VIABILITY OF SPERMATOZOA OF CHICKENS UNDER VARIOUS ENVIRONMENTAL CONDITIONS

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Clyne Samuel Shaffner

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CONTENTS

INTRODUCTION

How to increase the reproductive capacity of the most desirable males is an important problem in Poultry Husbandry. The process of artificial insemination is being attempted as a possible solution of the problem but the success of the process is dependent on the preservation of the viability of sperm over long periods of time. The problem of finding a suitable method of semen preservation is made especially difficult by the fact that there is no accurate way of determining fertilizing capacity other than by insemination.

In early research work it was supposed that a temperature very near that of the animal's body was needed to maintain the viability of the sperm. Later work, however, tends to indicate that spermatozoa reacts very much as a cold blooded animal, in that they survive in a rather wide range of temperatures. Whether or not the spermatozoan is a sufficiently complete organism to take nourishment from the supporting media is not definitely known. It is common knowledge that motility of the sperm continues to decrease until the time of death of the cell. In other small organisms inactivity tends to prolong life, hence, it might be reasoned that inactivition of the sperm cell might be a good way to prolong its life. With the above thoughts in mind this experiment was begun to study the effect of various environmental factors on the viability of spermatozoa of chickens.

REVIEW OF LITERATURE

It has been demonstrated by Munro (10) that mature sperm will live for three or four weeks in the isolated ductus deferens of the male. That spermatozoa live three to four weeks in the hen's oviduct is suggested by the fact that hens sometimes lay fertile eggs for as many as 29 days following a single mating. (Nicoloides)(12). These facts have led workers to believe that if the proper conditions were provided, life in vitro could be maintained similar lengths of time. Attempts to meet these requirements have mainly been concerned with the environmental factors of temperature and the supporting media.

Munro (9) accounts for the long life of spermatozoa in the oviduct by an interaction of the high body temperature of the bird and body fluids. He finds that sperm serum, blood serum, and uterine fluid support motility at 105°F., but that fluid from the magnum and infundiblum inhibit motility at that temperature. He suggests that the spermatozoa propel themselves part way up the oviduct, become immobile without functional impairment at the isthmus, and are carried on to the infundiblum by ciliary action. Various concentrations and dilutions of physiological salt were tried by Iskikawa (3) but in no case was he able to maintain viability for more than three or four hours. He also reports that blood serum was injurious and that motility was maintained somewhat longer in egg white than in physiological salt. Munro (7) in his discussion of the effect of densities mentions that synthetic diluents containing sodium sulphate, glucose, and peptone supported motility in vitro but affected fertilization capacity in proportion to amount used. Phillips (15) has been able to maintain functional viability of bull semen for as long as 300 hours by diluting the semen with an egg yolk solution buffered with sodium and potassium acid phosphate.

The temperature of storage is one of the more easily controlled factors of environment and temperature influences have received considerable attention. Temperatures varying from that of the body of the bird to near absolute zero have been tried. Munro (9) using a warm field microscope found that the greatest activity of fowl sperm occurred at about 28°C. and that immobilization sets in as the temperature approaches either freezing or body heat. Ishikawa (3) suggests that temperature somewhat below that of the hen's body as optimum for storage. Walton (14) experimenting with rabbit semen tried storage temperatures ranging from 0°C. to 45° C. He found that the period of survival increased as the temperature raised from 0° C. to a maximum of 15° C. From 15° C. to 45° C., the survival period decreased and death occurred at 45° C. Gonzaga and Valenzuela (2) suggest a temperature of 2° C. for storage of the semen of the stallion. Investigating the effect of temperature on the survival of bull spermatozoa Komatosa (5) found that no injury occurred between 2° C. and 52° C. Phillips as previously quoted, recommends a storage temperature of 7° C. for bull semen.

