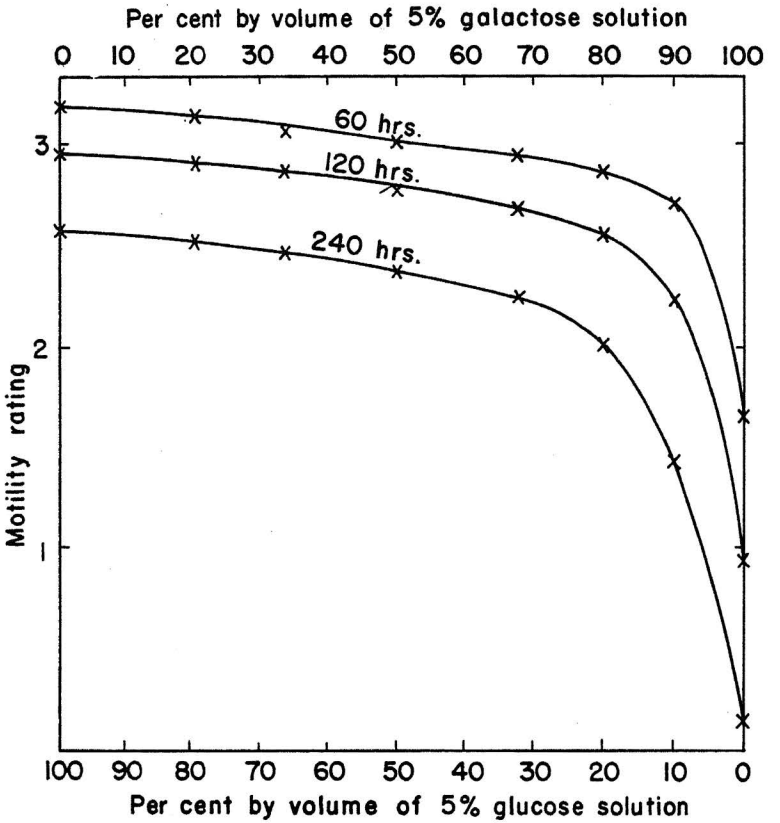
In order to establish in a more precise manner the cause of the increased survival, the following experiment was designed. A diluter used as a control was composed of one part egg yolk to four parts of a mixture of 1 volume of isosmotic $NaHCO_3$ solution plus 4 volumes of isosmotic glucose solution. In the experimental media, the glucose solution was replaced by different proportions of an isosmotic sucrose solution. The results of eighteen such comparisons are summarized in Graph 2. There was a gradual, but not pronounced, decrease in the duration of motility as the percentage of glucose solution in the diluter

was decreased. This drop in motility with decreasing glucose concentration was not rapid until the percentage of glucose solution in the diluter reached a low value. The rapid decline in motility when the 5% glucose solution comprised one-fifth or less of the total volume might be due to the utilization of all of the glucose present. Each 10% increase in the glucose solution by volume (in Graph 2) corresponds to an increase of 20 mg. of glucose in the diluter. The semen samples in these experiments had an average number of spermatozoa of 743 million.



Graph 2.— The effect upon the motility rating of spermatozoa during storage of replacing part of the glucose solution, in a medium containing 1 part egg yolk to 4 parts of a mixture of (1 $NaHCO_3$ plus 4 glucose), with isosmotic sucrose solution.

