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Survival of Bovine Spermatozoa in Media Containing a Lipoprotein Complex Isolated From Spermatozoa and Other Sources

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ABSTRACT

Semen specimens in diluting media containing a lipoprotein complex extracted from spermatozoa, testicular tissue, or thymus gland were subjected to three distinct survival tests. Portions of these semen specimens were subjected to the survival tests in a basic control diluter, which contained neither egg yolk nor lipoprotein. Other portions were tested in an egg yolk-citrate control diluter and in the basic diluter with a preparation of pulverized spermatozoa added.

The diluter containing the pulverized spermatozoa yielded results in all three survival tests similar to those obtained with the basic control diluter. Apparently, the lipoprotein complex in the spermatozoa cannot protect other spermatozoa unless it is first released from a bound condition by the alkaline extraction procedure.

In all tests the egg yolk-citrate diluter was superior to those containing the lipoprotein complex from all three tissues, except during a two-day storage test. In the two-day storage test the egg yolk citrate diluter and that with the testicular lipoprotein complex gave identical results. Extraction of the lipoprotein complex of thymus gland origin with ether apparently removed a constituent or altered the complex so that it no longer protected the spermatozoa during the survival tests.

In all of the experiments the testicular lipoprotein complex, which may contain extractants in addition to the lipoprotein complex, was superior to the complex isolated from spermatozoa and thymus gland in maintaining spermatozoa motility and survival.

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INTRODUCTION

The atypical haploid cells, mammalian spermatozoa, were first observed in the 17th century by John Hamm of Arnhem. Since that time interest in these cells has stimulated fundamental investigations of their morphology and, later, of their physiology. It was not until the latter half of the 19th century that chemists began to isolate and characterize the constituents of spermatozoa and other cells. Interest in the mechanism of reproductive processes, in animal breeding, and in artificial insemination acted as stimuli to further research into the chemical constitution of mammalian spermatozoa, especially those of the farm animals.

With the advent of adequate techniques, the study of cellular chemistry, especially the chemistry and physiological activities of spermatozoa, has become a part of a relatively new field of biochemistry. Many cellular processes could not be explained entirely on the basis of existing morphological or physiological knowledge. Hence it became evident that the biochemist must study the complex chemistry of these cells prior to a final solution of these problems.

As physiological and chemical knowledge of mammalian spermatozoa accumulated, simple and complex lipid constituents became implicated more and more in the metabolic and physiological functioning of spermatozoa.

Kampschmidt, Mayer, and Herman (1953) isolated a lipoprotein from egg yolk which, when added to the diluting medium of bull spermatozoa, was able to protect the spermatozoa from the detrimental effects of adverse environmental conditions.

Thomas and Mayer (1949) and Orsini (1956) isolated a lipoprotein complex from boar and bull spermatozoa which comprised approximately half of the total mass of these cells. Hence, it was feasible that this lipoprotein complex from the sperm cell should also be capable of protecting these cells under adverse conditions. Perhaps, one of the functions of the spermatozoan lipoprotein complex may be its protective activities.

This investigation is mainly concerned with the lipoprotein complex of bull spermatozoa. A lipoprotein complex was also extracted from thymus gland tissue and bull testes tissue to compare the relative protective potentialities of lipoproteins from several sources.

In this investigation the semen samples were in a basic diluter to which the substances under investigation were added. Samples were subjected to cold shock, incubation, and storage tests. These treatments provided adverse environmental conditions detrimental to spermatozoan viability, and were excellent tests of the ability of the spermatozoa to withstand adverse conditions.

REVIEW OF LITERATURE

Storage of Spermatozoa

Preservation of the viability of spermatozoa for prolonged periods of time is a major problem in animal breeding. In semen plasma, the mixture of accessary gland secretions accompanying the spermatozoa at ejaculation, spermatozoan survival is of relatively short duration. Hence, if spermatozoa are to be stored for periods longer than two or three days, semen plasma alone is an inadequate medium; the addition of certain substances is necessary. Among these substances are ones which enable spermatozoa to withstand the adverse environmental conditions which develop during storage as a result of metabolic and other physical or chemical activities.

