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To the Graduate Council:

I am submitting to you a thesis written by Emory Gene Morrison entitled "Physiology of Jack Semen". I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Husbandry.

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Major Professor

We have read this thesis
and recommend its acceptance:

Arthur V. Jones

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Accepted for the Council

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Dean of the Graduate School

# PHYSIOLOGY OF JACK SEMEN

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A THESIS

Submitted to
The Graduate Council
of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the degree of
Master of Science

---

by

Emory Gene Morrison

December 1951

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E. G. M.

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# CHAPTER I

## INTRODUCTION

In 1937 a project to study the problems of jack stock and mule production was initiated at the Tennessee Experiment Station. As the work progressed, it became apparent that the possibilities of using artificial insemination in mule production needed to be studied. It was envisioned that with the decline in jack stock, it might become necessary for a small number of jacks to furnish service for a large number of mares to maintain the necessary mule population for farm work. To accomplish this, artificial breeding seemed to be the only alternative.

Most of the studies pertaining to the application of artificial insemination have been restricted to the dairy industry. Dairy bull semen can be transported long distances and used successfully after days of storage. Such is not the case with jack sperm. In undiluted semen, the sperm lose their motility within a few hours and even in diluted semen they do not retain their fertilizing capacity nearly as long as do bull sperm.

The sperm is a most sensitive and delicate organism and must be placed in some media to preserve its viability. One of the many problems in artificial insemination is finding a satisfactory diluent for the semen of a particular specie. This study was initiated in an attempt to find a diluent for jack semen in which the sperm could be stored for a longer period and at the same time maintain their fertilizing potentiality.

## CHAPTER II

## REVIEW OF THE LITERATURE

Artificial insemination is not a new development. Nichols (1945) reported a claim being made that it was successfully used by an Arab chief to inseminate a mare in the 14th century. During recent years, much interest has developed in artificial insemination with advancement being rather rapid and far reaching in some species of farm animals, namely, dairy cattle. Jack semen has been used very little in artificial breeding and data concerning it is rather scanty. The semen of stallions has been more widely studied, although the picture is far from complete and does not approach the progress made with bull semen. Due to the belief that the published material on stallion semen is applicable in many ways to jack semen and since the semen of both animals seem to have very similar properties, information regarding both is included in this review.

Anderson (1945) and Mann (1949) have made excellent reviews of the literature.

According to McKenzie, et. al. (1939) stallion semen is ejaculated in three fractions: (1) a clear, watery and sperm free portion; (2) a slightly viscous, whitish portion containing a high concentration of sperm; and (3) a very viscous portion containing very few sperm. Lambert and McKenzie (1940) did not find the third fraction in jacks except in old age, and stallions did not ordinarily produce it in the autumn. Milovanov (1933), Guerreiro (1940) and Sasaki and Nishikawa (1949) reported

that the seminal fluids were detrimental to sperm motility. Gonzoga and Valenzuela (1934) maintained the life of epididymal sperm obtained from castrated horses for several days, while ejaculated sperm collected from the vagina survived for only seven to nine hours. This substantiates the theory that accessory gland secretions are detrimental to the life of sperm.

Day (1940) reported that the third fraction varied with individuals and from collection to collection. Of four thoroughbred stallions one did not produce any of this fraction. Lambert and McKenzie (1940) found that the third fraction often made up to two-thirds of the entire volume of the ejaculate. The variations in volume of the third fraction were not due to the frequency of collection in a two year old Belgian stallion used by Davis and Cole (1939). However, Parsutin (1941), in a study using ten stallions and a "Teaser", was able to increase the amount of the accessory gland secretion by over sexual excitement in the case of a "teaser" stallion and by frequent collections in the case of the other stallions. By reducing the number of collections, the "normal sires" tended to clear up. A ration containing oats and/or sprouted corn also helped.

Stallion and jack semen are somewhat different from other species in physico-chemical properties. Milovanov (1934, 1936) according to Berliner (1942) considered the low glucose content to be one of the reasons why stallion semen did not store well. Bernstein (1933), Sergin (1937), and Davis and Cole (1939) found the glucose content to be 67-116 (82 average), 0-8 (3.5 average) and 4-18 mg. percent, respectively. Bernstein (1933b) according to Anderson (1945) found an average glucose

content of about 300 mg. percent (range from less than 100 to 400 mg. percent) in 92 samples of fresh bull semen. Sugar was present in both the sperm and the semen plasma; the glucose content in the latter was very variable and that of the former somewhat more constant. The smaller the number of sperm the lower was the glucose content. The high glucose content in bull semen compared to the low content in stallion semen may be one of the reasons why bull semen has more longevity in storage than does stallion semen.

The lactic acid content of stallion semen was also found to be low (26.2 mg. percent) by Sergin (1937), and the increase within 24 hours was only 1.2 mg. percent. However, Sergin (1935) stated that boar and stallion sperm, during storage, showed a shift towards alkalinity and no lactic acid was produced. Boar and stallion sperm taken from ducts before ejaculation contained small amounts of lactic acid. Davis and Cole (1939) reported that the lactic acid content was low in stallion semen which had aged, while in bull semen according to Bernstein (1933), the concentration of lactic acid was 40-50 mg. percent and increased during storage.

Iljasov (1933) according to Anderson (1945) found the creatinine and creatine content of stallion semen to be 3.7 and 6.2 mg. percent respectively. Creatine was predominant in the semen of the stallion, but that of the bull contained less creatine than creatinine. Whole semen was much richer in creatinine than semen plasma, and sperm contained more creatinine than creatine. This would explain the difference in content of these substances in the semen of the bull and stallion, since bull semen has a much high sperm concentration.

In observations on two half-bred Brabancon stallions, Voloskova (1938) found lecithin in both sperm and seminal fluid, and cholesterol only in the former. This author reported that the cholesterol content of semen may thus serve as an index of density. Following intensive sexual activity, the lipoid level of semen decreased and a three to six day rest period was required to restore it to the normal level. The lecithin content also affected motility and survival of sperm; given a moderate sexual activity, survival was longest if the cholesterol:lecithin ratio was one.

Sergin (1935) reported that the average content of carbonic acid per 100 ml. of stallion semen was 24 ml. The osmotic pressure in terms of the depression of the freezing point as given by Bernstein and Sergin (1936) was 0.595 for stallion semen. Bogart and Mayer (1950) considered the buffering capacity of stallion semen adequate for storage and did not find it necessary to add a buffer since in no case was buffer plus egg-yolk diluent equal to the glucose egg-yolk diluent alone. However, Sergin (1935) found that the buffering capacity of semen is lower than that of blood. In the semen of the bull and ram, it is two to three times greater than in that of the boar, stallion, and rabbit. Stored sperm of the boar and stallion showed a considerable change of buffering capacity within a pH of six to eight, which suggests a destruction of the carbonate buffer. Anderson (1945) reported that stallion semen is very rich in phosphates, both organic and inorganic, however, great variations occur within animals of the same species and among different ejaculates from the same animal.

Bernstein (1933) found the ammonia content of fresh stallion semen to be low (not over 2.7 mg. percent) which is ascribed to the presence of dead sperm.

Little is known at present about the role of vitamins in sperm metabolism. Phillips, et. al. (1940) reported the presence of high levels of ascorbic acid in fresh bull semen; three to eight mg. per 100 ml. with an average of 6.19. VanDemark and Salisbury (1944) reported the thiamine, riboflavin, pantothenic acid, and niacin contents of fresh bull semen to be 0.089, 0.209, 0.371, and 0.363 mg. per 100 ml. respectively. The concentrations of all four vitamins were correlated with the number of sperm; with the exception of pantothenic acid, the vitamins determined were significantly correlated with initial sperm motility. This seemed to correspond with the metabolic activity concerned in promoting the motility of sperm. Thus, not only sperm numbers were concerned in the variation in the concentration of these three vitamins, but the relative motility of the sperm also was greater in the presence of increased quantities of thiamine, riboflavin, and niacin. Riboflavin content and niacin concentration showed a significant positive correlation, which seemed probable since they are closely related with enzyme systems in metabolism. Thiamine and riboflavin were also associated with the trend in pantothenic acid concentration.

In studying metabolism in stallion semen Nesmejanova (1939) considered the lipoid content to be the difference between whole sperm and sperm serum. This lipoid content was about 42.0 mg. percent of semen volume. During the life of the sperm the lipoids were gradually used up,

some passed into the sperm serum but a considerable part was destroyed. Apparently the destruction of the lipoids occurred through oxidation and represented a source of energy. The intensity of the process seemed to be associated with carbohydrate metabolism, notably glycogen content and rate of glycolysis.

Sergin (1939) reported that when semen was diluted so as to have the same sperm concentration, oxidation was the same for stallion, ram, bull and boar. This author also reported that undiluted sperm live longer with free access to oxygen as compared with restricted aeration. Oxygen according to Sergin (1938) penetrates only a given depth and that two layers, therefore, are present in semen. Due to sperm motility, there is a constant shifting of sperm from an aerobic to an anaerobic condition, and as the sperm age intermixing become less and less as sperm motility decrease. In experiments with different shapes and sizes of containers, Milovanov, et. al. (1939) found that undiluted sperm survive badly at all degrees of aeration, while diluted sperm with an anabiotic diluent survive inversely in proportion to aeration. This led them to recommend replacing the oxidative process with the more profitable one of reduction. Milovanov (1940) reported that under anaerobic conditions sperm can maintain for a long period not only motility but also complete fertilizing capacity. This author states that to store semen outside the organism it is best to maintain an anaerobic regime, providing the sperm with sugar and removing the surplus of accumulating hydrogen ions. According to Davis and Cole (1939) stallion semen apparently has a different type of metabolism to that of muscle or semen of other species, as shown in

its low lactic acid and glucose contents. Their preliminary results indicated that probably the source of energy is, at least indirectly, linked with protein metabolism. Ivanov (1943) reported that both respiration and glycolysis are capable of furnishing the energy for the motility of sperm cells, their relative importance depending on the species; absence of respiration and glycolysis leads rapidly to the suppression of sperm motility. Bogart and Mayer (1950) reported that studies using diluents containing glucose or sucrose alone or in combination with egg yolk seemed to indicate that the superior results obtained with glucose plus egg yolk diluent are not necessarily due to the utilization of glucose for energy production, since sucrose gave comparable results, but that the beneficial effects of both glucose and sucrose are probably due to their lack of ionization in solution.

Lardy, et. al. (1949) found a metabolic regulator to be present in bull semen. It was observed that epididymal sperm had an appreciably lower rate of endogenous respiration than ejaculated sperm, and also that glycolysis was more rapid under anaerobic conditions in epididymal sperm. These authors found a material that was bound in the epididymal sperm and which was liberated in an active form soon after ejaculation. If a small amount of this substance was added to epididymal sperm, an increased rate of respiration and aerobic glycolysis occurred. They reasoned that the regulator stimulates cellular respiration and aerobic glycolysis while preventing utilization of energy.

Sergin (1935) found the pH of fresh stallion semen to be 7.23 and a shift toward alkalinity occurred upon storage. He attributed this

shift to the loss of $CO_2$ into the atmosphere, absence of lactic acid formation, and the formation of ammonia. Davis and Cole (1939) found a definite increase in pH of undiluted semen with lowered temperature, especially from body temperature to room temperature and to a lesser extent to storage temperature of 15° or lower. They found the average increase in pH ranged from 0.6 to 1.2 units. Gonzaga and Valenzuela (1934), in studies on ejaculated semen obtained directly as a residual emission after the stallion had served a mare and semen obtained from the epididymis of castrated stallions, found the pH to be 7.3 and 7.9 respectively. Guerreiro (1940) reported that the pH of stallion semen obtained from an artificial vagina to be 7.5 to 7.6. The serum alone had a pH of 7.75. Donkey sperm had a pH of 7.9 to 8.0, the serum alone being 0.2 higher when similarly collected. Soliven and Gonzaga (1940) stated that a decrease in percent of motile stallion sperm was always accompanied by a decrease in pH, complete loss of motility occurring at pH 4.6 to 4.7, even with storage at a very low temperature.