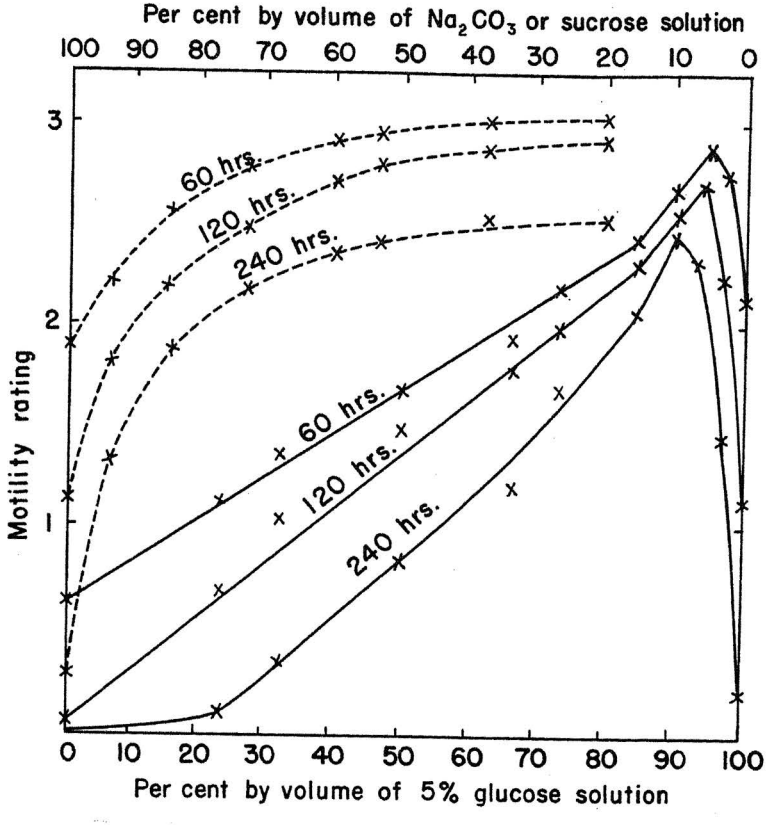
Graph 3 shows results quite similar to those of Graph 2. In this instance the 5% glucose solution was replaced with an isosmotic galactose solution. Both the galactose and sucrose are sugars which are not



Graph 3.— The effect upon the motility rating of spermatozoa during storage of replacing part of the glucose solution, in a medium containing 1 part egg yolk to 4 parts of a mixture of (1 Na_2HCO_3 plus 4 glucose), with galactose.

metabolized by spermatozoa. On the basis of these results, it is evident that some metabolizable sugar is necessary for maintaining a high level of motility, and that the sugar must be present in sufficient quantities for the maintenance of glycolytic activities throughout the storage period.

Graph 4 combines the data presented in Graphs 1 and 2. The data from Graph 2 have been recalculated to represent the per cent of glucose present in the entire diluting medium instead of the per cent of total sugar solution present as glucose solution. These two graphs cannot be compared directly since they do not represent the same group of semen samples, but the slopes of these curves may be compared. Again, from the broken line curves, it is apparent that a slight decrease in motility rating occurs as the glucose solution is replaced



Graph 4.— A comparison of the effects of the concentration of glucose and the electrolyte solutions upon the duration of spermatozoan motility during storage. (The solid lines in the graph represent results with media containing varying proportions of Na_2CO_3 and glucose solution. The broken lines represent media containing a constant 20% of $NaHCO_3$ solution and varying amounts of sucrose and glucose solution.)

with sucrose solution, but the decline in the curve is not rapid until the point is reached where very small amounts of glucose are present. If, however, the glucose solution is replaced with Na_2CO_3 solution, the decline in duration of motility is rapid. These results suggest that a small amount of glucose and buffer are important for prolonged storage, and that the electrolytes are more detrimental than sucrose as substitutes for glucose. Electrolytes, then, should be kept at a minimum in storage media for bull semen.

As a further check on the effect of a high concentration of electrolytes on spermatozoa during storage, a portion of the glucose in the standard diluter, composed of one part egg yolk to four parts of a

mixture of 1 part of isosmotic $NaHCO_3$ solution plus 4 parts of isosmotic glucose solution, was replaced with either an isosmotic solution of NaCl, Na_2SO_4, or of sucrose. In these experiments the buffering capacity should remain almost constant, whereas the concentrations of electrolytes and of glucose vary. In no experiment was the isotonic glucose solution lowered below 20 per cent by volume. The following mixtures were used to replace the glucose in the above standard medium:

80% by volume of 10% sucrose plus 20% by volume of 5% glucose
66⅔% by volume of 10% sucrose plus 33⅓% by volume of 5% glucose
50% by volume of 10% sucrose plus 50% by volume of 5% glucose
33⅓% by volume of 10% sucrose plus 66⅔% by volume of 5% glucose
20% by volume of 10% sucrose plus 80% by volume of 5% glucose
80% by volume of 0.9% NaCl plus 20% by volume of 5% glucose
66⅔% by volume of 0.9% NaCl plus 33⅓% by volume of 5% glucose
50% by volume of 0.9% NaCl plus 50% by volume of 5% glucose
33⅓% by volume of 0.9% NaCl plus 66⅔% by volume of 5% glucose
20% by volume of 0.9% NaCl plus 80% by volume of 5% glucose
80% by volume of 2.2% Na_2SO_4 plus 20% by volume of 5% glucose
66⅔% by volume of 2.2% Na_2SO_4 plus 33⅓% by volume of 5% glucose
50% by volume of 2.2% Na_2SO_4 plus 50% by volume of 5% glucose
33⅓% by volume of 2.2% Na_2SO_4 plus 66⅔% by volume of 5% glucose
20% by volume of 2.2% Na_2SO_4 plus 80% by volume of 5% glucose