Phillips (1939) introduced the egg yolk-buffer medium, which not only increased the volume of the semen samples but aided the sperm cells to resist the adverse storage conditions.

Phillips (1940) showed further that the yolk-buffer diluent maintained the activity of spermatozoa at a level equivalent to that of the original semen for a period dependent upon the quality of the original semen sample.

The egg yolk-buffer mixtures not only prolonged the survival but maintained the fertilizing capacity of bull spermatozoa, according to Phillips and Lardy (1940). They reported occurrences of pregnancy with semen stored with egg yolk-buffer for 180 hours. Data on over 1400 inseminations in an artificial insemination association indicated that, when proper precautions are taken in handling the semen, it can be diluted and stored up to 4 days with results as satisfactory as those with semen used on the first day of collection (Anderson, 1945).

Easley, Mayer, and Bogart (1942), Lasley and Mayer (1944), and Mayer and Lasley (1945) have definitely established the presence in egg yolk of a distinct factor which has the property of protecting spermatozoan survival during storage and other adverse conditions such as rapid cooling.

"Cold Shock" of Spermatozoa

Milovanov (1934) found that in a glucose-phosphate medium, spermatozoan motility ceased completely at 0° C if rapidly cooled to this temperature. He termed the harmful effect of rapid cooling "temperature shock."

The phenomenon of temperature or cold shock of sperm cells has received much attention since Milovanov's early experiments. At the Missouri Experiment Station workers have used this phenomenon as the basis of a simple technique for determining the relative resistance of spermatozoa in a semen specimen to the adverse effects of rapid cooling. In performing this test, a small portion of semen in a vial is placed in a water bath at 0° C for a 10-minute period. The percentages of live spermatozoa in the specimen prior and subsequent to the 10-minute cold shock are determined by a differential "live-dead" staining technique (Easley, *et al.,* 1942; Squires, *et al.,* 1951).

Gladcinova (1937), Milovanov (1934), and Walton (1930) showed that cold shock was definitely injurious to spermatozoa and that only a small percentage of the spermatozoa survived rapid cooling in semen with no protecting agent.

Experimenting with range bulls in Arizona, Lasley and Bogart (1943) found that some spermatozoa in a semen specimen were protected from injury if egg yolk was present in the diluter. When the semen was diluted with an egg yolk-buffer solution, the spermatozoa were protected in some manner so that a large percentage of them were able to survive either a 10-minute cold shock or the conditions encountered during storage at 5° C.

Lasley, Easley, and Bogart (1942) reported that egg yolk-buffer diluent increased the ability of spermatozoa to withstand cold shock by an average of 400 percent and increased storage time approximately 600 percent over non-diluted semen. They also found that spermatozoa from some bulls "resisted" cold shock better than that from others. Their results indicated the cold shock technique could be used as an index of the ability of the spermatozoa to survive during storage at 5° C in an egg yolk-butter diluent.

That the percentage of "live" or unstained spermatozoa surviving a 10-minute cold shock in semen diluted with an egg yolk-buffer diluent was significantly correlated with fertility was reported by Swanson and Herman (1944) and Lasley and Bogart (1943).

According to Herman and Madden (1943) the spermatozoa surviving the cold shock may be expected to maintain motility for two to five days under proper storage conditions.

Incubation of Spermatozoa

Spermatozoa exhibit rather vigorous metabolic activities during the period of storage. The rate of these metabolic reactions is dependent upon the concentration of spermatozoa, the relative vigor of the individual cells and the in-

cubation temperature. Several other factors are responsible for the progressive decrease in metabolic activities during the storage period. These include the available energy supply, osmotic pressure, pH, and the resistance of the spermatozoa to adverse conditions.