Gladcinova (1937) found stallion semen very sensitive to temperature shock. Bogart and Mayer (1950) used this fact in a study of the "resistance factor" of egg yolk. They found that egg yolk tended to reduce the sensitivity of stallion sperm to cold shock. Skatkin (1940) found that the harmful effects of temperature were due to the rate of decline in temperature rather than temperature itself. Nishikawa and Sugie (1949) reported that the maximum and minimum temperatures at which stallion sperm can survive are 54° to 56° C. and -17° to -18° C., respectively. Optimum storage temperature appeared to be 2° to 4° C. and

the cooling process should be extended over a period of 30 to 40 minutes. Milovanov, et. al. (1939) found that temperature did not influence survival in undiluted sperm, but on dilution reduced temperature has a favorable effect, viz. at eight to ten degrees survival is twice as long as at 20°. Further reduction of temperature to three to six degrees had no effect. Chang (1943) reported that storage of diluted pony stallion semen at 1° C. for 24 hours was little superior to storage at 10° C. Buiko-Rogalevic (1949) stated that motility was maximal when diluted stallion semen was stored at 0° C., and decreased progressively when the semen was stored at 5° and 10° C. Skatkin (1940) concluded that the divergence of opinion of various authors as regards the optimal temperature for storage of stallion sperm is due to the different methods of cooling.

Milovanov, et. al. (1939) reported that undiluted stallion sperm usually perish in three to four hours. Parsutin (1940) found a direct correlation between sperm survival in vitro and its fertility. Therefore, a diluent for stallion and jack semen is necessary to store and maintain the fertility of the sperm. Milovanov (1934, 1936) as reported by Berliner (1942) suggested several dilutors for all species of farm animals that were made up according to the physiological and chemical properties of the different semen types. The problem for stallion semen was to induce anabiosis, supplement the low glucose content and to strengthen the weak protective sperm capsule. To accomplish this, Milovanov combined glucose with sodium sulfate and a small amount of peptone in one series (SGL) and glucose with potassium sodium tartrate and peptone in another series (TGL). The TGL dilutor was later modified by adding

small amounts of tartaric and tannic acid to hasten the onset of anabiosis. This dilutor, TGL-5, gave good storage results and sperm were maintained up to five days. Milovanov, et. al. (1939) modified the tartrate dilutor again and when used in a 1:3 ratio it gave 11 to 12 fold increase in survival time as compared to undiluted semen.

Milovanov, et. al. (1939) concluded that with proper dilutors it was possible to preserve the fertilizing capacity of stallion semen for a period of time comparable with other farm animals.

Gonzaga and Valenzuela (1934) reported that the longest period of survival of stallion semen (96 hours at 0 - 2° C.) was obtained in a glucose-peptone-tartrate solution (pH 7.20), 90 percent of the spermatozoa being very active on the second day, 50 percent on the third, and 15 percent on the fourth. In a glucose-sulfate-peptone dilutor (SGH-2), the spermatozca survived for 30-48 hours at 0-2° C. and 7-10° C. Both of these diluents were more effective than phosphate diluent, buffer with five percent dextrose, Locke's solution, Ringer's solution, glucose-phosphate or Tyrode solution.

Berliner, et. al. (1938) using Milovanov's TGL-5 (glucose 64.8 g., K-Na tartrate 3.38 g., tannin 0.02 g., tartaric acid 0.1 g., peptone 2.0 g. dissolved in 1000 ml. of distilled water and filtered) with fresh stallion semen in a ratio of 1:1 had practically 95 percent active motility at 48 hours and 50 percent at 120 hours when reheated to body temperature. Rumjancevo (1940) found a glucose dilutor (7 or 7.2 g. of anhydrous glucose in 100 ml. of distilled water), when added in a 1:3 ratio to jack sperm, was found to be more effective in preserving the

viability of sperm in vitro than either the sulphate or tartrate dilutors. Parsutin (1940) and Nishikawa and Sasaki (1946) reported that the optimum concentration of the glucose solution for stallion sperm survival in vitro was 7.0 and 5.0 to 6.0 percent, respectively. Sergin and Gladcinova (1938) concluded that their best diluent is one composed of glucose 5.80 g. and anhydrous sodium sulphate 0.34 g. per 100 ml. of distilled water. Sasaki and Nishikawa (1949) obtained fairly good results with 5.25 percent glucose solution and ten percent cane sugar solution, and with glucose solutions buffered with phosphate, citrate, sulphate buffers or egg yolk extract. Mihailov (1949) diluted stallion semen with boiled milk from the mare, sheep, cow, and goat and obtained a sperm survival time of 156, 132, 108, and 108 hours, respectively; the optimum dilution with mare's milk was 1:4 to 1:25 and with sheep's, cow's, and goat's milk was 1:3 to 1:8. Soliven and Gonzaga (1940) collected semen at the Alabang Stock Farm, diluted with the Russian tartrate and sulfate dilutors with various proteins, protein derivatives, and substances containing protein being added, and transported it to Manila in ice boxes where it was stored at 2 to $12^{0}$ C. and microscopically examined daily. All sperm were generally found to be dead within 24 hours in the tartrate and sulfate diluents and none of the protein substances had any effect except forms of lecithin and water soluble extract from the yolk of hen or duck eggs. Vitamins A, D, B, and C added in combination were favorable but no significant influence on sperm longevity was noted when any of these were added singly.

Lardy and Phillips (1939) reported that the yolk buffer (equal volumes of fresh egg yolk and sterilized phosphate buffer) developed for preservation of bull sperm was likewise effective in preserving sperm of other species. Stallion sperm had a motility of three (with five point scale) at 24 hours, two at 48-72 and 96 hours, and one at 150-200 hours. Guerreiro (1940), using an egg yolk medium, obtained a survival time of 66 hours.

Berliner (1942), in a comparison of dilutors, found the addition of egg yolk to the Russian dilutor TGL (glucose 5.76 g., K-Na tartrate 0.67 g.) was superior to all other dilutors tried. Gelatin capsules were satisfactorily used as a means of preparing the buffer salt; a one-half ounce capsule containing the glucose and Rochelle salt, which can be stored and used as needed, is dissolved in 100 ml. of water and an egg yolk added. This dilutor can be pasteurized. Guerreiro (1940), using various diluents of Milovanov, found that the addition of gelatin to the dilutors had no effect on survival time of sperm. However, his gelatinised fluids became heavily contaminated with bacteria. Chang (1943) in comparing eight dilutors found all dilutors improved storage capacity but the effect of 0.9 percent Na Cl was slight. Glucose alone was beneficial but gluco-sulfate, gluco-tartrate, TGL-6, and glucose phosphate were better and similar to each other. At low temperature the most satisfactory dilutors were glucose-yolk phosphate and glucose-yolk tartrate.

Gotze and Rosenberger (1944) reported that stallion sperm viability was considerably prolonged (48-180 hours) by diluting with egg yolk-dextrose in distilled water, using a five to six percent dextrose

solution and one to two percent egg yolk, according to the rate of dilution, and storing at 2° to 6° C. They also found that the addition of 20 percent inactivated stallion serum was even more effective than egg yolk. Buiko-Rogalevic (1949) reported that stallion sperm motility was preserved for eight to thirteen days when semen was diluted with egg yolk glucose, and for two and one-half days when it was diluted with glucose. The optimal concentration of egg yolk in the diluent was 0.6 to 0.8 percent, and the optimal ratio of semen to diluent was 1:2-3; motility decreased rapidly as the proportion of diluent increased. Murphree, et. al. (1949) reported the effects of dilutors composed of glucose; combinations of glucose and sodium potassium tartrate; combinations of glucose, sodium potassium tartrate, and egg yolk; and combinations of glucose and egg yolk upon the maintenance of motility of jack sperm. In all of the samples studied, the combination of five percent glucose with egg yolk gave superior motility up to 144 hours.

Bogart and Mayer (1950) reported that experiments with various diluents alone and in combination with egg yolk indicated the presence of a factor or factors in egg yolk which enable stallion sperm to acquire resistance to adverse conditions. In checking the motility, percent alive, and percent to survive cold shock, the percentage of resistant sperm markedly increased, as compared to undiluted semen, when one part of semen was diluted with four parts of an egg yolk diluent. Their best results were obtained with a diluent containing equal parts of egg yolk and five percent glucose.

Patrick (1949) found that by using a final concentration of 1:25,000 potassium permanganate on jack semen diluted with egg yolk-glucose, the rate of motility was increased above that of samples diluted in the same manner but without the addition of potassium permanganate. This was confirmed by Good (1950).

Camici and Polestra (1939) and Chang (1943) obtained better conservation results with horse sperm by centrifuging and removing the supernatant liquid and diluting than by diluting alone. Walton (1938) obtained fertile inseminations with sperm, twice diluted, centrifuged, and stored for 24 hours at 0° C.

## CHAPTER III

## EXPERIMENTAL PROCEDURE AND RESULTS

Two jacks of the Tennessee Agricultural Experiment Station were used in this study. The animals were kept on grass in separate outside paddocks as much as weather permitted. The ration consisted of two parts oats to one part corn with free access to hay while in the barn. Semen collections were made by the use of an artificial vagina. The jacks were used alternately in collections, using each jack at least once a week. A mare mule was used for the jacks to mount. One jack showed a preference for the mule over two mares that were available and refused to serve the mares. The other jack showed interest in both the mule and mares so the mule was used in order to get both jacks to serve. The mule was placed in a breeding pit but at first refused to stand. Injections of diethylstilbestrol were given to the mule and after a few services little trouble was encountered. The jacks served even though the mule was not in heat, however, when the mule showed signs of heat the jacks seemed to work a bit faster.

After semen collections were made and the animals returned to their paddocks, the semen was taken to the laboratory without delay. The bottle of semen, already having one protective cover, was wrapped again to prevent temperature shock. The time that elapsed between semen collection and dilution was from 30 to 45 minutes.

Dilutors were always prepared fresh just prior to the time of collection and allowed to stand at room temperature until used. Fresh

eggs were obtained from the University Poultry Department. In most cases the eggs used were laid that day and in no cases were the eggs more than one day old. The egg yolk was separated from the albumin by removing as much white as possible with the egg shell, the remaining albumin being removed by rolling the yolk on filter paper. The yolk membrane was then ruptured by means of a stirring rod and the pure egg yolk collected in a small beaker.

Initial sperm motility was determined immediately upon arrival at the laboratory prior to dilution. Semen dilution was made in a graduated cylinder and mixing was accomplished by stirring with a glass rod. In dilutors containing glucose, the anhydrous form was always used. The dilution rate was a one to one ratio in all cases. After the diluted semen was put into small test tubes, the tubes were placed in a beaker of tap water having a temperature of about 26° C. The beaker containing the samples was then placed in a refrigerator where the temperature was about 8° C. The cooling of the water caused the diluted samples to cool slowly and thereby reduced the chance of temperature shock during cooling.

At 24 hour intervals, each semen sample was examined microscopically for motility. This continued for 240 hours or until the sperm showed no evidence of life. Motility was observed by placing a drop of semen on a clean glass slide, covering the drop with a cover slip, and examining under the low power objective (100x magnification). Since motility rating is influenced by temperature, the slides were held in a warming stage maintained at 37° C. This precaution was taken to prevent the temperature factor from influencing the results. Motility was designated

as zero or by a number from one to five inclusive, the five denoting maximum motility.

The data on sperm motility after 96 hours of storage were analyzed statistically using the analysis of variance technique and the "t" test for paired differences (Snedecor, 1946).