Sub-zero temperatures initiated by some method of quick freezing have been suggested by Goetz (13) as a possible means of maintaining life over long periods of time. He estimates that in a storage temperature of -252° C. a cell would age only about a minute in 10,000 years. With this same thought in mind, Joknel (4) found that a few human spermatozoa recovered motility after being frozen and stored at -79° C. for 40 days, at -196° C. for 52 hours, and at -269.5° C. for 3 hours. Luyet and Hodapp (6) reporting the resistance of frog spermatozoa to freezing state that about 20 per cent resume motion after having been submerged in liquid air and then immediately warmed in a water bath at 20° C. Nelson (11) found that no spermatozoa of chickens resumed motion after being quick frozen to -164° C. by

immersion in liquid air.

No reports were found of fertility having been produced by spermatozoa that had been subjected to temperatures below freezing.

PURPOSE

It is the purpose of this study to further the knowledge of the factors influencing the activity of fowl spermatozoa in vitro and to determine if possible, the most satisfactory way of maintaining the fertilizing capacity.

METHODS AND MATERIALS

Semen for the study was secured as needed from six barred rock males, housed in a battery built for that purpose. Infertile hens for use in verifying the fertilizing capacity of the various samples were of two groups. One, a lot of yearling hens being trapnested in a common laying house and having been separated from males for more than five months and the other a lot of fall pullets, having never been with males and housed in isolated laying batteries but in the same room with the males which were being used for semen production. No hen was inseminated more than once.

Semen was collected from the males in the manner described by Burrows and Quinn (1) and a composite sample made. Samples were collected in warmed glassware at room temperatures usually between 8:00 a.m. and 9:00 a.m.

Slides for microscopic work were stained with carbol fuchsin and methylene blue by the technique recommended by Munro (8).

To conserve the original supply of energy of the spermatozoa by decreasing its motility seemed a plausible way to preserve viability. The factors thought to have some effect on motility included osmotic pressure, viscosity of the supporting media, temperature, H-ion concentration, density of the spermatozoa, the presence of certain elements, or any combination of these factors. In this experiment it was decided to work with the factors

of osmotic pressure, viscosity of the media and temperature.

RESULTS

EFFECT OF DEHYDRATION

In preliminary investigations it was found that motility could be reduced and even stopped by diluting fresh samples of semen with various concentrations of sugar solutions. Movement was resumed when the sample was again diluted with physiological salt or with distilled water. Assuming that the sugar might act as a nutrient to the cell and at the same time tend to reduce its movement probably by dehydration, a series of experiments were conducted to determine the effects of different concentrations of the two sugars sucrose and levulose. Since there is no good way of accurately measuring motility as seen under the microscope, a system of relative measurements was adopted. The number four being used to represent the activity as observed in a freshly collected sample, and 0 the point at which no further motion could be observed.

The various concentrations indicated were used diluting the original sample with two times its volume of diluent. Samples were placed in test tubes, stoppered and stored at 5° C.

Table No. 1.

		Storage Time in Hours										
No.	Diluent	6	24	56	80	105	170	194	218	266	290	314
1	1 M Sucrose	1	1	*0								
2	3/4 M "	1	1	1	*0							
3	1/2 M "	4	3	2	1	*1	*1	*1	*1	*0		
4	1/4 M "	4	3	3	3	2	1	1	1	0		
5	3/4 M Levulose	3	3	3	3	3	2	2	2	1	1	0
6	1/2 M "	4	4	3	3	3	2	2	2	1	*0	
**7	1 M " Phy. Salt	*2	*2	*1	*1	*1	*0					
**8	1/2 M " " "	3	2	*1	*1	*0						
9	Control	4	3	2	1	0						

* Diluted with physiological salt

** Samples 7 and 8 were made using physiological salt. (.9% NACL) in place of pure water.

It is interesting to note that in the above experiment motility in all samples, either stopped completely or was greatly reduced for a short period following the making of the dilutions. From the above table it would appear that the dilution with 1/2 to 3/4 M levulose solution had lengthened the life of the spermatozoa, as determined by motility. This increase in length of life later proved to be of no practical value since the dilution destroyed the fertilizing power. This is in agreement with the findings of Munro (7) in which he reported that synthetic diluents were harmful in proportion to amount used.