Incubation of spermatozoa at temperatures of 37° C or higher for short periods of time provides a rapid means of determining their potential survival time at storage temperatures near 5° C. Higher temperatures increase the rate of the various metabolic activities and all related biological process of the sperm cells. The rate of one of these activities at the incubation temperature may serve as a measure of the relative survival time of the spermatozoa at 5° C.

The longevity and viability of spermatozoa during storage is a good index of the fertilizing capacity of the spermatozoa according to Herman and Madden (1953). Therefore, the incubation test is a means of ascertaining the storage potentialities and fertilizing capacity of spermatozoa. The method is simple, requiring one hour or less for its completion.

For example, Beck and Salisbury (1943) showed that the decrease in motility of spermatozoa in bull semen diluted with a yolk-citrate medium and stored 10 days at 5° C., after having been brought to storage temperatures in steps of 5° drop each 10 minutes, was positively and significantly correlated with the decrease in motility for similar samples stored in water baths for one hour at 46.5° C. They further showed that spermatozoa in an egg yolk diluent survived the incubation period better than those without egg yolk in the diluting medium.

Components of Egg Yolk Which Favorably Affect or Protect Spermatozoan Survival

From the preceding discussion, it is evident that egg yolk may be providing more than the resistance factor to the spermatozoa during incubation or storage. Bogart and Mayer (1950) suggested that egg yolk might contribute two distinct factors—one which protects the spermatozoa against adverse environmental conditions (a resistance factor) and a second which contributes in some manner to the maintenance of spermatozoa survival (a storage factor).

Lardy and Phillips (1941) found while testing various isolated components of egg yolk that the component responsible for the maintenance of spermatozoan motility was lecithin. They considered phospholipids to be the substrate, in the absence of a metabolizable sugar, for the non-glycolytic activities of these cells.

In 1945, Mayer and Lasley were able to isolate from egg yolk a small fraction soluble in phosphate buffer which would protect a higher proportion of the spermatozoa from temperature shock than would whole egg yolk.

A mixture of lipoproteins was insolated from egg yolk by Kampschmidt, Mayer, and Herman (1953). The lipoproteins were active both as resistance factors and as storage factors. The isolated phospholipid constituents of the egg yolk lipoproteins were active as a resistance factor, but were inferior to the intact

lipoproteins as storage factors. The phospholipids of the egg yolk could be replaced either by pure lecithin or cephalin. These experiments indicate that other egg yolk constituents, in addition to those required for the protection of spermatozoa from temperature shock, are probably needed during the prolonged storage of bull spermatozoa at low temperatures.

Although previous studies in this laboratory and elsewhere had shown that cold shock resulted in a lowering of the percentage of live spermatozoa, no evidence regarding the nature of the detrimental effects of rapid cooling was available. The work of Ryan (cited by Mayer, 1955) showed that cold shock adversely affected the metabolic activities of bull and ram spermatozoa. Aerobic glycolysis was affected to the greatest extent, but both anaerobic glycolysis and endogenous respiration were reduced markedly.

In 1957, Bialy *et al.* extended the use of lipoprotein to frozen semen. They concluded that a 5 percent level of added lipoprotein increased the spermatozoan survival rate approximately 5 percentage units over that of a control during the low temperature freezing of semen.

Chemical Nature of Lipoproteins

The term lipoprotein refers to a conjugated molecule comprised of a protein and a phospholipid. The phospholipid is usually cephalin or lecithin, but other lipid substances may be present. The protein-lipid bonds are not easily split by treatment with ether, but alcohol, especially after heat denaturation of the protein, breaks the lipid-protein bond.

Lecithin and cephalin are phospholipids. Upon hydrolysis lecithin yields fatty acids, glycerol, phosphoric acid, and the nitrogenous base, choline. Choline constitutes about 15 percent of the lecithin molecule. Cephalins yield the same substances except that ethanolamine, serine, or inositol replaces choline as the base.

It is assumed that the membrane of cells in mammalian tissues is a lipoprotein structure of some type.