## Semen Characteristics

The volume of the ejaculates was measured in all cases in a graduate cylinder and sperm concentration was determined by using the hemocytometer method. Immediately after arrival at the laboratory the percent of live sperm was determined, using the Fast Green-Eosin staining technique (Mayer, et. al., 1951). Two hundred sperm were counted in determining the percentage of live sperm.

The average volume (73.3 ml.) and initial percent alive (86 percent) are in agreement with data reported by other workers, however, the average concentration (492,391/mm.$^3$) is somewhat higher than in other reports (Table I).

Determinations of pH values were made with a Model G Beckman potentiometer with a glass electrode on both diluted and undiluted semen samples that were less than one, 24, 48, and 72 hours old, using a five ml. sample for each pH determination. All pH readings were made to the nearest tenth. The results show that the pH of diluted semen decreased slightly during the first three days of storage and that undiluted semen remained consistently more alkaline during a similar interval (Table II). This is in agreement with reports (Sergin, 1935, 1937) (Davis and Cole,

TABLE I

SEMEN CHARACTERISTICS

| | Number of Ejaculates | Range | Average |
|---|---|---|---|
| Volume | 123 | 40-135 ml. | 73.3 ml. |
| Concentration | 133 | 140,000-980,000/mm.$^3$ | 492,391/mm.$^3$ |
| Initial Percent Alive | 100 | 55-94 | 86 |

TABLE II

pH OF JACK SEMEN

| | Number of Ejaculates | Age of Semen (hrs.) | Average pH | Range |
|---|---|---|---|---|
| Diluted* | | | | |
| | 14 | <1 (undiluted) | 7.20 | 7.00-7.85 |
| | 10 | <1 | 6.71 | 6.65-6.80 |
| | 12 | 24 | 6.65 | 6.50-6.80 |
| | 11 | 48 | 6.62 | 6.40-6.80 |
| | 6 | 72 | 6.56 | 6.40-6.75 |
| Undiluted | | | | |
| | 14 | <1 | 7.20 | 7.00-7.85 |
| | 15 | 24 | 7.35 | 6.95-7.90 |
| | 11 | 48 | 7.45 | 7.00-7.90 |
| | 13 | 72 | 7.42 | 7.00-7.85 |

*Diluted with one part of egg yolk to four parts of five percent glucose solution in a one to one ratio with semen.

1939) that little if any lactic acid is produced in stallion semen. These results confirm the suggestion (Bogart and Mayer, 1950) that a dilutor for jack semen need not contain a buffer for good storage since the diluted samples contained no buffer and all maintained motility for several days while the undiluted samples showed no motility at the end of the first 24 hours of storage. Apparently jack semen has a reasonably high inherent buffering capacity since little change in pH was observed even in the undiluted semen during 72 hours of storage.

## The Effect of Egg Yolk and Various Amounts of Glucose on Sperm Motility

Ten ejaculates were used in this study comparing five different dilutions with an undiluted control. The components of the five diluents were as follows: (1) five percent glucose solution; (2) one part of egg yolk to one part of five percent glucose solution; (3) one part of egg yolk to four parts of five percent glucose solution; (4) one part of egg yolk to five parts of five percent glucose solution; and (5) one part of egg yolk to ten parts of five percent glucose solution.

The data show that dilutors are necessary for maintaining the motility of jack sperm since the undiluted samples showed no motility the day after collection (Figure 1). Motility must be maintained if any degree of success is to be expected in producing pregnancy with stored semen. It is quite obvious that the dilutors containing egg yolk were superior to that without egg yolk (Table III). One part of egg yolk to four, five, or ten parts of the glucose solution gave better results in maintaining motility

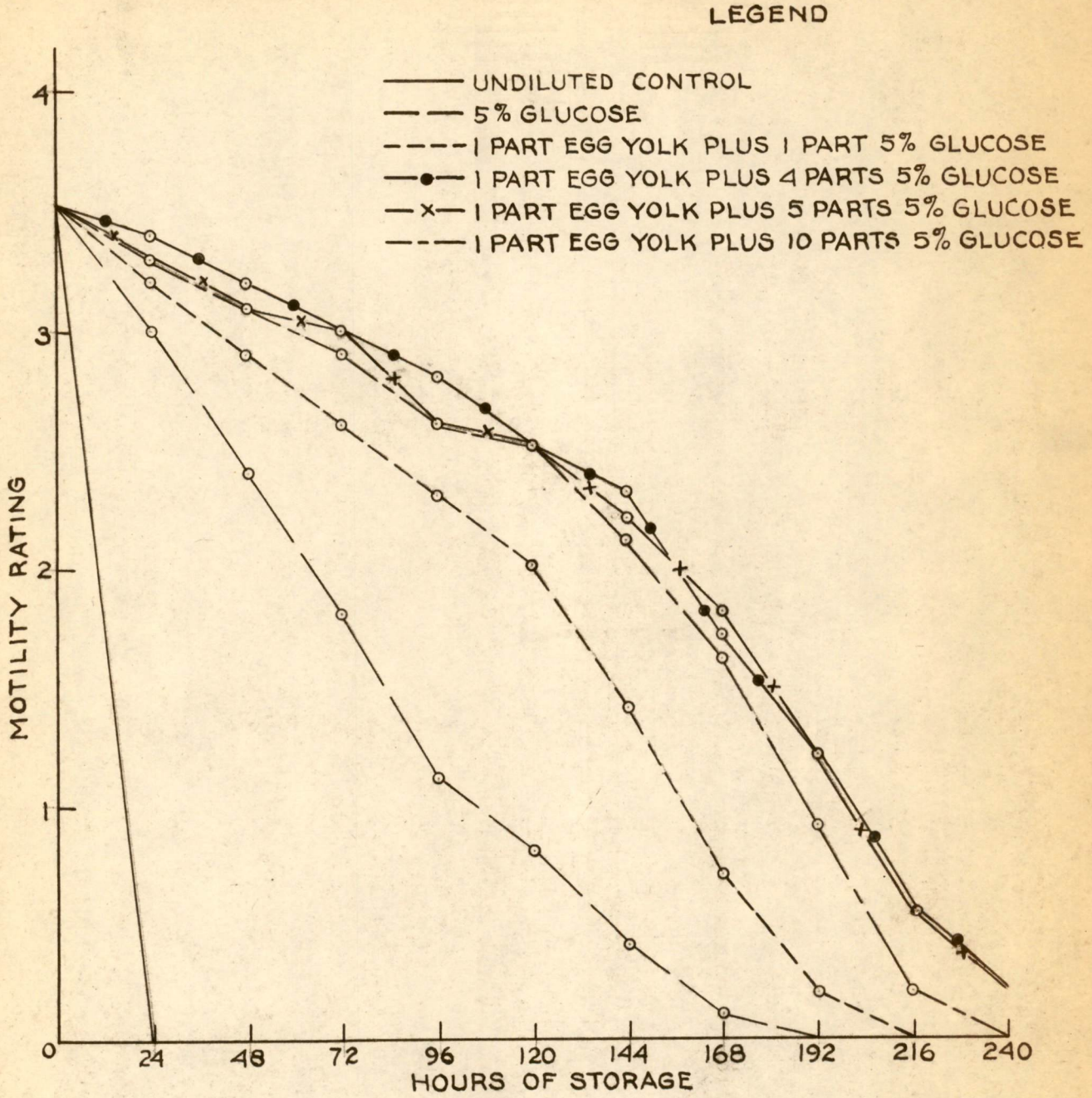



FIGURE 1. THE EFFECT OF EGG YOLK AND VARIOUS AMOUNTS OF GLUCOSE ON SPERM MOTILITY.

TABLE III

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 96 HOUR OLD SPERM DILUTED WITH VARIOUS LEVELS OF EGG YOLK GLUCOSE

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 59 | 71.40 | |
| Between subclasses | 5 | 62.00 | 12.40** |
| Undiluted vs. diluted | 1 | 43.32 | 43.32** |
| Diluted with egg yolk vs. diluted without egg yolk | 1 | 17.40 | 17.40** |
| Between levels of egg yolk-glucose | 3 | 1.28 | 0.43 |
| Within subclasses | 54 | 9.40 | 0.17 |

**Highly significant, $P < .01$.

than equal parts of egg yolk and glucose solution, however, the difference was not significant. Due to the slight advantage shown by the dilutor containing four parts of the glucose solution, it (one part of egg yolk to four parts of five percent glucose solution in a one to one ratio with semen) was used as the control dilutor in subsequent studies.

## The Effect of Sodium Potassium Tartrate-Egg Yolk-Glucose and Glucose-Egg Yolk-Phosphate on Sperm Motility

Thirteen ejaculates were used in this study. The dilutors were prepared as follows: (1) Control: One part of egg yolk to four parts of five percent glucose solution; (2) TGL: 5.76 g. of glucose plus 0.67 g. of Rochelle Salt in a one-half ounce gelatin capsule dissolved in 100 ml. of distilled water with one egg yolk added (Berliner, 1942); (3) GYP: One part of GYP (0.793 g. of $Na_2HPO_4$, 0.2 g. of $KH_2PO_4$, and 5 g. of glucose dissolved in 100 ml. of distilled water) plus four parts of egg yolk (Berliner, 1942). Fresh semen was diluted with equal volumes of these dilutors.

Sperm motility was not maintained as satisfactorily in either TGL or GYP as in the control dilutor (Figure 2). The GYP dilutor was less satisfactory than the TGL dilutor. All differences were highly significant (Table IV). However, it was difficult to see the individual sperm in the TGL dilutor and the motility estimates were harder to make.