Each of the above mixtures was compared in fifteen within-sample comparisons. The duration of motility of the spermatozoa after 60, 120 and 240 hours of storage is shown in Graphs 5, 6, and 7, respectively.

In this series of graphs the pH remained constant throughout and the osmotic conditions were identical at the beginning of each experiment. The per cent by volume of isosmotic glucose solution was varied from a minimum of 20 up to 100. It was possible under these standard conditions of an adequate amount of glucose, constant pH and similar osmotic conditions to compare the effects of added isosmotic electrolyte and non-electrolyte solutions. As shown in the above mixtures these comparisons were made by replacing from zero to 80% of the isosmotic glucose solution with isosmotic solutions of electrolytes (NaCl or Na_2SO_4) or non-electrolytes (sucrose). The replacement with isosmotic sucrose solution resulted in only a very slight decrease in the duration

of motility of the spermatozoa during prolonged storage. On the other hand, when isosmotic solutions of either of the electrolytes were used, there was a rapid decline in the motility of the spermatozoa during storage.

Importance of the type of cation used in the buffer salt on the viability of spermatozoa during storage. The carbonate anion has proven to be more satisfactory for spermatozoan storage than the phosphate or citrate ion because it can be used in smaller concentrations due to its superior buffering capacity. The carbonate anion was, therefore, used with various cations to check the effects of the latter upon spermatozoan viability during storage. Table 1 shows the averages of 14 within-sample comparisons using sodium, lithium, ammonium and potassium cations. Each of these carbonates was used as the buffering agent in the following medium: 1 part egg yolk to 4 of a mixture of (1 buffer to 4 glucose).

TABLE 1.— MOTILITY OF SPERMATOZOA WITH VARIOUS CARBONATE BUFFERS IN AN EGG YOLK-GLUCOSE-BUFFER MEDIUM*

Hours of storage	$NaHCO_3$	Na_2CO_3	Li_2CO_3	K_2CO_3	$(NH_4)_2CO_3$
60	2.78	2.66	1.21	2.59	2.71
120	2.71	2.61	0.64	2.54	2.59
240	2.38	2.21	0.31	2.19	2.12

*Standard error of any mean difference = .076

All of the buffer solutions were adjusted to a pH of 8 with CO_2 before being added to the medium. There were no significant differences in the pH values between these media containing the various carbonates either during or at the end of the storage period. The spermatozoa in the sodium carbonate medium were significantly lower ($P<.05$) in motility than those in the bicarbonate medium after 240 hrs. of storage. In the Li_2CO_3 medium spermatozoan motility was significantly lower ($P<.01$ than in any of the other carbonates throughout the 10-day storage period. An analysis of variance between the Na_2CO_3, K_2CO_3 and $(NH_4)_2CO_3$ showed no significant difference in the duration of spermatozoan motility in these media after 240 hours of storage. This analysis is presented in Table 2.

Two organic buffers, imidazole and tris (hydroxymethyl) amino ethane, were substituted for the $NaHCO_3$ in the following mixture: egg yolk 1 part to 4 parts of a mixture of (1 $NaHCO_3$ + 4 glucose). These organic buffers had been previously adjusted to a pH of 8 through

the use of Co_2. The averages of 14 comparisons of the motility ratings of spermatozoa during storage in these media are shown in Table 3.