Isolation of Lipoprotein From Mammalian Spermatozoa

Miescher (1897) was perhaps the first worker to extract lecithin from spermatozoa. He found that the ether-extractable material from salmon spermatozoa consisted of 50 percent lecithin, 14 percent cholesterol and 35 percent fat.

Thomas and Mayer (1949), experimenting with spermatozoa of the boar, obtained an acidic-type protein fraction precipitable at pH 6 and similar to a fraction previously isolated from thymus nuclei by Mayer and Gulick (1942). This fraction was obtained from the boar spermatozoa by extracting these cells with alkali followed by precipitation at about pH 6 with acetic acid.

The work of Wang *et al.* (1950) demonstrated that the pH 6 fraction or acidic protein of rat liver nuclei was essentially a lipoprotein comprised of phos-

pholipids, proteins, cholesterol, and possibly other substances. This lipoprotein complex behaved as a unit electrophoretically.

Lipe and Mayer (1955) showed by various chemical tests that the alkali-soluble acidic protein precipitable at pH 6, which had been isolated from boar spermatozoa by Thomas and Mayer (1949), was also a lipoprotein. It constituted 48 percent of the dry weight of boar spermatozoa.

Madera-Orsini (1956) isolated a similar lipoprotein fraction from bull spermatozoa. The spermatozoa were first washed with 1 molar sodium chloride and then washed with glass distilled water. The lipoprotein fraction was extracted with 0.01 N. sodium hydroxide solution and precipitated from solution at about pH 6 with dilute acetic acid. The lipoprotein fraction constituted about 42 percent of the dry weight of bovine spermatozoa. Chemical studies of this fraction showed it to be a complex molecule containing protein, phospholipid, cholesterol, glucosamine and glucuronic acid. The protein portion contained 0.5 percent phosphorus, 14.6 percent nitrogen and a high percentage of basic amino acids. The lipid was shown to be tightly bound in the complex but could be extracted with a chloroform-methanol mixture. Electrophoretically, this lipoprotein complex also behaved as a single entity possessing a low electrical charge.

MATERIALS AND METHODS

Collection of Semen

Semen from dairy bulls of the Holstein, Jersey, and Brown Swiss breeds, was obtained from the University of Missouri Dairy Department. The semen was collected with an artificial vagina and transported to the laboratory as quickly as possible. In the laboratory some of the semen specimens were pooled. The spermatozoa were separated from the semen plasma by centrifugation and the lipoprotein-carbohydrate complex to be used in subsequent experiments was extracted from the spermatozoa. The remainder of the semen specimens were used in the comparative "survival test" experiments, which included the cold shock technique, incubation at 46.5° C for one hour, and storage at temperatures near 5° C for 10 days.

Evaluation Tests

A. Motility Determination: The motility was examined under the low power objective (10 X) of the microscope and rated on a scale from 5 to 0, 5 signifying maximum motility and 0 indicating complete absence of motility.

B. Differentiation of Live from Dead Spermatozoa: A stain described by Mayer *et al.* (1951) was used to differentiate live spermatozoa from dead. Using this stain, the dead cells stained blue whereas the live cells remained unstained. The cells were counted under a magnification of 880X.

Survival Tests

Since the advent of artificial insemination several techniques have been used for assessing the relative ability of substances in the semen diluent to maintain the life activities of spermatozoa. Three survival tests were selected for this investigation. They tested the ability of the factor being investigated to maintain spermatozoan survival under three different types of adverse environments. The tests were: (1) incubation at 46.5° C for 1 hour, a temperature at which spermatozoan metabolic activities are at a maximum rate; (2) storage at temperatures near 5° C for 10 days, a prolonged storage period at a low temperature during which changes in pH, osmotic pressure, and other factors enhance the detrimental effects of the low temperature; and (3) the cold shock technique in which the cells are subjected to sudden and rapid cooling to 0° C and then maintained at this low temperature for 10 minutes, a rather drastic test of survival under adverse conditions.