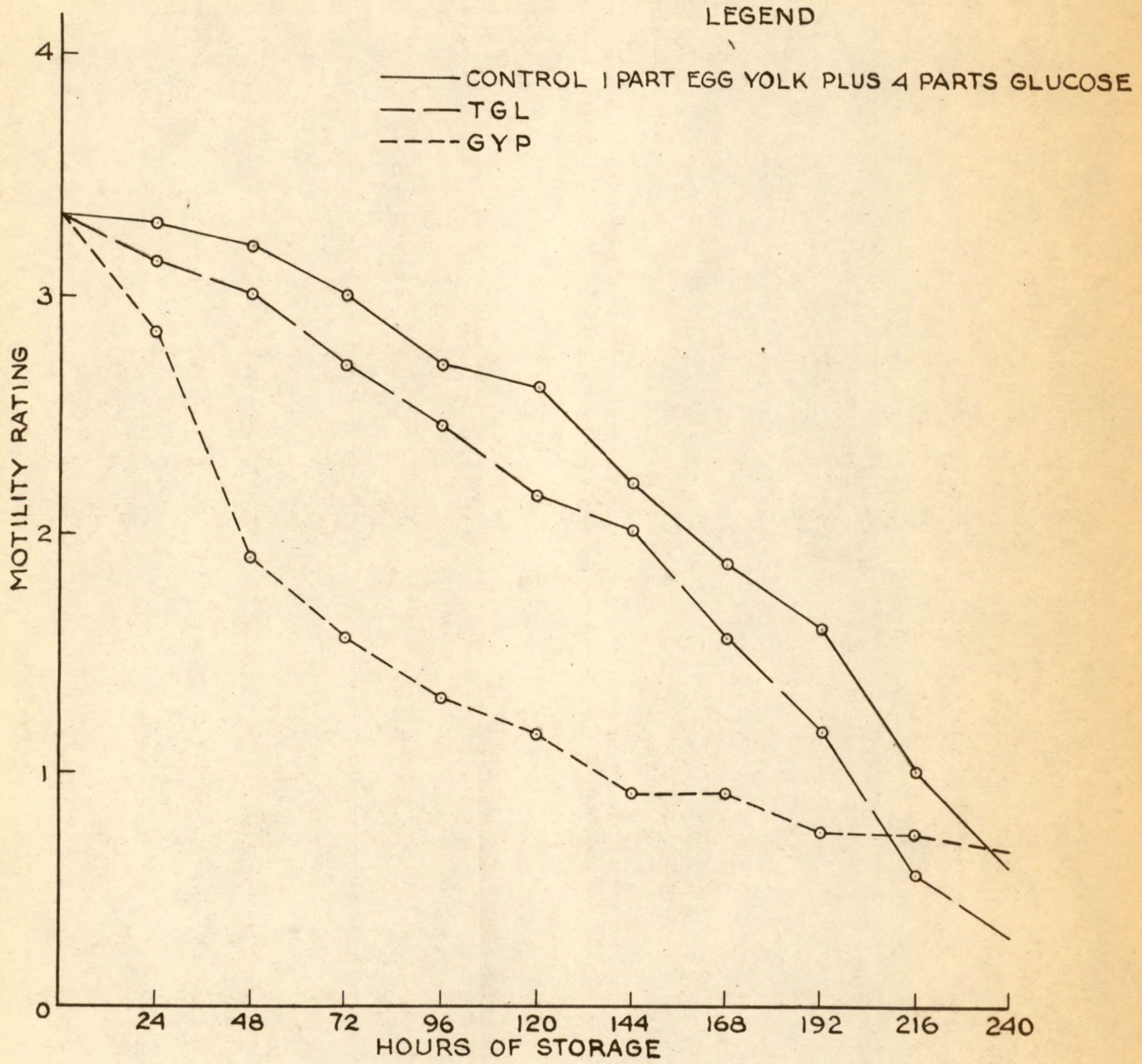



FIGURE 2. THE EFFECT OF TGL AND GYP ON SPERM MOTILITY.

TABLE IV

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 96 HOUR OLD SPERM IN EGG YOLK-GLUCOSE, TGL AND GYP DILUTORS

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 38 | 23.08 | |
| Between subclasses | 5 | 15.14 | 3.03** |
| Egg yolk-glucose vs. TGL and GYP | 1 | 5.66 | 5.66** |
| Between jacks | 1 | 0.79 | 0.79* |
| Interaction jack and (TGL, GYP) | 1 | 0.02 | 0.02 |
| Between TGL and GYP | 1 | 8.65 | 8.65** |
| Interaction jack and TGL and GYP | 1 | 0.02 | 0.02 |
| Between ejaculates of same jack | 11 | 4.25 | 0.39* |
| Interaction (TGL, GYP) and ejaculate (error) | 22 | 3.69 | 0.17 |

*Significant, $P < .05$.
**Highly significant, $P < .01$.

## The Effect of Various Amounts of Ascorbic Acid in Combination with Egg Yolk-Glucose on Sperm Motility

Although Soliven and Gonzaga (1940) found no significant influence on stallion sperm longevity in storage when vitamins A, B, C, and D were added singly, combinations of these vitamins did favorably influence maintenance of motility. Consequently, it was decided to study the effect on jack sperm motility during storage of some vitamins known to be involved in cellular metabolism. The possibility was also considered that the vitamins might exert varying effects when used in different diluents; the above authors used diluents different from the one used in this experiment.

The role of ascorbic acid is not completely understood, but one of the main theories concerning its role in tissue metabolism is that it serves as a hydrogen-transport agent between unidentified metabolites and other carriers or molecular oxygen, by the way of two or more oxidase systems (Gortner, 1949).

Ten ejaculates were used in this study in comparing the effects of the addition of various amounts of ascorbic acid to diluted semen. Semen samples diluted with egg yolk-glucose containing one, three, five, and ten mg. of added ascorbic acid were compared with a diluted control containing no additional ascorbic acid. All samples contained ten ml. of diluted semen and the vitamins were weighed on an analytical balance just prior to semen collection.

The results showed no effect attributable to vitamin C during the first 96 hours of storage (Figure 3). Three and ten mg. apparently

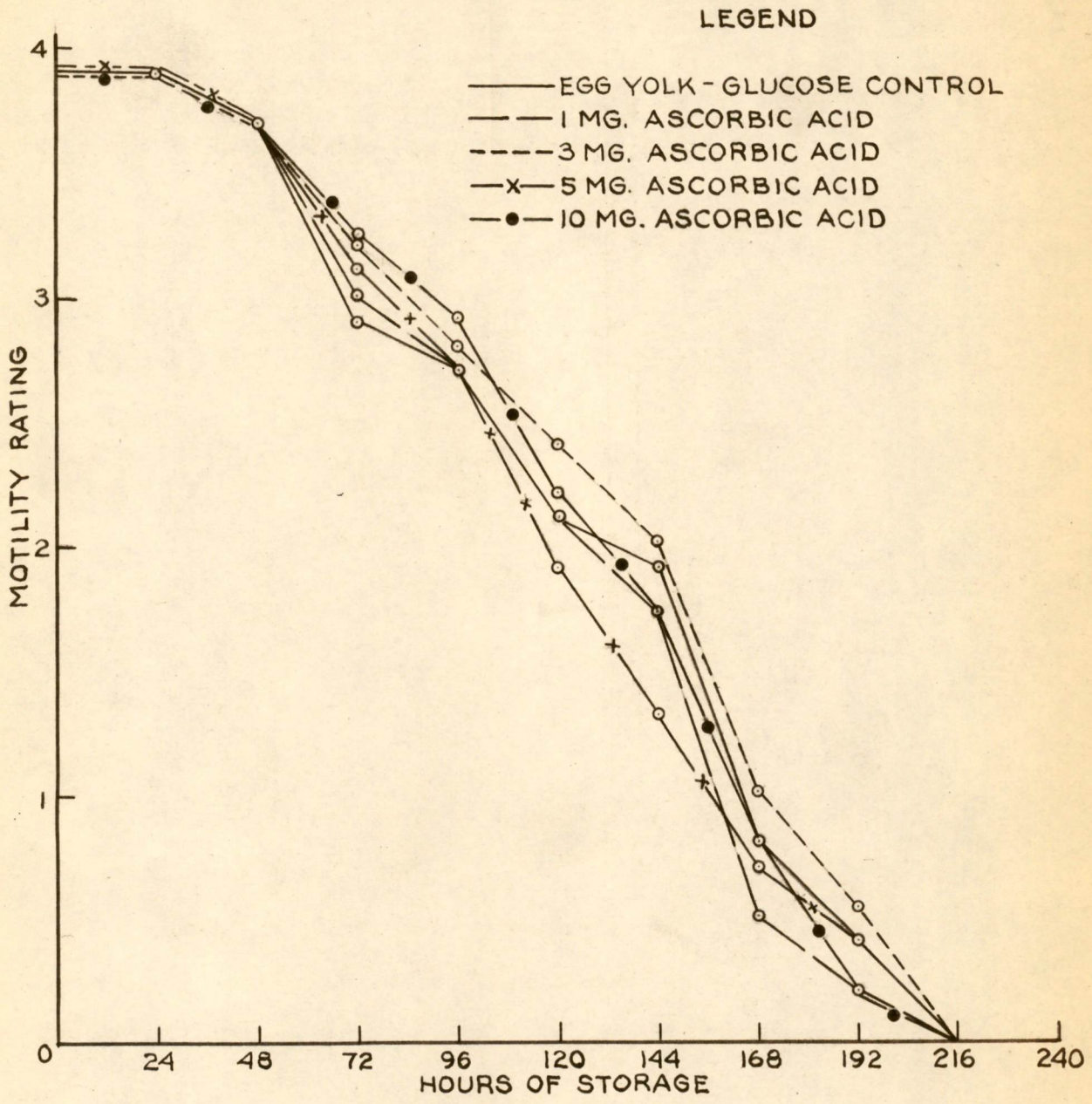



FIGURE 3. THE EFFECT OF VARIOUS AMOUNTS OF ASCORBIC ACID IN COMBINATION WITH EGG YOLK - GLUCOSE ON SPERM MOTILITY.

exerted some stimulatory effect during longer intervals of storage, however, the differences were not significant (Table V). It is not clear why the sample containing five mg. had a lower motility through the major part of the storage period than did the samples containing three or ten milligrams.

### The Effect of Various Amounts of Thiamine in Combination with Egg Yolk-Glucose on Sperm Motility

Thiamine is closely associated with carbohydrate metabolism, apparently forming part of an enzyme system which is necessary for the decarboxylation of pyruvic acid, an intermediate compound of carbohydrate metabolism (Anderson, 1950).

Fifteen ejaculates were used in this study. Samples containing one, three, five, ten, and twenty mg. of added thiamine hydro-chloride were compared with a diluted control containing no additional thiamine. All samples contained ten ml. of diluted semen.

All samples containing additional thiamine except the 20 mg. sample showed higher motility than the control from the 48th to the 120th hours of storage (Figure 4). The differences between all levels of thiamine were highly significant at 96 hours of storage, however, this was due to the low motility in the 20 mg. sample (Table VI). The "t" test for paired differences showed no level of thiamine to be significantly better than the control in maintaining motility.

TABLE V

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 96 HOUR OLD SPERM DILUTED WITH EGG YOLK-GLUCOSE AND VARYING LEVELS OF ASCORBIC ACID

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 49 | 41.12 | |
| Between subclasses | 9 | 0.72 | 0.08 |
| Control vs. treated | 1 | 0.04 | 0.04 |
| Between jacks | 1 | 0.08 | 0.08 |
| Interaction jack and treated | 1 | 0.05 | 0.05 |
| Between levels | 3 | 0.28 | 0.09 |
| Interaction jack and level | 3 | 0.27 | 0.09 |
| Between ejaculates of same jack | 8 | 37.84 | 4.73** |
| Interaction treated and ejaculates (error) | 32 | 2.56 | 0.08 |