TABLE 2.— ANALYSIS OF VARIANCE BETWEEN THE MOTILITY RATINGS OF SPERMATOZOA AFTER 240 HOURS OF STORAGE IN SODIUM, POTASSIUM AND AMMONIUM CARBONATE

Sources	D.F.	x_2	Mean Square
Total	41	13.3630	
Treatments	2	.0678	.0339
Samples	13	11.8006	
Treatment x Sample	26	1.4946	.0574

TABLE 3.— DURATION OF MOTILITY OF SPERMATOZOA WITH ORGANIC BUFFERS IN THE STORAGE MEDIUM*

Hours of storage	$NaHCO_3$	Imidazole	Tris (hydrozymethyl) amino ethane
60	2.91	2.76	2.51
120	2.60	2.48	2.00
240	2.27	2.06	1.75

*Standard error of any mean difference = 0.13

A small portion of the significantly lower motility after prolonged storage with organic buffers might be accounted for by failure to exactly adjust the pH with CO_2. This was demonstrated in Table 1 with $NaHCO_3$ as compared to Na_2CO_3, motility in the latter being slightly lower. Actual determinations of osmotic pressure were not made with these organic buffers. The quantities used for isosmotic solutions were calculated from available data on ionization constants. Thus, osmotic relationships may not be as exact as in the $NaHCO_3$ or Na_2CO_3 media. It is interesting that the $NaHCO_3$ buffer gave storage results superior to those obtained with the two organic buffers.

SUMMARY AND DISCUSSION

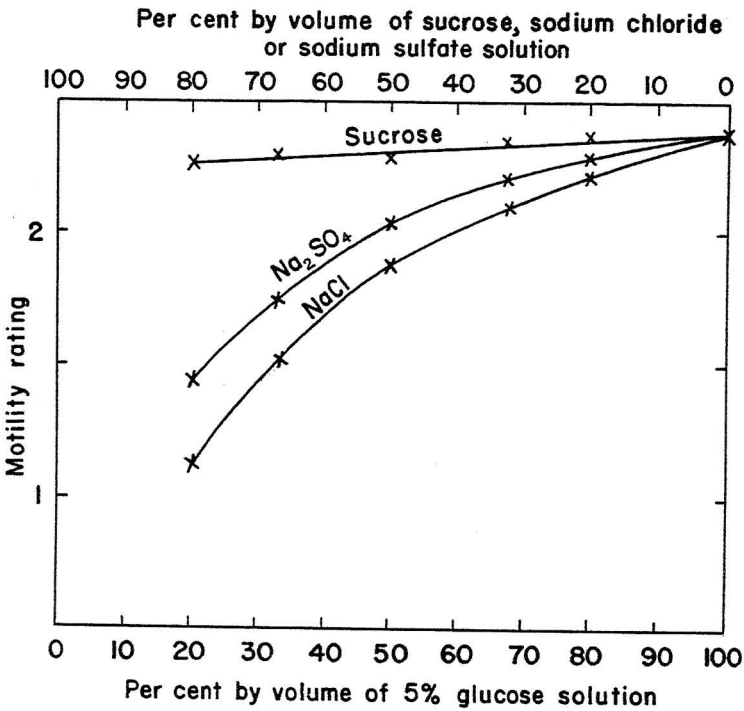
The experimental endeavors to prolong the viability of spermatozoa *in vitro* provide many intricate problems. The most successful method for the prolongation of spermatozoan viability is low temperature storage, which greatly reduces metabolic rate and makes possible the study of the effects of various substances and of physical changes in the environmental medium over rather long periods of time.

Although these cells are capable of oxidative respiratory activities, anaerobic glycolysis, or of aerobic glycolysis, previous investigations have shown that they preferably obtain energy by glycolytic activities, During shipping or storage, conditions are favorable for anaerobic

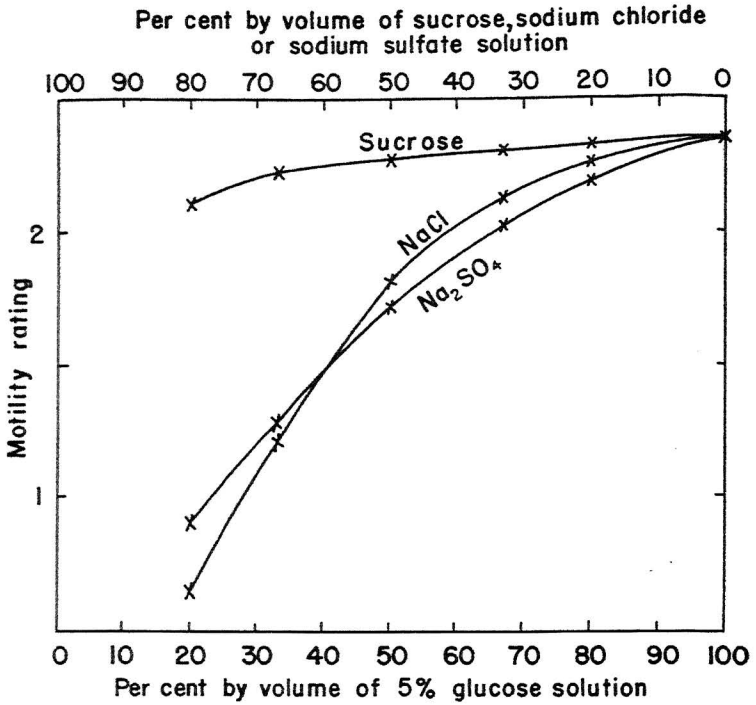
glycolytic activities utilizing the sugars of the seminal plasma or metabolizable sugar added to the diluting medium. The end product of glycolysis is lactic acid which progressively increases in concentration and progressively lowers pH. Maintenance of the pH level during storage is dependent upon buffer salts in the medium. The presence of buffer salts in the environment of spermatozoa raises problems of osmotic relations, ion concentration and buffering capacity.