EFFECT OF INCREASED VISCOSITY

If it may be assumed that the senescence encountered in the aforementioned procedure was the result of expended energy, any procedure which would reduce activity might prolong viability. The addition of gelatin to the sperm suspension media seemed to be one means of increasing viscosity and reducing motion. The gelatin was added but appeared to have no effect on the spermatozoa. When gelatin (4 % by weight) was added to 1/2 M. levulose solutions, a gelatin mixture resulted that set within an hour after being placed in the refrigerator yet was easily liquified by warming to body temperature. Samples of freshly collected semen were diluted with the levulose gelatin mixture and stored in a refrigerator at 5° C. Samples were removed periodically from storage and liquified to verify motility. Spermatozoa so treated, remained viable only three days.

EFFECT OF QUICK FREEZING

Following the thought expressed by other authors that spermatozoa react very much as cold blooded animals, it was reasoned that perhaps quick freezing would not destroy life.

In the first attempt to quick freeze, spermatozoa as collected were

placed in a test tube and immersed in a mixture of solid CO^2 and alcohol (-76^o C.). The samples were thawed very slowly by packing in ice and allowing the ice to melt gradually. With this procedure no cells resumed motion. From the suggestion of Luyet and Hodapp (6) it was thought that partial dehydration of the spermatozoa might improve the technique. It is shown in Table I that the effect of 1/2 to 3/4 M. levulose solutions was not harmful to the life of the cell, but the extent of dehydration was not measured. To improve the technique, fresh samples were diluted with 3/4 M. levulose solutions prior to freezing the cell. To make freezing as rapid as possible, empty test tubes were pre-cooled in the refrigerant and the sample blown from a capiliary pipette into the cold test tube. In this way freezing was brought about almost instantly. When samples so frozen were warmed by immersion in a water bath at 37^o C., an occasional cell resumed motion. In further trial it was found that the warmer the water bath used, or the more rapid the thawing, the better were the results. To help in the process of quick thawing small tinfoil envelops .015 inches thick and about 5 cm. square were constructed to replace test tubes as containers. The diluted semen sample was then placed in the envelop, the sides of the envelop pressed together (to make the sample as thin as possible) and then immerged in the refrigerant. For prolonged storage these envelops were removed from the alcohol CO^2 mixture, quickly blotted between pieces of chilled blotter paper (to remove excess alcohol) and then stored between pieces of dry ice in a small box lined with Celotex. Envelopes of .005 in thickness were thought to give a slightly better result but were found objectionable in that small pin holes often developed letting in enough alcohol to spoil the sample. To determine the maximum temperature that could be used for thawing envelops containing fresh semen were immersed in water baths of various temperatures. It was found that no noticeable damage occurred

with temperatures as high as 45° C. In thawing frozen samples they were removed from the refrigerant or from storage, as the case might be, and immediately dipped into water at a temperature of 45° C. Samples allowed to thaw by exposure to air at room temperature showed no motility. Since alcohol absorbs more CO° at lower temperatures than at high, both freezing and thawing were somewhat delayed by the formation of bubbles when the warm envelop was placed in cold alcohol or when the cold envelop with its adhering alcohol was placed in the warm water. By using a double bath of alcohol with dry ice in the outside bath only, this trouble was overcome. However, further work has proven that contact freezing between flat surfaces of dry ice gives results as satisfactory as freezing by use of a secondary refrigerant. It was found that dehydration could be brought about either by adding sugar directly to the semen or by mixing the semen and a sugar solution and then removing the excess water by centrifruging, vacuum drying, or by absorption by filter paper. The method found most suitable was that of adding sugar (.12 grams of levulose per gram of semen) directly to the sample.

Quick freezing by the best technique so far developed, that is, dehydrating with levulose and freezing by the method previously described, apparently kills or immobilizes approximately 65 to 75% of the sample. Motility of a sample that had been frozen to -76° C continued for five days following thawing and storage at 0 to 1° C.