**Highly significant, $P < .01$.

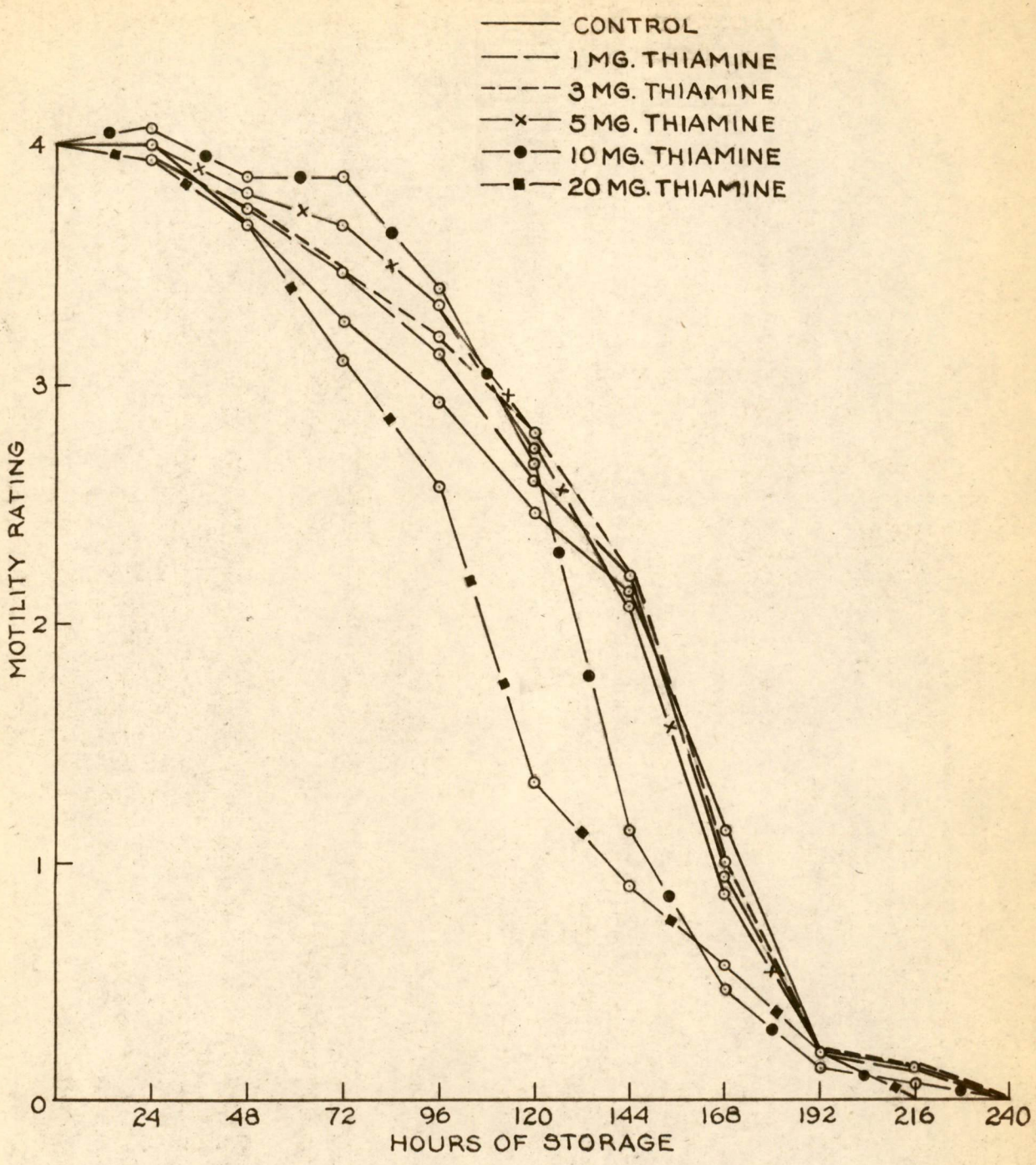



FIGURE 4. THE EFFECT OF VARIOUS AMOUNTS OF THIAMINE IN COMBINATION WITH EGG YOLK-GLUCOSE ON SPERM MOTILITY.

TABLE VI

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 96 HOUR OLD SPERM DILUTED WITH EGG YOLK-GLUCOSE AND VARYING LEVELS OF THIAMINE

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 89 | 58.10 | |
| Between subclasses | 11 | 10.06 | 0.91** |
| Control vs. treated | 1 | 0.50 | 0.50 |
| Between jacks | 1 | 0.35 | 0.35 |
| Interaction jack and treated | 1 | 0.32 | 0.32 |
| Between levels | 4 | 6.01 | 1.50** |
| Interaction jack and level | 4 | 2.88 | 0.72 |
| Between ejaculates of same jack | 13 | 29.29 | 2.25** |
| Interaction treated and ejaculates (error) | 65 | 18.75 | 0.29 |

**Highly significant, $P < .01$.

## The Effect of Various Amounts of Riboflavin in Combination with Egg Yolk-Glucose in Sperm Motility

Riboflavin is essential in the synthesis of the flavoproteins which function as intermediary catalysts in vital dehydrogenation processes (Gortner, 1949).

Twenty ejaculates were used in this study. Samples containing one, three, five, and ten mg. of added riboflavin were compared with a diluted control with no additional riboflavin. All samples contained ten ml. of diluted semen. The data showed no beneficial effect on sperm motility due to the addition of riboflavin at any level to diluted semen (Figure 5 and Table VII). The riboflavin had a low solubility and the individual sperm were difficult to see in the samples containing five and ten mg. of added riboflavin.

## The Effect of Various Amounts of Pyridoxine in Combination with Egg Yolk-Glucose on Sperm Motility

Little is known about the function of pyridoxine although it has been shown that it plays a role in the metabolism of proteins (Gortner, 1949).

Twenty ejaculates were used in this study. Samples with ten ml. of diluted semen containing one, three, five, and ten mg. of added pyridoxine hydrochloride were compared with a diluted control with no additional pyridoxine.

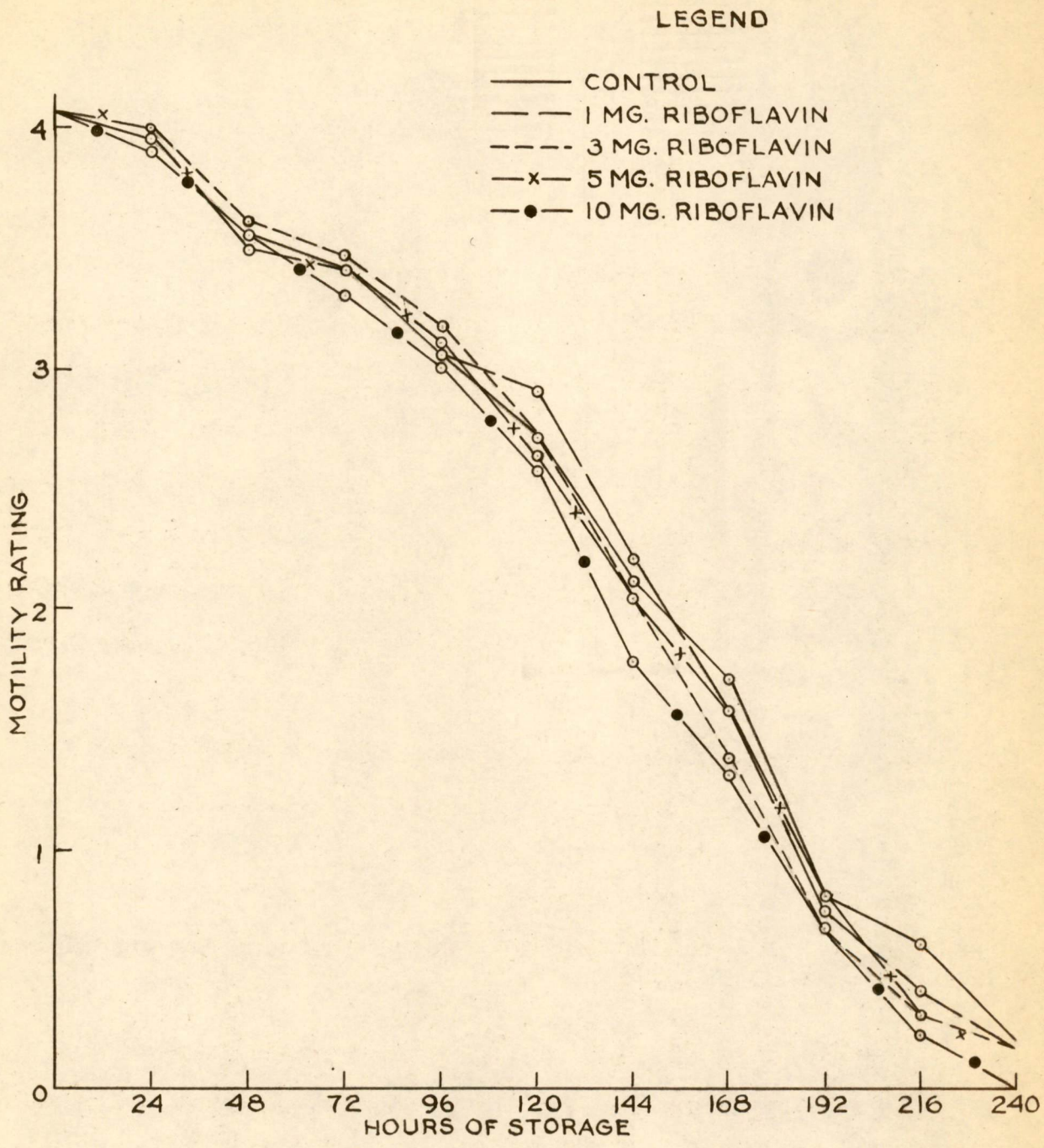



FIGURE 5. THE EFFECT OF VARIOUS AMOUNTS OF RIBOFLAVIN IN COMBINATION WITH EGG YOLK - GLUCOSE ON SPERM MOTILITY.

TABLE VII

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 96 HOUR OLD SPERM DILUTED WITH EGG YOLK-GLUCOSE AND VARYING LEVELS OF RIBOFLAVIN

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 99 | 38.51 | |
| Between subclasses | 9 | 5.61 | 0.62** |
| Control vs. treated | 1 | 0.01 | 0.01 |
| Between jacks | 1 | 4.41 | 4.41** |
| Interaction jack and treated | 1 | 0.49 | 0.49* |
| Between levels | 3 | 0.25 | 0.08 |
| Interaction jack and level | 3 | 0.45 | 0.15 |
| Between ejaculates of same jack | 18 | 26.50 | 1.47** |
| Interaction treated and ejaculates (error) | 72 | 6.40 | 0.09 |

*Significant, $P < .05$.
**Highly significant, $P < .01$.

Pyridoxine added to diluted semen had a stimulating effect on sperm motility during the first 72 hours of storage (Figure 6). The differences between all levels of pyridoxine were highly significant at 96 hours of storage, however, this was due to the lower motility in the 10 mg. sample (Table VIII). The samples containing three or five mg. of additional pyridoxine showed a highly significant higher degree of motility than did the control sample at the end of 96 hours of storage ("t" test).

## The Effect of Various Amounts of Niacin in Combination with Egg Yolk-Glucose on Sperm Motility

Niacin plays an important role in carbohydrate metabolism. Coenzyme I and coenzyme II, both of which are involved in carbohydrate metabolism, are derivatives of nicotinic acid amide, the amide of niacin (Harrow, 1947).

Five ejaculates were used in this study. Samples with ten ml. of diluted jack semen containing one, three, five, and ten mg. of added niacin were compared with a diluted control with no additional niacin.

The results showed that the addition of small amounts of niacin to diluted semen had a stimulating effect on sperm motility at the end of 96 hours of storage (Figure 7). Although the differences were not significant (Table IX), the fact that all samples containing added niacin showed a higher level of sperm motility than the control suggest that niacin was beneficial. It is possible that had more ejaculates been studied, a significant effect would have been found especially at the five and ten mg. levels.