In addition to buffer salts and sugars as constituents of diluting media with the concomitant problems accompanying their presence, it is generally agreed that egg yolk, boiled milk, chick embryo or factors provided by these biological materials are necessary for the successful storage of mammalian spermatozoa.

Diluting media now used in the practice of artificial insemination are yielding satisfactory results, perhaps due to the addition of the complex biological substances mentioned above. However, since we lack knowledge regarding the exact physical, chemical and physio-



Graph 5.— The effect of electrolytes in a diluter composed of 1 part egg yolk to 4 parts of a mixture of (1 part $NaHCO_3$ solution plus 4 parts of glucose and sucrose, NaCl or Na_2SO_4 solutions) on the motility of spermatozoa after 60 hours of storage.



Graph 6.— The effect of electrolytes in a diluter composed of 1 part egg yolk to 4 parts of a mixture of (1 part of $NaHCO_3$ solution plus 4 parts of glucose and sucrose, NaCl or Na_2SO_4 solutions) on the motility of spermatozoa after 120 hours of storage.

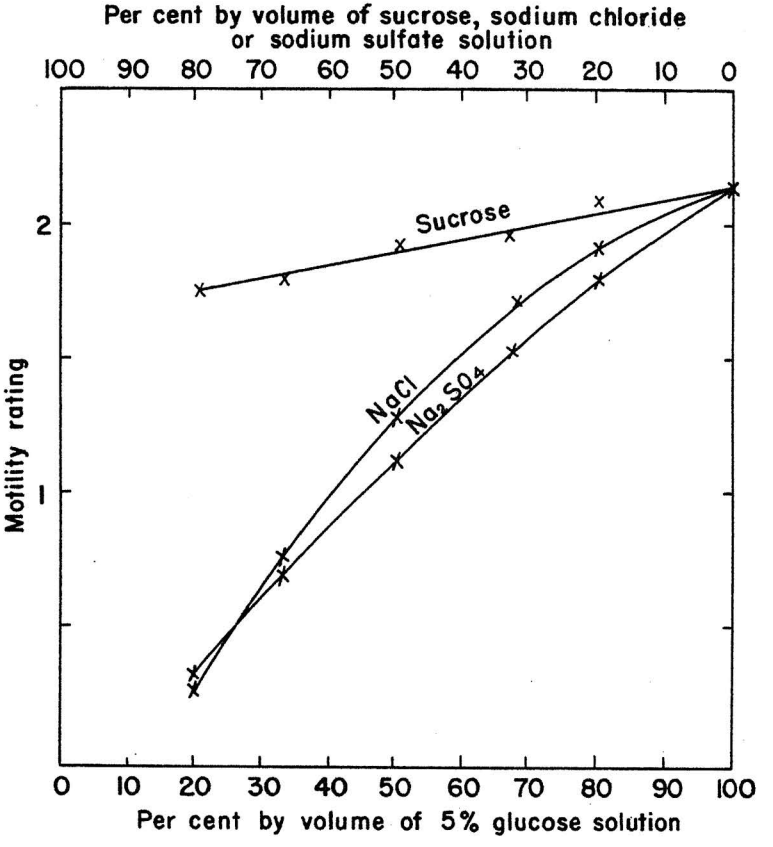
logical requirements of spermatozoa, we are not certain whether present day diluters actually meet all the requirements necessary for storage of these cells under the exacting conditions which might be provided by a hypothetical "ideal" diluter.