The temperatures of -6° C., -12° C., -24° C and -76° C. were tried as storage temperatures for the frozen sample. The only one of these temperatures at which viability persisted for longer than a few hours was that of -76° C. (storage between cakes of dry ice). In an attempt to determine how long viability could be maintained in the frozen condition several samples were frozen and stored between cakes of dry ice. Samples were

thawed periodically to verify motility. Semen stored in dry ice remained viable 24 days when by accident the samples were allowed to thaw. Time did not permit a duplication of this part of the work, hence the maximum length of time that semen will remain viable in the frozen state was not determined.

From a comparison of Fig. 1 and Fig. 2 it will be noted that freezing and holding at -76° C has little, if any, destructive action on the cells. Inasmuch as there seem to be as many cells recover motility upon thawing after 24 days storage as there did upon thawing immediately following freezing, it is hard to predict how long life could be maintained in the frozen condition.

In studying the effect of quick freezing at various temperatures it was found that when either fresh or dehydrated samples were frozen to -6° C. (a temperature at which semen is a solid) and then quickly thawed no effect on motility could be noted. As colder temperatures were used, more and more sperm were rendered immobile until a maximum of effect appeared to be reached at -15° C. Temperatures between 15° C. and -76° C showed no gradient effect, in other words, a temperature of -15° C. was as harmful as a temperature of -76° C. Because of this fact, and for reason of simplicity, the only temperature tried for prolonged storage was that of -76° C.

Samples which were frozen and thawed as many as three times, still showed approximately 15% of the cells to be motile.

As previously mentioned, quick freezing to only a few degrees below zero has the advantage that it results in little apparent injury to the cell as judged by motility; but when semen is stored at that temperature crystals form and destroy the cells. The rate at which the cells were destroyed in samples diluted 2 to 1 with 3/4 levulose, frozen to -8° C., and held at -6° C. is indicated by the table below.