Dilution of the Semen Samples

Prior to performing the storage, incubation, and cold shock tests on the semen, all samples were diluted with a basic diluent to which the substance under test was added.

In previous investigations at the Missouri Agricultural Experiment Station, it was shown that egg yolk contained a lipid component which protected mammalian spermatozoa from adverse environmental conditions, including rapid changes in temperature. This factor was designated as the "resistance factor." Boiled milk, chick embryos, follicular fluid, lipoproteins from egg yolk and phospholipids from a variety of sources were also shown to act as a resistance factor. As stated previously, the prime purpose of the present investigation was to ascertain the effects of the lipoprotein complex of spermatozoa and other cells upon bovine spermatozoa during periods of adverse environmental conditions. In addition to the lipoprotein complex extracted from spermatozoa, this complex was also isolated from thymus gland and bull testis tissues. A comparative study of the complex from three different cell types would determine whether there was any difference in the ability of this complex from the different cells to protect bovine spermatozoa under the experimental conditions. In some cases a preparation of pulverized spermatozoa was added to the diluter prior to incubation or cold shock to compare effects of the extracted lipoprotein complex with effects of that bound in the spermatozoon.

Egg yolk, shown to be valuable in a semen diluent during long periods of storage by Mayer and Lasley (1945) and Lasley and Mayer (1944), was also used in some of the diluters as a control. These control samples enabled a comparison between the effects of egg yolk and those of the lipoprotein complexes.

The basic components of the diluters, in addition to the lipoprotein fractions, were:

1. 4 percent sodium citrate solution.
2. 5 percent glucose solution.
3. 10 percent sucrose solution.

The ratios of these constituents by volume in the diluters used in the survival tests and to which egg yolk or one of the lipoprotein preparations was added were:

1. 20 percent of the sodium citrate solution.
2. 15 percent glucose solution.
3. 45 percent sucrose solution.
4. 20 percent of the egg yolk or suspension of the lipoprotein fraction being investigated.

The lipoprotein complexes, regardless of source, were all handled in the same manner. A 0.25 gram portion of the complex was placed in a glass tissue grinder with 1 ml. of a 4 percent solution of sodium citrate. The two were thoroughly mixed to form a fine suspension. The suspension was added to the diluter to equal 20 percent by volume of the total diluter. The pulverized spermatozoa preparation was added to the diluter by a procedure similar to the one employed with the lipoprotein complex.

A basic control diluter which contained no egg yolk or lipoprotein complex was comprised of:

1. 25 percent by vlume of the sodium citrate solution.
2. 56 percent by volume of the sucrose solution.
3. 19 percent by volume of the glucose solution.

In all cases, the semen and the diluter were combined in a 1 to 10 ratio, one part semen to 10 parts diluter.

Extraction of the Lipoprotein Complex From Spermatozoa

The method of extracting the lipoprotein complex from spermatozoa was essentially the same as that described by Orsini (1956) and given here in less detail.

The pooled bull semen specimens were centrifuged for 15 minutes. The separated semen plasma was removed. Spermatozoa were then washed and recentrifuged four times using 1 M sodium chloride as the wash solution. The purpose of this washing was to remove any semen plasma and plasma protein adhering to the spermatozoa. After the fourth washing, the supernatant was tested with a few drops of 10 percent trichloroacetic acid to assure completeness of the washing procedure. If the supernatant was free of plasma proteins, no precipitate formed upon addition of the acid and this indicated that all protein had been removed. If a positive trichloroacetic acid precipitation was noted, the spermatozoa were rewashed with 1 M sodium chloride solution until a negative test resulted.

Sperm cells were further washed with glass double-distilled water to remove any traces of the sodium chloride remaining from the preceding washes. This process was continued until the water washes were free of chloride ions. A 0.5 M silver nitrate solution was used to test for the presence of the chloride ion.