It is from this viewpoint that the present and similar investigations have been undertaken at the Missouri Agricultural Experiment Station.

Previous work at this station had shown that Na ions, and perhaps other ions, produced by the ionizable buffer salts in the diluting medium were detrimental to spermatozoan viability. In this investigation, the first experiments were a study of the relative effects of varying the concentrations of ionizable inorganic buffer salts, electrolytes, and the non-electrolytes, the sugars. The results of preliminary experiments showed that an increase in glucose solution accompanied by a proportionate decrease in buffer solution in the storage medium improved the storage capacity of bull spermatozoa. The results of this

series of comparisons between media with different relative amounts of a buffer salt, sodium carbonate, and a sugar, glucose, were graphically illustrated (Graph 1).

These results are somewhat unsatisfactory for deciding on a ratio of electrolytes and non-electrolytes, since it was necessary to make adjustment in the pH in order to maintain a suitable medium. There might be and undoubtedly are effects due to variations in hydrogen ion concentration in addition to variations in electrolyte concentration. It is difficult to determine whether the improved storage of spermatozoa is due to the increased glucose or the decreased electrolyte concentration.



Graph 7.— The effect of electrolytes in a diluter composed of 1 part egg yolk to 4 parts of a mixture of (1 part $NaHCO_3$ solution plus 4 parts glucose and sucrose, NaCl or Na_2SO_4 solutions) on the motility of spermatozoa after 240 hours of storage.

In order to determine whether the increased glucose, or metabolizable sugar, was an important factor, part of the glucose solution was replaced with sucrose or galactose solution. The results obtained, as shown in Graphs 2 and 3, indicate that addition of a small but metabolically adequate amount of glucose is beneficial and necessary. If the glucose is present in adequate quantities for metabolic activities osmotic equilibrium can be established with glucose, galactose or sucrose with equally satisfactory results.

It was possible from these preliminary studies to determine a close approximation of the minimum amounts of glucose, as the metabolite, and sodium bicarbonate, as the buffer, for optimum storage of spermatozoa. After the addition of these minimum amounts of glucose and $NaHCO_3$ solutions, the rest of the medium could be composed either of an electrolyte or of a non-electrolyte. Thus, comparisons could be made which would definitely show the effect of electrolytes on spermatozoan survival during storage. In order to add both electrolytes and non-electrolytes which would affect neither the buffering capacity nor the available metabolites to any appreciable extent, sucrose was chosen as the non-electrolyte while NaCl and Na_2SO_4 were used as the electrolytes. Graphs 5, 6, and 7 show only a very slight decrease in spermatozoan survival when isosmotic sucrose solution was used to make up the remainder of the medium. If isosmotic solutions of either of the electrolytes were used, a rapid decline in motility occurred with increased concentrations of these salts. Thus, with increased electrolytes the motility declines even though an optimum amount of glucose and buffer are present.

The studies on the types of cations best suited for spermatozoan storage were not entirely satisfactory, since the cations were added in very low concentrations making exact comparisons impossible. The results do show that the lithium carbonate was definitely toxic to bovine spermatozoa. This is in agreement with the work of McLeod, *et al.* (1949) who found lithium toxic to human spermatozoa.

On the assumption that some of the organic buffers might possibly give adequate buffering, without exhibiting the detrimental effects of the ionizable inorganic buffers, comparisons were made between these buffers and $NaHCO_3$. However, in the low concentrations necessary for adequate buffering, the two organic buffers used were less satisfactory than sodium bicarbonate.

In summary, it has been demonstrated that electrolytes are definitely detrimental to bull spermatozoa during a prolonged storage period. Therefore, when added to the medium as buffers salts, they should be present in the lowest concentrations adequate for the maintenance of pH during the storage or shipping period. Although the quantity of

metabolizable sugar necessary as the substrate for glycolytic activities is small, it is important that sufficient substrate be present to meet the needs of the spermatozoa throughout the entire storage or shipping period. The requirements for metabolizable sugars will vary slightly with the concentration of spermatozoa in the stored specimen of semen. The osmotic balance between spermatozoa and medium can be accomplished with non-metabolizable sugars or with glucose. In addition, the storage factor or factors can be supplied by egg yolk, boiled milk, chick embryo or certain lipoprotein complexes present in egg yolk (Kampschmidt *et al.*, 1952).