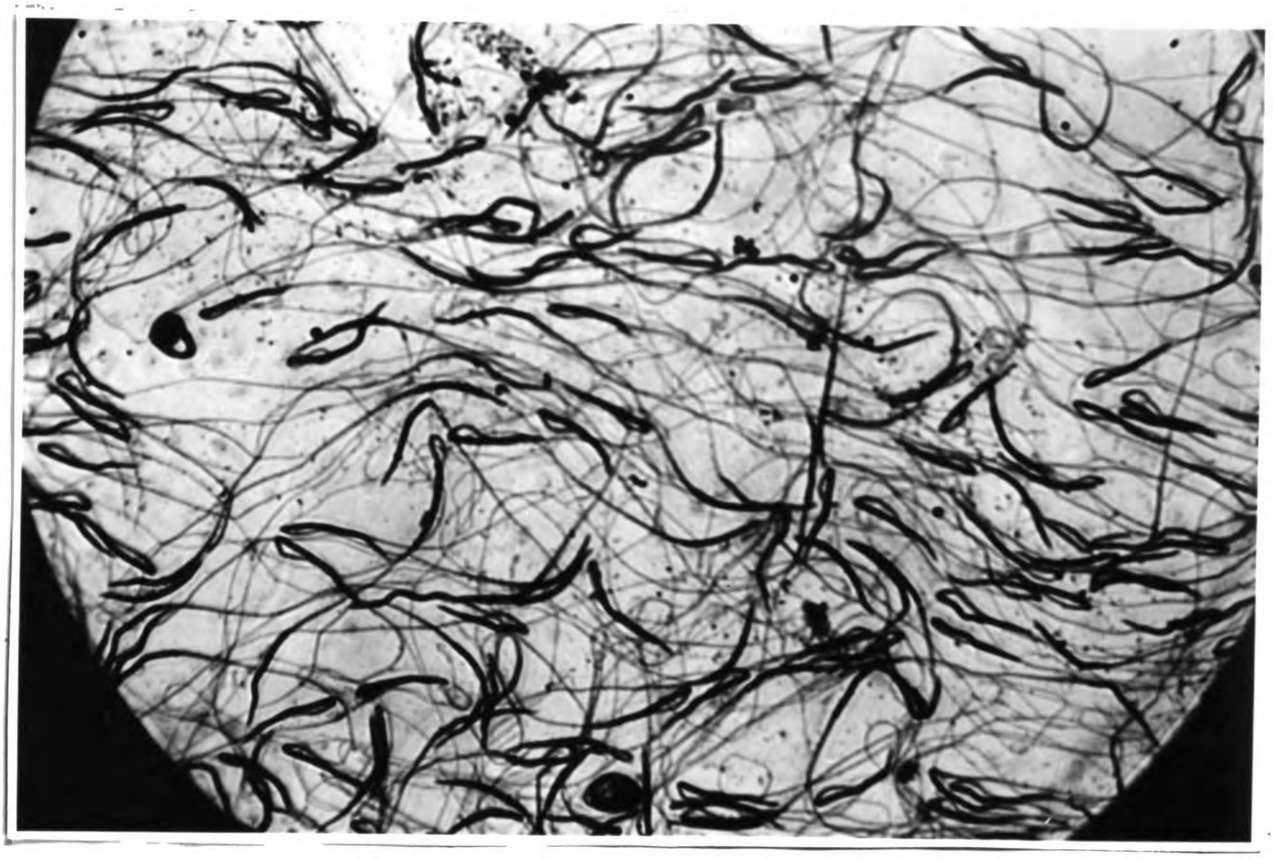

Fig. 1 Microscopic slide of an untreated sample of chicken Spermatozoa.
Mag. 1-1000