The washed spermatozoa were mixed with 0.01 M sodium hydroxide solution and mascerated in a small ground glass tissue grinder. The resulting mixture was then poured into a flask and stirred for 30 minutes.

After the 30-minute extraction period the mixture was centrifuged for 15 minutes. The supernatant was removed and used for the precipitation of the lipoprotein complex.

For the precipitation of the lipoprotein complex, 5 percent acetic acid was slowly added to the supernatant until a fine precipitate formed at a pH of 5.8 to 6.0. After this occurred the extract was centrifuged for 15 minutes to remove the precipitate. At the end of the centrifugation period, the supernatant was discarded and the remaining residue, the lipoprotein complex, was washed twice with glass double-distilled water and then placed in a freezer until it was used for the experiments. The lipoprotein fraction was never stored longer than 8 hours before being used.

Extraction of the Lipoprotein Complex From the Thymus Gland

The glands were obtained from young beef cattle. They were first washed with distilled water and then homogenized in a 4 percent solution of sodium citrate in a Vir Tis "45" homogenizer. The lipoprotein fraction was extracted by the procedure described above.

Extraction of Lipoprotein Complex From Bull Testes

The testes used in this study were from Hereford bulls, approximately two years old. The homogenization method and subsequent procedure were the same as those used with the thymus gland tissues.

RESULTS AND DISCUSSION

The semen samples plus diluters containing the lipoproteins were subjected to the rigorous conditions of three survival tests. Each of the survival tests was a measure of the ability of the sperm cells in a particular diluter to survive adverse environmental conditions. Each of these survival tests represented a different type of adverse conditions. Hence, the results should be an adequate criterion of the protective capabilities of the various substances. results of the three survival tests are presented separately.

Cold Shock

Table 1 gives motility ratings of the spermatozoa and the percent of live or unstained spermatozoa in the various experimental diluters after subjection to

TABLE 1--COMPARISON OF MOTILITY AND PERCENT LIVE SPERMATOZOA AFTER SUBJECTION TO 0° C. FOR 10 MINUTES IN THE VARIOUS DILUTERS

Diluter	Motility Rating	Percent Live
Egg Yolk	2.5	68
Spermatozoan lipoprotein	1.5	48
Testicular lipoprotein	2.0	65
Thymus gland lipoprotein	1.5	50
Thymus gland ether-extracted lipoprotein	1.0	44
Pulverized spermatozoa	0.5	34
Control	0.5	30

0° C for 10 minutes. The diluent containing egg yolk maintained the highest motility as well as the highest number of live spermatozoa. The diluents containing lipoprotein from the various sources all showed much better results than those in which no lipoprotein complex was present, i.e. the control diluter (sodium citrate, glucose, and sucrose) and the diluter containing pulverized spermatozoa. Extraction of the lipoprotein complex obtained from thymus glands with ether lessened the effectiveness of this complex in the diluent.

The difference in the results observed with the testicular lipoprotein and with the spermatozoan lipoprotein as diluter constituents cannot be explained at this time. It may be due to a difference in the type of phospholipid united with the protein of the lipoprotein molecule or to extraneous substances extracted with the lipoprotein from the testicular tissues.

Incubation

Table 2 shows the motility ratings and percentage of live spermatozoa obtained on experimental samples of diluted semen after incubation at 46.5° C for 1 hour. As was the case after cold shock treatment, the egg yolk-citrate diluter maintained the highest motility as well as highest percentage of live spermatozoa after incubation.