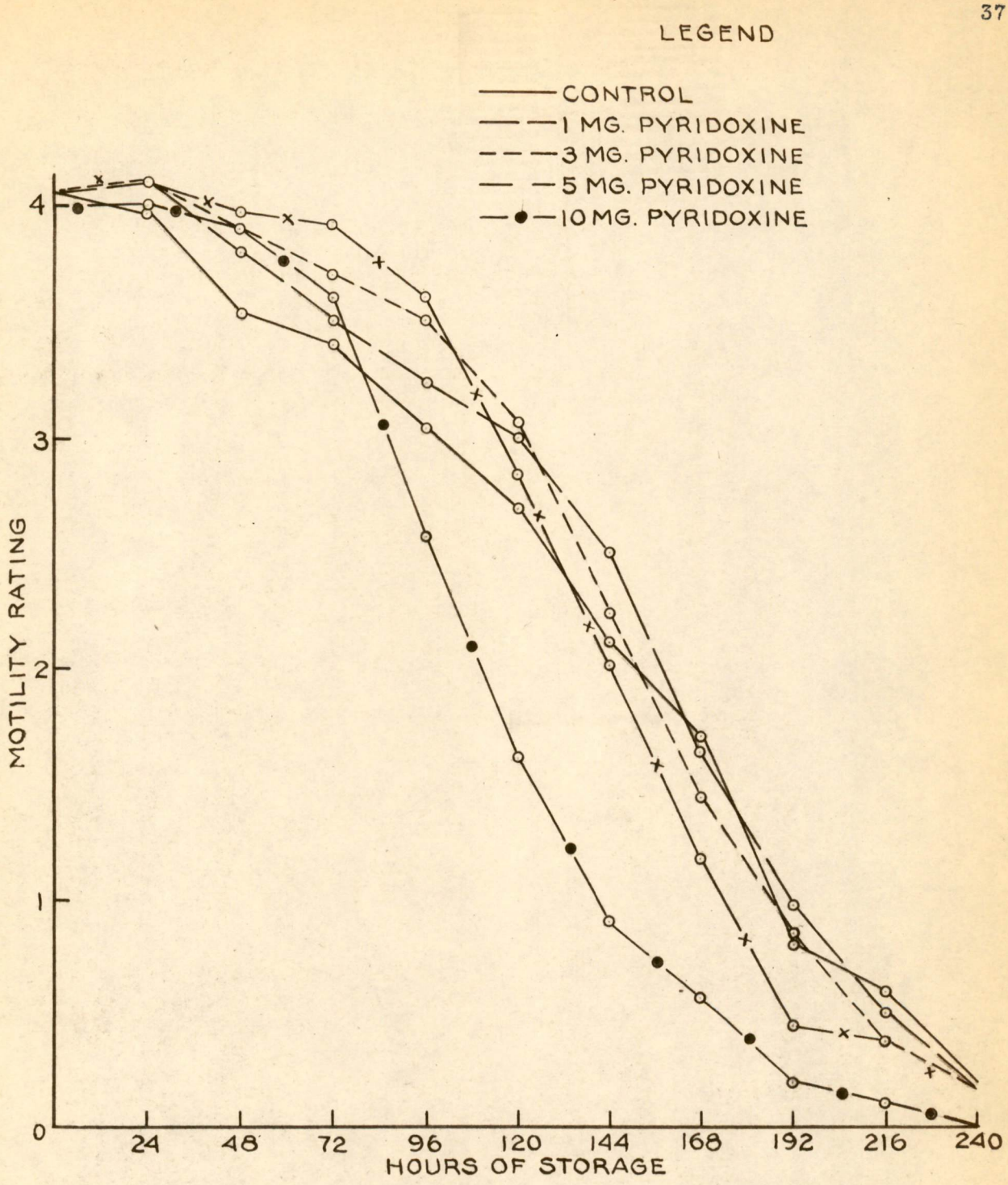



FIGURE 6. THE EFFECT OF VARIOUS AMOUNTS OF PYRIDOXINE IN COMBINATION WITH EGG YOLK-GLUCOSE ON SPERM MOTILITY.

TABLE VIII

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 96 HOUR OLD SPERM DILUTED WITH EGG YOLK-GLUCOSE AND VARYING LEVELS OF PYRIDOXINE

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 99 | 61.39 | |
| Between subclasses | 9 | 20.29 | 2.25** |
| Control vs. treated | 1 | 0.49 | 0.49 |
| Between jacks | 1 | 2.89 | 2.89* |
| Interaction jack and treated | 1 | 0.81 | 0.81 |
| Between levels | 3 | 13.90 | 4.63** |
| Interaction jack and level | 3 | 2.20 | 0.73 |
| Between ejaculates of same jack | 18 | 10.10 | 0.56 |
| Interaction treated and ejaculates (error) | 72 | 31.00 | 0.43 |

*Significant, $P < .05$.

**Highly significant, $P < .01$.

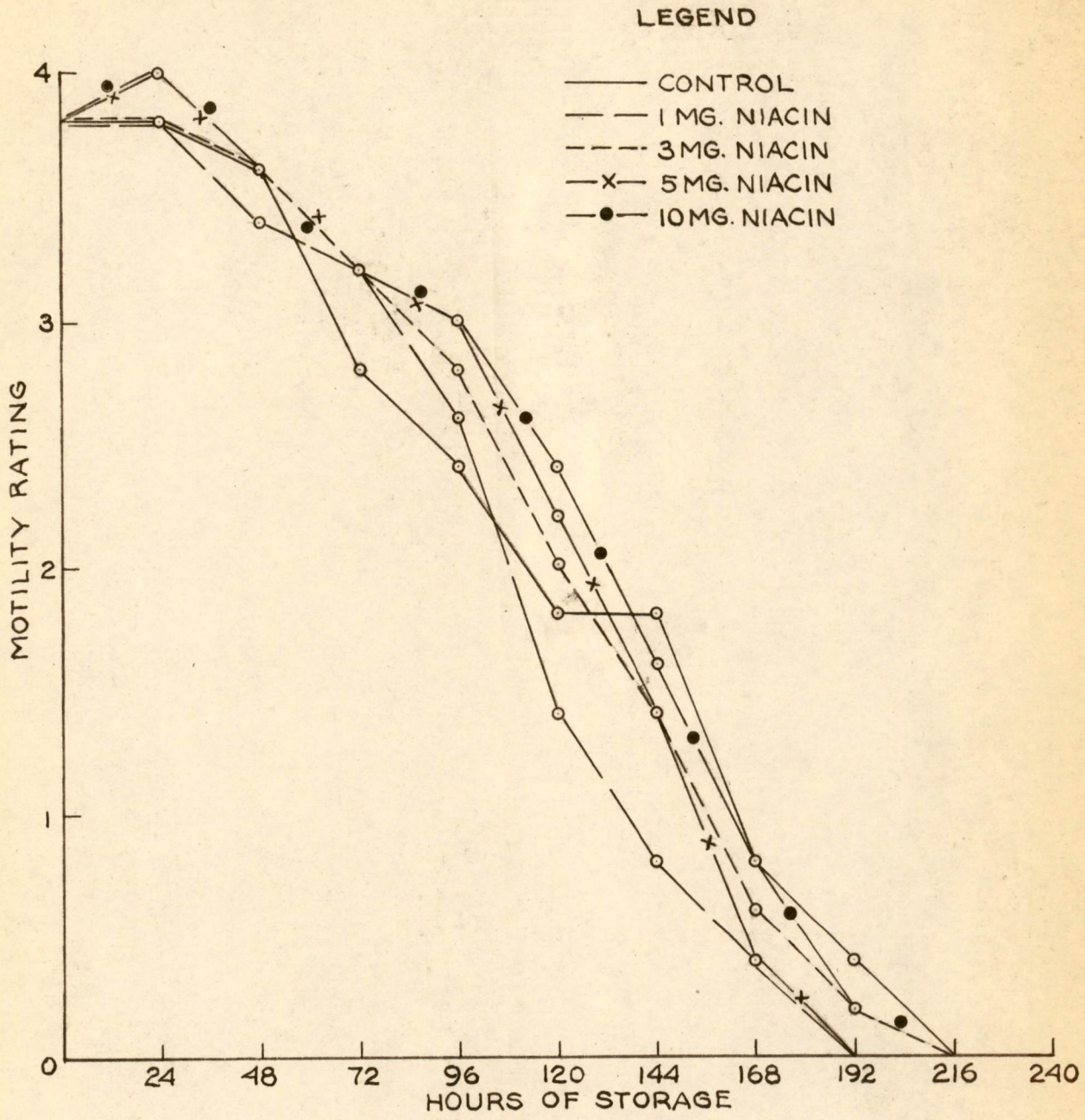



FIGURE 7. THE EFFECT OF VARIOUS AMOUNTS OF NIACIN IN COMBINATION WITH EGG YOLK-GLUCOSE ON SPERM MOTILITY.

TABLE IX

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 96 HOUR OLD SPERM DILUTED WITH EGG YOLK-GLUCOSE AND VARYING LEVELS OF NIACIN

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 24 | 26.56 | |
| Between subclasses | 4 | 1.36 | 0.34 |
| Control vs. treated | 1 | 0.81 | 0.81 |
| Between levels | 3 | 0.55 | 0.18 |
| Within subclasses | 20 | 25.20 | 1.26 |

## The Effect of Potassium Permanganate in Combination with Egg Yolk-Glucose on Sperm Motility

Patrick (1949) and Good (1950) found that by using a final concentration of 1:25,000 potassium permanganate in jack semen diluted with egg yolk-glucose, the sperm motility was increased above that of semen diluted in the same manner but without potassium permanganate. This study was undertaken to see if the results of the above authors could be confirmed and also to see if potassium permanganate, when added 48 or 72 hours in the storage period, would maintain a higher level of sperm motility for a longer period than when the permanganate was added at the time of initial dilution.

Fourteen ejaculates were used in this study. The samples were made up as follows: (1) a control sample diluted with egg yolk-glucose; (2) a sample of egg yolk-glucose diluted semen containing a final concentration of 1:25,000 potassium permanganate added at the time of initial dilution; (3) a sample of egg yolk-glucose diluted semen containing a final concentration of 1:50,000 potassium permanganate added at the time of initial dilution; (4) a sample of egg yolk-glucose diluted semen containing a final concentration of 1:25,000 potassium permanganate added to 48 hour old diluted semen; and (5) a sample of egg yolk-glucose diluted semen containing a final concentration of 1:25,000 potassium permanganate added to 72 hour old diluted semen. All samples contained ten ml. of diluted semen.

The results show that samples of diluted semen with additions of either 1:25,000 or 1:50,000 concentrations of potassium permanganate

maintained a higher level of sperm motility throughout most of the storage period than did the control sample with no additional permanganate, however, the differences at the end of 96 hours of the storage period were not significant (Figure 8 and Table X).

After 120 hours of storage, all samples containing permanganate showed a significantly higher degree of motility than the control (Table XI). Using the "t" test, only the samples containing permanganate added to a final concentration of 1:50,000 at the time of initial dilution and 1:25,000 added at 48 hours after initial dilution showed a significant advantage over the control.

## The Effect of Potassium Permanganate and Some Vitamins in Combination with Egg Yolk-Glucose on Sperm Motility

Since some of the vitamin compounds had a stimulating effect on sperm motility, the possibility was considered that vitamins together with potassium permanganate, when added to diluted semen, would have an even greater stimulatory effect on sperm motility than either of the compounds when added singly. This study was initiated to examine this possibility.

Ten ejaculates were used in comparing the following dilutors: (1) a control diluted with egg yolk-glucose; (2) the addition of a 1:50,000 concentration of potassium permanganate plus five mg. of thiamine hydrochloride to diluted semen at the time of dilution; (3) the addition of a 1:50,000 concentration of potassium permanganate plus ten mg. of thiamine hydrochloride to diluted semen at the time of dilution; and (4) the addition of a 1:50,000 concentration of potassium permanganate plus three mg.