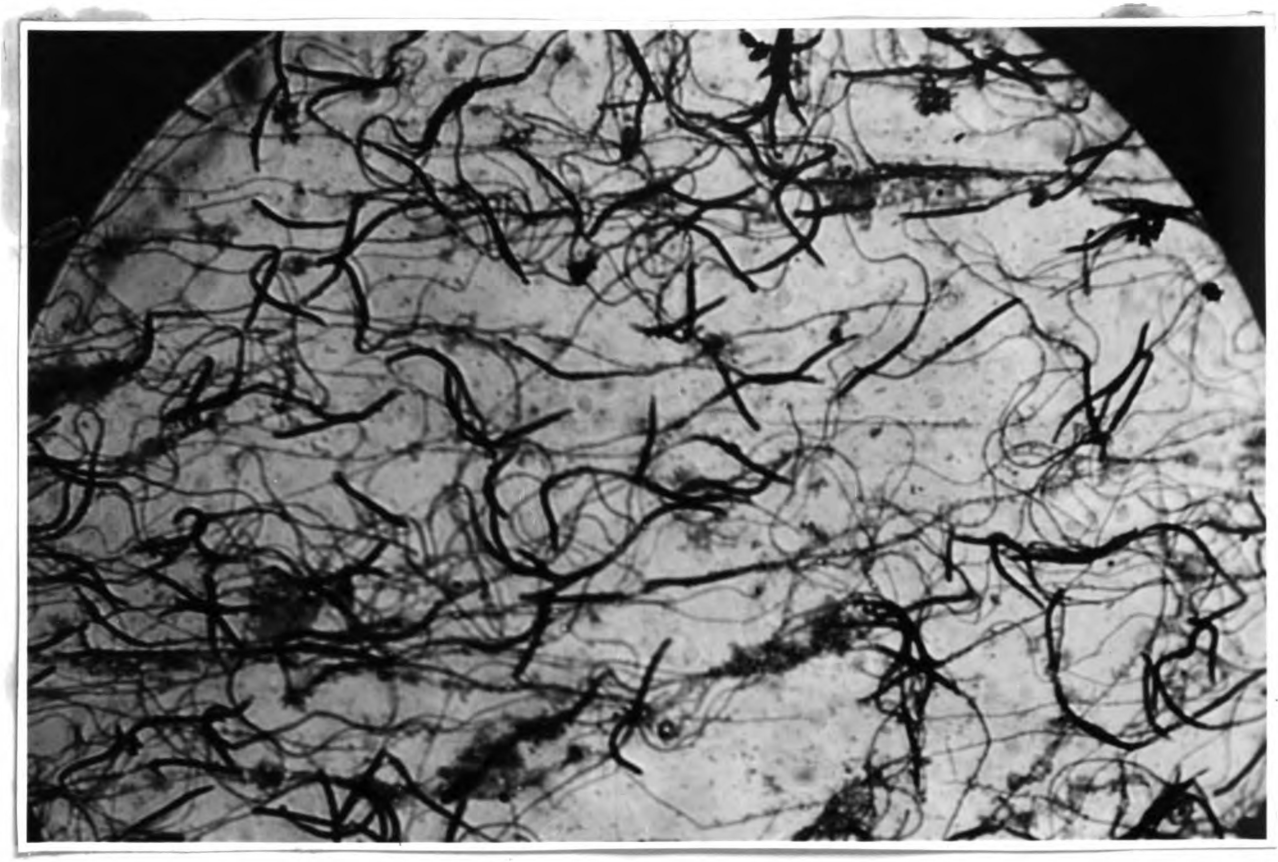

Fig. 2 Microscopic slide of a sample of chicken spermatozoa quick frozen and held 48 hours at -76° C. Mag. 1-1000