LITERATURE CITED

Bogart, Ralph and Dennis T. Mayer. 1950. *The effects of egg yolk on the various physical and chemical factors detrimental to spermatozoan viability.* J. of Animal Sci. 9:143-152.

Comstock, R. F. 1939. *A study of the mammalian sperm cell. I. Variations in the glycolytic power of spermatozoa and their relation to motility and its duration.* J. Expt. Zool. 81:147-164.

Dubincik, J. 1934. *The influence of physico-chemical factors on vitality of spermatozoa.* Ginekologija 3:79-86 (Abs.) Animal Breed. Abst. 4:256, 1936.

Easley, Glynden T., D. T. Mayer and Ralph Bogart. 1942. *Influence of diluters, rates of cooling, and storage temperature on survival of bull sperm.* Am. J. Vet. Research 3:358-363.

Henle, Gertrude and Charles Zittle. 1942. *A. Studies of the metabolism of bovine epididymal spermatozoa.* Am. J. Physiol. 136:70-78.

Herman, H. A. and A. C. Ragsdale. 1939. *Artificial insemination of dairy cows.* Mo. Agr. Expt. Sta. Bull. 407.

Kampschmidt, Ralph F., Dennis T. Mayer, Harry A. Herman and Gordon E. Dickerson. 1951. *Viability of bull spermatozoa as influenced by electrolyte concentration, buffer efficiency and added glucose in storage media.* J. Dairy Sci. 34:45-51.

Kampschmidt, Ralph F., Dennis T. Mayer and Harry Herman. 1952. *Lipid and lipoprotein constituents of egg yolk in the resistance and storage of bull spermatozoa.* (In press.)

Lambert, W. V. and Fred F. McKenzie. 1940. *Artificial insemination in livestock breeding.* Circular No. 567. U.S.D.A.

Lardy, H. A. and Paul H. Phillips. 1941. *The interrelation of oxidative and glycolytic processes as sources of energy for bull spermatozoa.* Am. J. Physiol. 133:602-609.

Lardy, H. A. and Paul H. Phillips. 1942. *The relation of certain fundamentals of sperm metabolism to the problem of semen storage for artificial insemination.* J. Animal Sci. 1:344.

Lasley, John F., G. E. Easley and Fred F. McKenzie. 1942. *A staining method for the differentiation of dead and live spermatozoa. I. Applicability to staining of ram spermatozoa.* Anat. Record 82:167-173.

Lasley, John F. and Ralph Bogart. 1943. *Some factors influencing reproductive efficiency of range cattle under artificial and natural breeding conditions.* Mo. Agr. Expt. Sta. Res. Bull. 376.

Lasley, John F. and Dennis T. Mayer. 1944. *A variable physiological factor necessary for the survival of bull spermatozoa.* J. Animal Sci. 3:121-135.

MacLeod, John. 1941. *Sperm motility and glycolysis.* Endocrinology 29:583-591.

MacLeod, John, Roy C. Swan and Gerard A. Aitken, Jr. 1949. *Lithium, its effect on human spermatozoa, rat testicular tissue and upon rats in vivo.* Am. J. Physiol. 157(2):177-183.

Mann, Thomas. 1948. *Fructose content and fructolysis in semen. I. Practical application in the evaluation of semen quality.* J. Agri. Sci. 38:323-332.

Mann, Thomas. 1949. *Metabolism of semen.* Enzymology 9:329-390.

Mayer, Dennis T., C. Dale Squiers, Ralph Bogart and Mohammed M. Oloufa. 1950. *The technique for characterizing mammalian spermatozoa as dead or living by differential staining.* J. of Animal Sci. 10:226-235.

Milovanov, V. K. 1933. *Three years work on diluters for sperm of livestock.* (Abs.) Animal Breed. Abst. 1:153.

Moore, Burton, H. and Dennis T. Mayer. 1941. *The concentration and metabolism of sugar in ram semen.* Mo. Agr. Expt. Sta. Res. Bull. 338.

Moore, Burton, H. and Dennis T. Mayer. 1951. *Characteristics of the substrate and media essential for metabolism and motility of ram, boar and stallion spermatozoa.* (In press.)

Salisbury, G. W. and N. L. VanDemark. 1945. *Stimulation of livability and glycolysis by additions of glucose to the egg yolk-citrate diluent for ejaculated bovine semen. Am. J. Physiol.* 143:692-700.