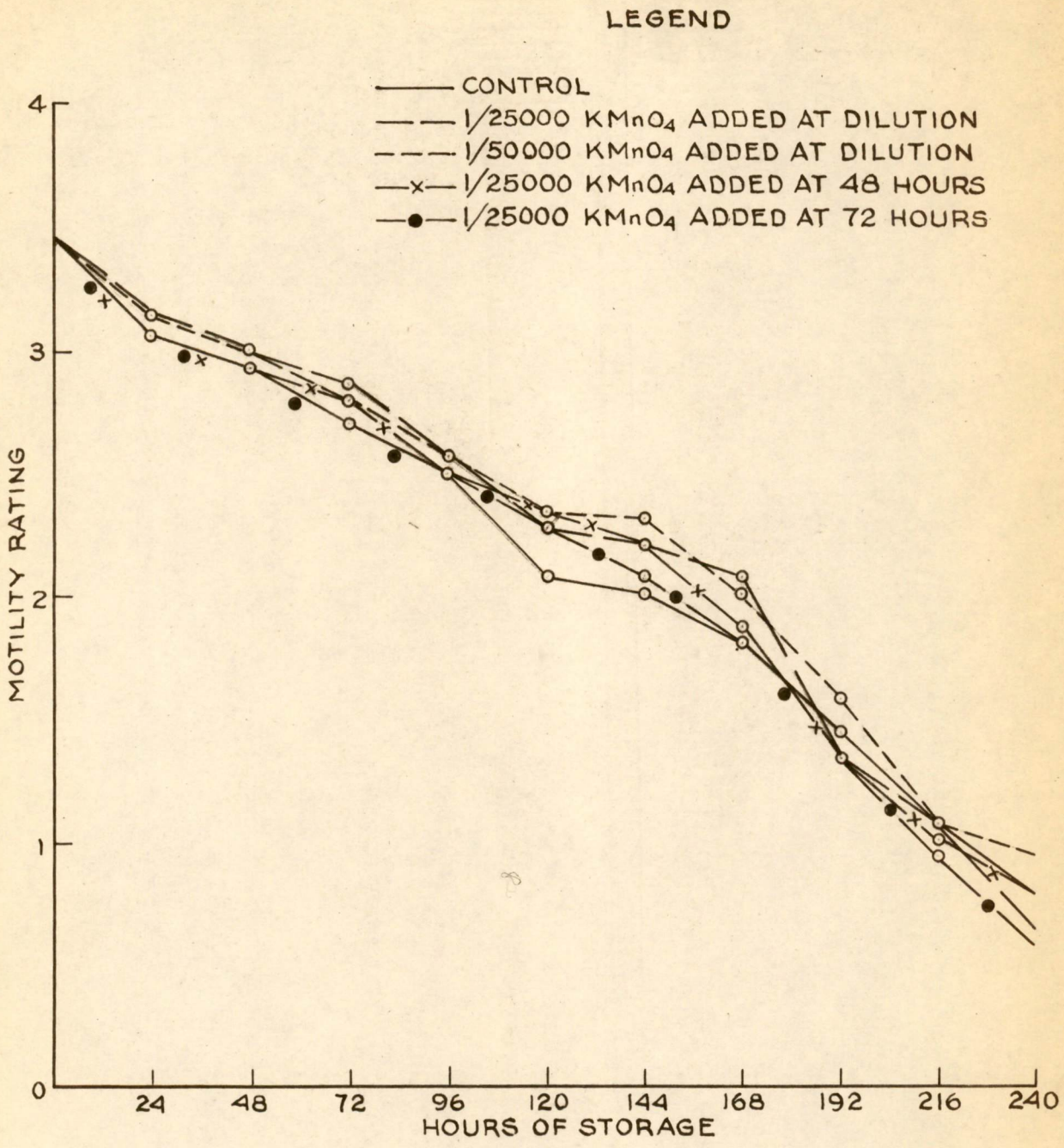



FIGURE 8. THE EFFECT OF POTASSIUM PERMANGANATE IN COMBINATION WITH EGG YOLK-GLUCOSE ON SPERM MOTILITY.

TABLE X

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 96 HOUR OLD SPERM DILUTED WITH EGG YOLK-GLUCOSE AND POTASSIUM PERMANGANATE

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 69 | 17.44 | |
| Between subclasses | 9 | 0.34 | 0.04 |
| Control vs. treated | 1 | 0.01 | 0.01 |
| Between jacks | 1 | 0.20 | 0.20 |
| Interaction jack and treated | 1 | 0.01 | 0.01 |
| Between treatments | 3 | 0.07 | 0.02 |
| Interaction jack and treatments | 3 | 0.05 | 0.02 |
| Between ejaculates of same jack | 12 | 12.84 | 1.07** |
| Interaction treatments and ejaculates (error) | 48 | 4.26 | 0.09 |

**Highly significant, P $<$.01.

TABLE XI

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 120 HOUR OLD SPERM DILUTED WITH EGG YOLK-GLUCOSE AND POTASSIUM PERMANGANATE

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 69 | 19.84 | |
| Between subclasses | 9 | 2.51 | 0.28* |
| Control vs. treated | 1 | 0.70 | 0.70* |
| Between jacks | 1 | 0.89 | 0.89** |
| Interaction jack and treated | 1 | 0.06 | 0.06 |
| Between treatments | 3 | 0.07 | 0.02 |
| Interaction jack and treatments | 3 | 0.79 | 0.26 |
| Between ejaculates of same jack | 12 | 12.55 | 1.05** |
| Interaction treatments and ejaculates (error) | 48 | 4.78 | 0.0996 |

*Significant, $P < .05$.
**Highly significant, $P < .01$.

of ascorbic acid to diluted semen at the time of dilution. All samples contained ten ml. of diluted semen.

The data show that all samples containing either five or ten mg. of thiamine or three mg. of ascorbic acid and a final concentration of 1:50,000 potassium permanganate maintained a higher level of sperm motility during the early part of the storage period (Figure 9), however, the differences were not significant after 96 hours of storage (Table XII).

## The Effect of Various Other Substances on Sperm Motility

In addition to the diluents previously discussed, the effects of the following combinations were also studied: (1) egg yolk and cane sugar; (2) egg yolk and sodium citrate; (3) egg yolk-glucose and sodium citrate; (4) egg yolk and gum arabic; (5) egg yolk and gum ghatti; (6) glucose and gum arabic; (7) glucose and gum ghatti; (8) glucose and lecithin; (9) varying quantities of lecithin; and (10) the addition of potassium permanganate to the TGL and GYP dilutors. Although in diluents (3), (4), (5), and (10) sperm motility was maintained longer than in the undiluted semen, none were as satisfactory as the egg yolk-glucose dilutor.

## The Effect of Centrifugation On Sperm Motility

Several workers (Camici and Polestra, 1939, Chang, 1943) obtained better storage results with stallion sperm by centrifuging and diluting

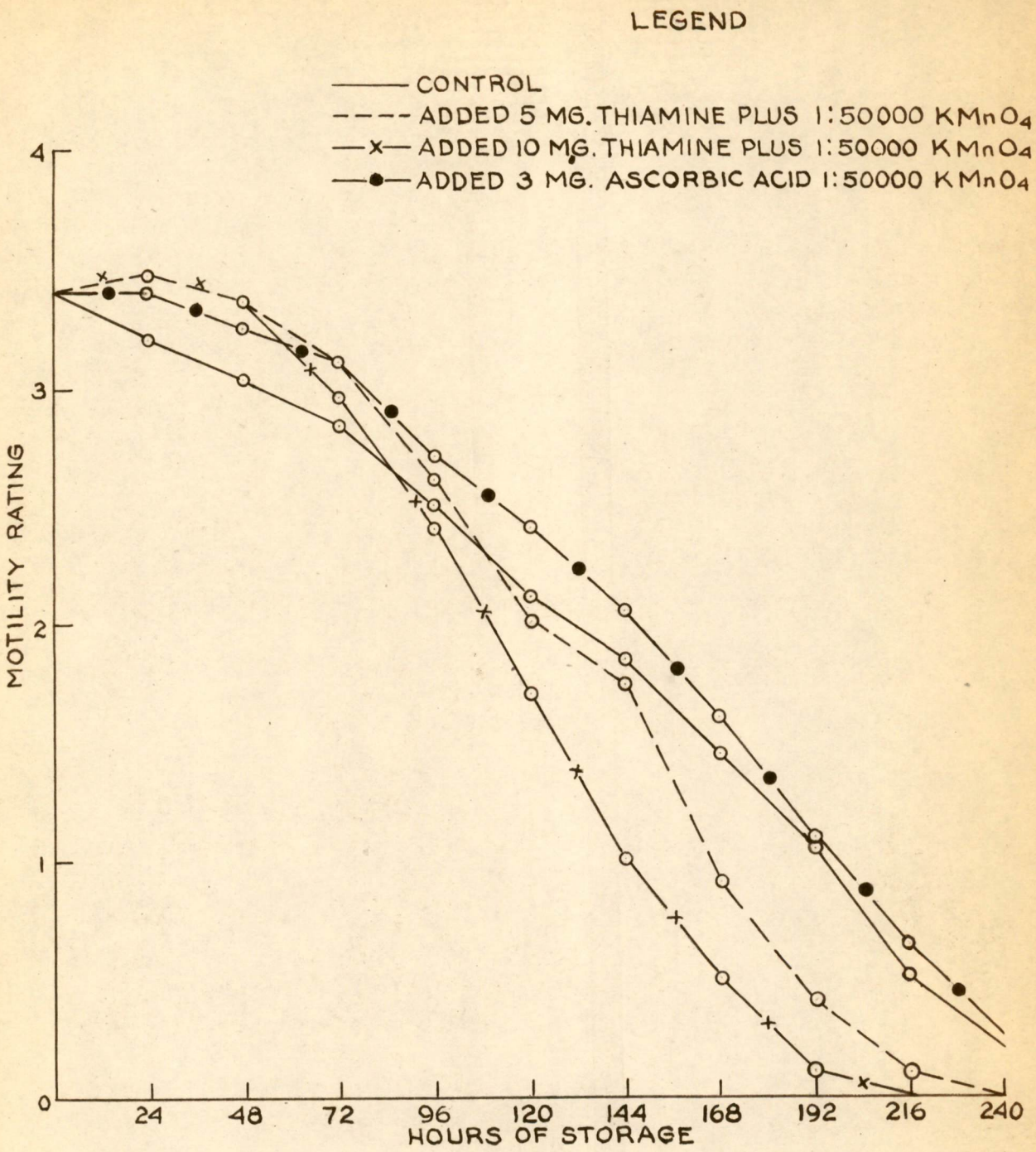



FIGURE 9. THE EFFECT OF POTASSIUM PERMANGANATE AND SOME VITAMINS IN COMBINATION WITH EGG YOLK-GLUCOSE ON SPERM MOTILITY.

TABLE XII

ANALYSIS OF VARIANCE OF MOTILITY RATINGS OF 96 HOUR OLD SPERM DILUTED WITH EGG YOLK-GLUCOSE, VITAMINS, AND POTASSIUM PERMANGANATE

| Source of Variance | Degrees of Freedom | Sum of Squares | Mean Squares |
|---|---|---|---|
| Total | 39 | 21.98 | |
| Between subclasses | 7 | 4.78 | 0.68 |
| Control vs. treated | 1 | 0.08 | 0.08 |
| Between jacks | 1 | 0.63 | 0.63 |
| Interaction jack and treated | 1 | 0.07 | 0.07 |
| Between treatments | 2 | 2.60 | 1.30 |
| Interaction jack and treatments | 2 | 1.40 | 0.70 |
| Between ejaculates of same jack | 8 | 7.10 | 0.89 |
| Interaction treatments and ejaculates (error) | 24 | 10.10 | 0.42 |

and removing a part of the seminal fluid than by diluting uncentrifuged semen. An attempt was made to see if the same was true for jack sperm.

Semen was collected and diluted with egg yolk-glucose as previously described. In the first method tried, the same semen samples were centrifuged after 24, 48, 72, and 96 hours of storage, the supernatant liquid removed and a sufficient amount of fresh egg yolk-glucose diluent to maintain the original volume was added after each centrifugation.

In the second method, the effect of a single centrifugation after varying periods of storage was studied, i. e., samples centrifuged after 24 hours or 48 hours or after 72 hours of storage. After centrifuging and removal of the supernatant fluid, a sufficient amount of fresh dilutor to maintain the original volume was added to each sample. A non-centrifuged sample diluted in the same ratio as the centrifuged samples served as a control. In no case did the centrifuged sperm maintain a level of motility as high or for as long a period as did the control. A majority of the centrifuged samples maintained a fair degree of motility for 72 or 96 hours but showed none thereafter.

## Fertility in Mares Artificially Inseminated with Diluted Semen

The final test of the efficacy of any semen dilutor is the production of pregnancy through the use of semen diluted with that dilutor. Due to inadequate numbers of mares in the Agricultural Experiment Station herds to test any single dilutor, it was necessary to enlist the cooperation of farmers in the Knoxville area. Mare owners within a 45 mile radius of the Knoxville station were contacted and agreed to notify the

inseminator when a mare was first observed in heat. Upon notification that a mare was in heat, diluted semen was taken to the farm and used to inseminate the mare. The semen was prepared for transportation by wrapping 20 ml. test tubes of diluted semen and placing them in a thermos bottle containing ice. Two ten ml. gelatin capsules of semen were inserted into the cervix of the mare. Mares were inseminated only once in a heat period, usually on the third or fourth day after the first sign of estrus.