TABLE 2--COMPARISON OF MOTILITY AND PERCENT LIVE SPERMATOZOA OF SPERMATOZOA INCUBATED AT 46.5° C. FOR 1 HOUR IN THE VARIOUS DILUTERS

Diluter	Motility Rating	Percent Live
Egg yolk	1.5	48
Spermatozoan lipoprotein	1.0	32
Testicular lipoprotein	1.0	39
Thymus gland lipoprotein	1.0	28
Thymus gland ether-extracted lipoprotein	0.0	17
Pulverized spermatozoa	0.0	14
Control	0.0	16

Note Table 2 that three of the diluters failed to maintain the motility of the spermatozoa during the one-hour period of incubation. Two of these, the control diluter and the one containing the pulverized spermatozoa, did not contain an isolated lipoprotein complex. In the third, the diluter did contain the isolated thymus gland lipoprotein complex which had been extracted with ether prior to its utilization in these experimens. These results suggest that the ether altered the complex or removed some important substance from the lipoprotein complex. The favorable results with the other three lipoprotein-containing diluters demonstrates the ability of the lipoprotein complex from a variety of sources to protect bovine spermatozoa during incubation at 46.5° C for one hour.

Storage

In the storage experiments only four of the diluters previously described were used: the egg yolk-citrate, control, spermatozoan lipoprotein-citrate, and the testicular lipoprotein diluters. The storage was for a period of 10 days with one exception; that in which the testicular lipoprotein diluter was being tested. It was stored for two days only.

Table 3 gives motility ratings and percentage-of-live-spermatozoa determinations in the various diluters during the storage period. The diluters containing

TABLE 3--MOTILITY RATINGS AND PERCENT LIVE SPERMATOZOA DURING A PERIOD OF STORAGE AT 3-6° C. IN VARIOUS DILUTERS

Days Storage	Egg Yolk-Citrate		Control		Spermatozoan Lipoprotein	
	Motility	% Live	Motility	% Live	Motility	% Live
1	3.0	75	1.5	48	2.5	67
2	3.0	73	1.5	46	2.0	66
3	2.5	73	1.0	44	2.0	66
4	2.5	70	0.5	43	2.0	63
5	2.0	69	0.5	40	1.5	58
6	2.0	68	0.0	37	1.5	55
7	1.5	62	0.0	33	1.0	50
8	1.5	60	0.0	30	1.0	49
9	1.0	58	0.0	29	0.5	47
10	1.0	55	0.0	25	0.5	45

the lipoprotein complex again gave better results than the basic control diluter. Some degree of motility was observed in all diluters containing the lipoprotein up to and including the 10th day, whereas motility ceased in the control diluter after the 5th day of storage.

Table 4 demonstrates an important difference between the results obtained during storage and those obtained with the cold shock and incubation tests. Note that little or no difference existed between storage capabilities of semen in the egg yolk-citrate diluter and in the diluter containing the testicular lipoprotein complex during a two-day storage period. In the cold shock and incubation

tests the egg yolk-citrate diluter proved superior. However, two-day storage may not subject the spermatozoa to environmental conditions as rigorous as those provided during cold shock or incubation.

TABLE 4--MOTILITY RATINGS AND PERCENT LIVE SPERMATOZOA DURING A PERIOD OF STORAGE AT 3-6° C. IN EGG YOLK-CITRATE AND TESTICULAR LIPOPROTEIN-CITRATE

Day	Egg Yolk-Citrate		Testicular Lipoprotein Citrate	
	Motility	% Live	Motility	% Live
1	3.0	77	3.0	75
2	2.5	73	2.5	70

The diluter containing the lipoprotein complex from spermatozoan sources did not maintain spermatozoan survival (percent live cells) as well as the testicular lipoprotein medium. This may be explained on the basis that the testicular lipoprotein preparation contains some factor which is not present in the spermatozoan lipoprotein complex. The nature of this factor is not known at present but the evidence suggests that these two lipoprotein preparations may differ in some manner. The difference in the effects of these two lipoproteins upon spermatozoan survival during storage may be due to (1) a contaminant in the testicular lipoprotein, (2) a difference in chemical constitution, (3) different stereochemical configurations or (4) to a difference in physical characteristics. In subsequent investigations these differences in the lipoproteins from the two sources may be ascertained and, possibly, yield information regarding the nature of the environmental requirements of spermatozoa for maximum survival.

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