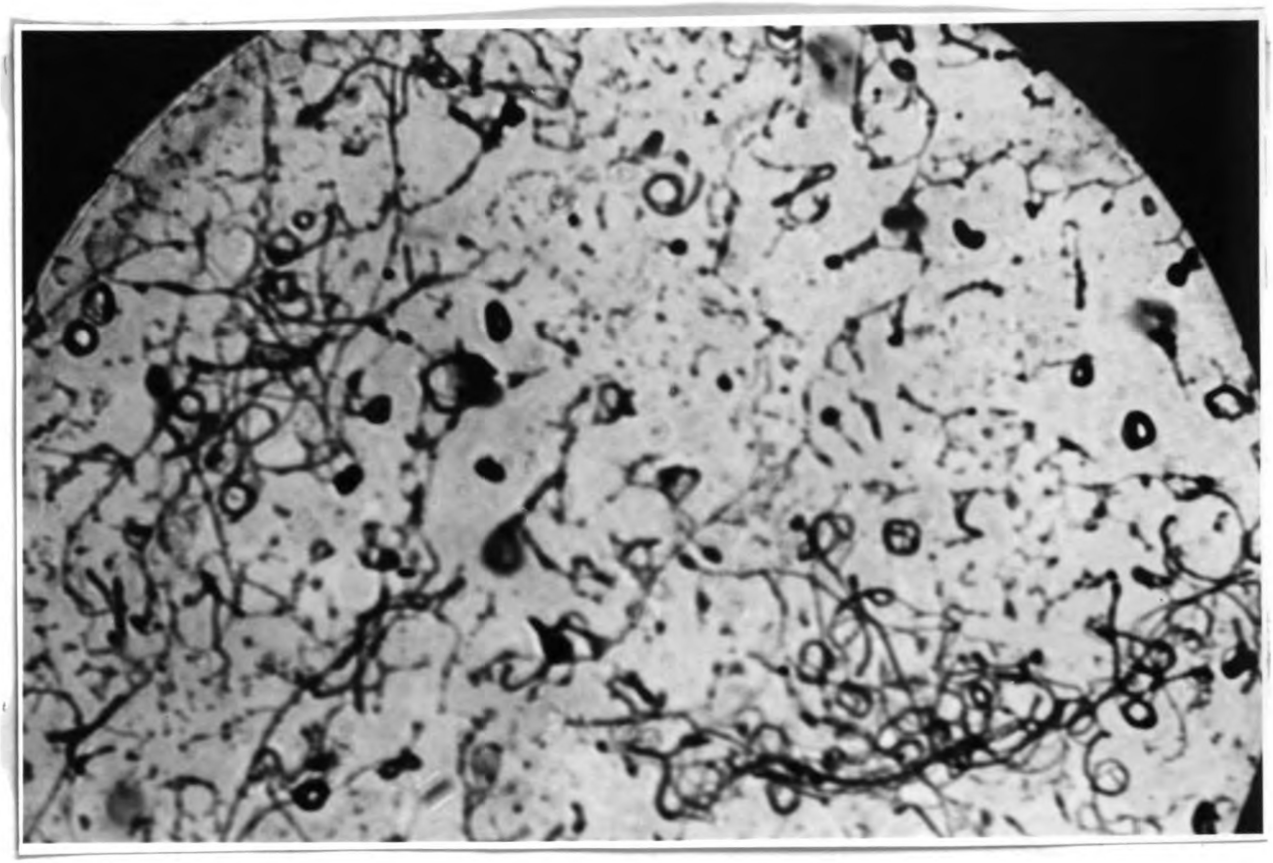

Fig. 3 Microscopic slide of a sample of chicken spermatozoa frozen by natural methods and held 12 hours at -10° C.
Mag. 1-1000

Table No. 2

Length of holding	Motility on thawing
0 (Check)	4
10 Min.	3
20 "	3
30 "	2
40 "	2
55 "	1
70 "	1

Little damage to the cells could be observed after 70 minutes storage at -6° C., however, after storage for 12 hours at this temperature, cellular destruction was serious enough to make the sample comparable to that shown in Fig. No. 3.

It was generally noted that the length of time motility persisted in vitro was inversely proportional to the temperature, provided that temperature was above zero. By means of the following table the length of time motility persisted in undiluted samples held at various temperatures is shown.

Table No. 3

Temperature	Length of time
37° C.	4 hrs.
28° C.	28 hrs.
6° C.(Regrigerator Temp.)	7 days
0-1° C.(Ice water bath)	14 days

Viability of Sperm as Measured by Fertilizing Ability

If increasing the length of life of the spermatozoa is to be of any practical value they must, of course, retain their fertilizing capacity. The results of this experiment are in agreement with those of Munro (7) where he reports that motility did not indicate fertilizing capacity. No fertile eggs were produced by hens inseminated with semen that has been frozen to -76° C. However, as good a fertility resulted from insemination with semen frozen to -6° C. and held 1/2 minute as there did from untreated semen. To eliminate the necessity of spermatozoa

traveling the full length of the oviduct it was decided to place it nearer the supposed site of fertilization. Incisions were made in three hens in a manner similar to that used in caponizing and semen that had been held at -76° C. for 48 hours injected into the oviduct near the infundiblum. But one egg was produced by these birds until six days after the operation. Neither the egg produced on the day following the operation nor those produced after a lapse of six days were fertile. Unfortunately no controls were run with untreated semen hence it is not known whether the semen or the technique was at fault.

DISCUSSION

Spermatozoa were found to remain viable longer if diluted with sugar solution than when not diluted (table No. 1). The reason for this increase in length of life was not determined but it was thought that it may have resulted from a combination of several factors. The decreased rate of movement probably caused the cell to have a reduced metabolic rate or a reduced metabolic rate may have been responsible for the reduced movement. The rate of auto intoxication, if such exists, would be reduced by the fact that there were fewer cells per cubic centimeter of supporting media. Whether or not the sugar in the supporting media may have been partially available to the cell as nourishment is still problematical. The dilutions found to be most favorable to life were 1/4 to 1/2 M. concentration of sucrose, a disaccharide, and 1/2 to 3/4 M. concentration of levulose, a monosaccharide. This would tend to indicate a solution very nearly isotonic with the cell as being essential. The amount of dehydration that took place could not be measured. However, the fact that movement was temporarily retarded following the diluting indicated that there was some change to which the cell soon became accustomed. This decrease in movement was not noticed following dilution with physiological salt.

The length of the cells in a media containing sugar and gelatin was much shorter than that in a media containing only sugar, or the natural sperm media. Since gelatin exerts little osmotic pressure, death of the cell probably did not result from direct dehydration. Upon setting, the gelatin absorbs and holds an increasing amount of water as "bound water". It seems possible that the cell was thus depleted of cell water and was, as a result, rendered immobile.