Six mares were inseminated at the Middle Tennessee Station, Columbia, Tennessee, with semen shipped from the Knoxville station, a distance of 250 miles. Morning collections were diluted and shipped by air from Knoxville to Nashville where the semen was transported by train to the station. The semen was used for insemination the following morning after being shipped making it 24 hours old at the time of use.

All mares inseminated in the Knoxville area during the 1950 and 1951 breeding seasons were tested for pregnancy between 57 and 111 days after insemination. The blood serum method of Hart and Cole (1932) was used. The discrepency between the results of this test and the actual foaling results (Table XIII) is much greater than that reported by Mayer (1944). This author reported that the method was correct in 82.2 percent of the mares which subsequently foaled.

Only two of 17 mares inseminated during the 1950 breeding season gave a positive test while five actually foaled. The results were not available for five others, due either to disposal of the mare prior to foaling time or failure of the owner to furnish the necessary information.

No pregnancy tests were made on the six mares inseminated with semen shipped to Columbia, however, three foaled and one was disposed of

TABLE XIII

ARTIFICIAL INSEMINATION RESULTS

| Year | Number of Mares | Age of Semen (hrs.) | Pregnancy Test Result* | Foaling Result |
|---|---|---|---|---|
| 1950 | | | | |
| Dilutor - Egg yolk-glucose plus 3 mg. ascorbic acid/10 ml. diluted semen plus 1:50,000 $KMnO_4$. | | | | |
| | 11 | 3 | 1 Positive | 3 Foals |
| | | | 10 Negative | 4 Negative |
| | | | | 4? |
| | 6 | 24 | 1 Positive | 2 Foals |
| | | | 5 Negative | 3 Negative |
| | | | | 1? |
| | 6** | 24 | No Test | 3 Foals |
| | | | | 2 Negative |
| | | | | 1? |
| 1951 | | | | |
| Dilutor - Egg yolk-glucose plus 10 mg. thiamine/10 ml. diluted semen. | | | | |
| | 4 | 3 | 3 Positive | ? |
| | | | 1 Negative | ? |
| | 6 | 24 | 1 Positive | ? |
| | | | 5 Negative | ? |
| | 2 | 48 | 1 Positive | ? |
| | | | 1 Negative | ? |
| | 1 | 72 | 1 Negative | ? |
| Dilutor - Egg yolk-glucose plus 5 mg. pyridoxine/10 ml. diluted semen. | | | | |
| | 2 | 3 | 2 Negative | ? |

*Used immature femal rats for pregnancy test.

? Foaling results not obtained.

**Semen shipped to Columbia for insemination.

prior to foaling time.

During the 1951 breeding season five of 15 mares inseminated gave a positive pregnancy test, however, none of these mares have had time to foal (Table XIII). Therefore, the accuracy of these inseminations cannot be evaluated. On the basis of the results of the preceding year, it might be expected that more than five mares would foal.

# CHAPTER IV

## DISCUSSION

The average volume (73.3 ml.) and pH (7.20) per ejaculate obtained in this study are in agreement with data reported by other workers, however, the average concentration (492,391/mm.$^3$) is somewhat higher than in other reports (Anderson, 1945). The slight change in pH of both diluted and undiluted semen seems to agree with reports (Sergin, 1935, 1937) (Davis and Cole, 1939) that little if any lactic acid is produced in stallion semen. Jack semen apparently has a reasonably high inherent buffering capacity since little change in pH was noted in semen stored for 72 hours. This is in agreement with data reported by Bogart and Mayer (1950).

Dilutors are necessary for maintaining the motility of jack sperm since no motility was observed in any undiluted samples the day after collection, and the egg yolk-glucose dilutors were superior to all other dilutors studied. This is in agreement with the report of Bogart and Mayer (1950).

Confirming the report of Soliven and Gonzaga (1940) that no significant influence on sperm longevity was noted when vitamins A, B, C, and D were added singly, in this study the addition of various levels of ascorbic acid, niacin, riboflavin, or thiamine, when added singly to semen diluted with the basic egg yolk-glucose diluent, showed no significant influence on sperm motility at the end of 96 hours of storage when compared with the egg yolk-glucose control. However, the

addition of three or five mg. of pyridoxine to ten ml. of diluted semen did show a highly significant beneficial effect when compared with the egg yolk-glucose diluted control. The exact manner of the stimulatory effect of pyridoxine is not known.

Despite the fact that no significant beneficial effects on sperm motility, due to the addition of ascorbic acid, niacin, riboflavin, or thiamine to the diluted semen, were found at the end of 96 hours of storage, the data suggest that some stimulatory effects were exerted during shorter periods of storage. The decrease in motility after first being stimulated may have been due to the fact that because of excessive stimulation of motility during the first part of the storage period, the sperm may have used up their reserve energy and therefore were unable to maintain the higher degree of motility for a longer period of time. However, the addition of three or five mg. of pyridoxine to ten ml. of diluted semen did show a highly significant beneficial effect on sperm motility at the end of 96 hours of storage.

The reports of Patrick (1949) and Good (1950) that the addition of minute amounts of potassium permanganate to diluted jack semen prolonged sperm motility at a higher level were confirmed in this study. Good theorized that the permanganate may be oxidizing the metabolic regulator of Lardy, et. al. (1949). In this manner glycolysis is increased and energy utilization decreased so that motility is maintained over longer periods of storage. This may explain the fact that significant differences between the control and treated samples did not appear until sometime after 96 but by 120 hours of storage.

Although some workers (Camici and Polestra, 1939) (Chang, 1943) obtained better storage results with semen by removal of a part of the seminal fluid by centrifuging and then diluting than by diluting uncentrifuged semen, this was not true in this study. The mechanics of centrifugation could have produced a harmful effect on the sperm in some manner so that normal motility could not be maintained.

Due to insufficient data the fertility results obtained in this study are not conducive to recommending the use of any particular dilutor. However, due to the simplicity and ease of preparation, the egg yolk-glucose dilutor is recommended for practical use in the field. The addition of three or five mg. of pyridoxine to diluted semen is not recommended unless it is necessary to use semen stored for 72 hours or more. Likewise, it is not recommended to add potassium permanganate to diluted semen unless the situation requires the use of semen stored for 120 hours or longer. The necessity for accurately controlling the amount of added pyridoxine is indicated by the fact that three or five mg. was beneficial, whereas, ten mg. was definitely detrimental to maintenance of sperm motility. Although only an observation and not investigated further, it was noted that sperm motility was maintained better in those samples where the vitamins and permanganate were prepared just prior to collection as compared to preparation several days prior to use.

In this study the procedure of obtaining fertility data in mares inseminated with diluted semen leaves much to be desired. Adequate numbers of mares were not available for an intensive study of any one

dilutor. Furthermore, the fact that the majority of the mares were not used regularly in farm work and the inability of the owner to detect estrus in many cases prevented insemination at an optimum time in the heat period. Likewise, these same conditions prevented detection of return to estrus in those cases where the mare did not conceive on the first service. Inseminations on alternate days as long as the mare remains in heat has been recommended as the most effective procedure for obtaining a high conception rate (Andrews and McKenzie, 1941). The widely scattered locations of the mares used in this study made such a procedure impractical.

If artificial insemination of mares, under field conditions, is to result in a satisfactory conception rate the following conditions are highly desirable: (1) a single insemination must be closely timed with reference to the end of the estrual period; (2) successive inseminations must be made on alternate days during the heat period with relatively fresh semen; or (3) successive inseminations on alternate days during the heat period with semen from a single ejaculate. The latter condition would be possible only if satisfactory sperm motility was maintained for a period of four days or more. For this reason the primary emphasis was placed in the laboratory studies on sperm motility at the end of 96 hours of storage.

A factor which has not been discussed, but of which the investigator was cognizant, was the differential response of the semen in different ejaculates of the same jack as well as between jacks. Examination of the data in Tables IV, V, VI, VII, VIII, X, and XI demonstrate this fact. This

variable response between ejaculates is further demonstrated by the difference in time that any sperm remained motile in the control egg yolk-glucose dilutor (Example - Figures 2 and 3). This probably reflects season and other environmental influences (Anderson, 1945) upon the ability of the sperm to survive during storage. It would have been desirable to study the effects of all dilutors on motility in the same ejaculate. This was not done.

# CHAPTER V

## SUMMARY

In this study the average volume, concentration, initial percent of live sperm, and pH of fresh jack semen was 73.3 ml., 492,391/mm.$^3$, 86 percent, and 7.20 respectively. The pH of undiluted semen increased during 72 hours of storage from the initial 7.20 to 7.42. The pH of fresh semen (undiluted, pH 7.20) after diluting at a one to one ratio with an egg yolk-glucose dilutor (one part egg yolk to four parts of five percent glucose solution) was 6.71 and decreased to 6.56 after 72 hours of storage.

Semen diluted with the above basic egg yolk-glucose dilutor consistently maintained a higher degree of sperm motility for a longer period of time than did semen diluted with other dilutors containing egg yolk-glucose-phosphate (GYP), egg yolk-glucose-sodium potassium tartrate (TGL), egg yolk-glucose and sodium citrate, egg yolk and sodium citrate, egg yolk and sucrose, egg yolk and gum arabic, egg yolk and gum ghatti, glucose and gum arabic, glucose and gum ghatti, glucose and lecithin, or varying quantities of lecithin.

Fresh semen diluted with the basic egg yolk-glucose dilutor containing three or five mg. of added pyridoxine showed a highly significant higher level of sperm motility at the end of 96 hours of storage than did semen diluted with the basic egg yolk-glucose dilutor containing no added pyridoxine. Varying levels of ascorbic acid, niacin, riboflavin, or thiamine showed no significant beneficial effect on sperm motility at

the end of 96 hours of storage when compared with the basic egg yolk-glucose dilutor, however, there was some indication of beneficial effect during shorter periods of storage.

Fresh semen diluted with the egg yolk-glucose dilutor containing a sufficient amount of potassium permanganate to obtain a final concentration of 1:50,000 added at the time of initial dilution or of 1:25,000 added 48 hours after initial dilution showed a significantly higher level of sperm motility at the end of 120 hours of storage when compared with the basic egg yolk-glucose dilutor; however, no level of permanganate added to egg yolk-glucose diluted semen showed a significant beneficial effect at the end of 96 hours of storage. Combinations of ascorbic acid or thiamine and potassium permanganate when added to the basic dilutor showed no significant beneficial effect on sperm motility at the end of 96 hours of storage.

The removal of a part of the seminal fluid by centrifuging diluted semen followed by the addition of a sufficient quantity of dilutor to restore the original volume failed to maintain a satisfactory level of sperm motility.

Three of 11 mares inseminated with diluted semen, approximately three hours old at the time of use, produced foals. Five of 12 mares inseminated with diluted semen, approximately 24 hours old at the time of use, produced foals.

Three of four mares inseminated with diluted semen approximately three hours old, one of six mares inseminated with diluted semen approximately 24 hours old, and one of two mares inseminated with diluted semen

approximately 48 hours old at the time of use gave positive pregnancy tests, however, these mares have not had time to foal.

The disparity between the pregnancy tests and foaling results, in which only two of 17 mares tested yielded a positive test but five actually foaled, is not explained.

This study has demonstrated the practicality of the use of jack semen for artificial insemination of mares 24 hours or longer after collection. It is emphasized that although the conditions under which the fertility data obtained in this study were not ideal, these conditions very probably were comparable to those that would be encountered in field insemination of farm mares.

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