To the author's knowledge this is the first report of fertility having been produced by semen that had been frozen. At present, the fact that freezing to -6° C. does not noticeably effect fertilizing capacity is not of practical importance, because storage at that temperature soon results in severe damage to the cells; nevertheless, it is very interesting in that it indicates that the freezing technique may offer possibilities as a means of semen preservation. It is apparent that the physical phenomena of solidification of the media does not destroy fertilizing capacity and that the difference in results between freezing to -6° C. and to -76° C. was a measure of the effect of the temperature on the cell itself. That cellular damage following storage at -6° C. is caused by the formation of crystals has not been verified by microscopic examination of frozen mounts. However, this seemed the most logical explanation. The question that naturally arises is how crystal formation might be prevented. The only means found to date is that of decreasing the temperature. The minimum storage temperature which will prevent crystal formation in chicken semen was not determined, but from the literature on quick freezing of food products it would seem that a temperature of at least -30° C. is necessary. It has been shown from the data in this study that there is a maximum of damage to motility by a temperature of -15° C., hence it is seen that a temperature sufficiently low to prevent crystal formation will cause

considerable damage to motility. If in the future some method is devised to prevent crystal growth, freezing to a few degrees below zero C. may prove useful.

The question naturally arises as to why freezing to -76° C. kills some cells and not others. It was first thought that the cells that survived were in a most favorable position in relation to the mass. If this were the case, one would expect that following a second freezing and thawing but few cells would regain motion. The fact that approximately the same number of cells resume motion after being frozen and thawed twice as after one freezing would tend to indicate that there is a difference in the ability of cells to withstand low temperatures. Should the freezing technique be sufficiently developed so that the fertilizing capacity of the cells that regain motility is not impaired, it is possible that this selective action might have some genetic bearing. This would depend on whether or not there was correlation between the physical make-up of the cell and the genes it was carrying.

The results here given show that the fertilizing capacity of spermatozoa is easily damaged by dilution with synthetic diluents in that dilution with 1/2 to 3/4 M. levulose, while it did not affect motility, did render the semen practically sterile. This is in agreement with the reports of Munro (7). Whether this was caused by the dehydrating effect of the sugar or by toxicity to the cell was not determined. Therefore, the possibility still remains that the development of a better method of dehydration may improve the freezing technique.

It is also interesting to note that the rate of thawing appeared to be more important than the rate of freezing. Freezing the retaining envelops by contact between two pieces of dry ice gave results comparable to those secured by use of a secondary refrigerant. Although no attempt was made to

determine the rate of freezing one would naturally expect the latter method to be much faster. Contact freezing makes possible the use of very thin foil as retaining envelops and thus increasing the rate of thawing. A combination of some of the above factors may prove useful in future work.

SUMMARY

1. The osmotic pressure of the supporting media of chicken spermatozoa was increased by the addition of sugars and its effect on length of life recorded.

2. The length of life of spermatozoa as measured by motility was somewhat lengthened by diluting the sample with 1/2 to 3/4 M. levulose solutions.

3. Spermatozoa of chickens was subjected to various storage temperatures between 42° C. to -76° C. and the length of time that life, as determined by motility and fertilizing power, could be maintained was recorded.

4. The length of life of the cell in the unfrozen condition was found to be inversely proportional to the temperature at which the sample was stored.

5. Semen quick frozen to -6° C. and held 1/2 minute showed no apparent damage to either motility or cellular structure.

6. Fertile eggs were produced by hens that had been inseminated with semen that had been frozen to -6° C. and held 1/2 minute at that temperature.

7. Approximately 30% of the spermatozoa resumed motion after having been partially dehydrated and quick frozen to -76° C.

8. Life of the cells in the frozen condition (-76° C.) was maintained for 24 days. No apparent difference could be noted in the number of cells that regained motility after remaining frozen a few minutes or 24 days.

9. No fertile eggs were produced following inseminations with semen that had been frozen to -76° C.

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