The effect of antibiotics alone and with various diluents and hormones upon the in vitro survival and fertilising capacity of chicken semen.

BY

Shahid Husain Qureshi.

Thesis submitted to the faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirement for the degree of Doctor of Philosophy,

1953

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

ACKNOWLEDGMENTS

The author wishes to express his grateful appreciation to Dr. C.S. Shaffner for his keen interest and guidance throughout the prosecution of this study and in the preparation of the thesis.

He also wishes to record his appreciation to Dr. Morley A. Jull, Head of Poultry Department, University of Maryland, and to Dr. A. L. Brueckner, Director Livestock Sanitary Service, Maryland, for their help and encouragement in various ways during the course of this study.

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INTRODUCTION

Artificial insemination is very old and is believed to have been used by the Arabs as early as the fourteenth century. The first authentic accounts of the use of artificial insemination are from an Italian named Spallanzani (1780-1785), as cited by Gunsalis (1941) and Von Dremmelen (1951) who introduced it to the scientific world with his records of results in dogs. The main contribution on the application of the method in breeding farm animals was by Professor E.E. Ivanov of Moscow (1899-1930) cited by Von Dremmelen (1951), who successfully used it in horse breeding in the stables of the Czar, and was then made in charge of the Tsarist Institute of Animal Breeding in 1923. Although the work was largely academic, the results so obtained opened new avenues in the field of animal breeding:

(1) Protection of the sire from the risks of services;

(2) Increase of the possible number of progeny of sires;

(3) Production of progeny from parents unfit for copulation.

During the last few decades very rapid advances have been made in the methods of artificial breeding. This was generally attributed to the outstanding economic achievements which stimulated a world wide interest. About 1928 the Russians launched a large scale scheme of inseminating cattle which became popular by 1938. In 1930 it was introduced in

dairy cattle in Denmark and the first American Artificial Breeding Association was formed in 1938 in New Jersey.

REVIEW OF LITERATURE

The adoption of artificial insemination as a breeding method is necessary to solve many problems that confront the poultryman today. It would bring within his reach the maximum amount of benefit from the modern breeding methods, such as, the use of proven sires, fertilizing of hens in batteries, improving fertility rate in turkeys as well as increasing the possibility of bringing about crosses between breeds and species when natural mating is very difficult if not impossible.

Ivanov (1913) studied artificial insemination in fowls using semen from the vasa dererentia of killed males. Payne (1914) used artificial insemination to study the activity of spermatozoa in the oviduct of the fowl kept separated from the cock. Since then numerous research workers being provided with almost unlimited opportunity to conduct investigations have added much to our fundamental knowledge of the physiology of reproduction as well as accumulated information useful to the practical breeder.

Crew (1926) and Warren and Kilpatrick (1929) reported that under natural conditions 7 to 10 days are required for the semen of a newly introduced male to supplant that of a previous male, whereas only two days may be required with the use of artificial insemination (Warren and Gish, 1943). Consequently, artificial insemination would provide better facilities in a breeding program with double or triple shifts

when the use of 2 or 3 males in a single pen of females is desired in a breeding season.

This research was noticeably stimulated in 1935 when Burrows and Quinn (1935) and Burrows and Quinn (1937 and 1939) reported a cheap and harmless method of collection of semen easily and directly from the cock. It is now generally agreed that artificial collection of fowl semen in practice is best done by "milking" of the bulbous ducts or vasa deferentia after stimulation of the abdomen and vent by manipulation to bring about ejaculation (Burrows and Quinn, 1937 and 1939; Burrows and Marsden, 1938). This method is applied to turkeys (Burrows and Quinn, 1937, Burrows and Marsden, 1937 and 1938) and adopted to ducks, peacock, finches and canaries (Bonadonna, 1939) as well as to pigeons and doves (Owen, 1941).

Quinn and Burrows (1936) compared the results of artificial insemination with those of natural mating, when the male and the female differ greatly in body weights and demonstrated the possibilities of obtaining better fertility with artificial insemination in contrast to that when natural mating was used. Burrows and Marsden (1938) obtained 88.4 per cent fertility with artificial insemination in a flock of ten turkey hens and one old tom against 7.5 per cent fertility following natural mating.

The economic production of poultry and eggs is the determining factor for the survival of the industry and a breeder must, therefore, produce its product as efficiently

as possible. This is, perhaps, the main reason for the general adoption of artificial insemination in practical poultry breeding particularly the turkeys (Bonnier and Trullson, 1939). The reproductive power of the cock is at its maximum in his first season (9-15 months) which rapidly declines after a year (Munro, 1938). Therefore, it is of economic necessity to use artificial breeding in order to get the most out of an outstanding proven sire.

The cock "treads" the hens up to 30 to 40 times in a day during the breeding season (Heuser, 1916; Martin and Anderson, 1918; Penquite et.al, 1930). But 14 per cent of these copulations may not be accompanied by true ejaculates (Parker et. al., 1940). Thus the production of sperms and their distribution between different copulations are factors determining the value of the cock. Munro (1938b) found that a maximum of $1x10^8$ spermatozoa per insemination are required for good fertility. Parker et. al. (1942b) estimated that if such ejaculation were limited to this number, cocks would be capable of fertilizing 17 to 24 hens per day. But 20 per cent of the ejaculations contained less than $1x10^8$ sperms, it seemed that under natural conditions a few hens could be fertilized properly by one male in a day. Munro (1938) and Hammond (1940) reported that the lower the concentration of spermatozoa in the semen the larger is the portion of ejaculate required for an insemination with optimum chances of success. In order that artificial insemination be of real practical value there must be an

adequate number of fertile spermatozoa in the semen to be used and that it must be stored outside the body of the animal for periods long enough to transfer them to the female to be inseminated.

The storage of semen is an important aspect of artificial breeding. According to our present knowledge it is best stored in the pure state (Weber, 1936; Komarov and Gladcinov, 1937; Winburg, 1939; Green, Winter and Comstock, 1942). The keeping qualities of fowl semen have consistently been found to be relatively poor, notwithstanding the total absence of accessary secretions and the high concentration of spermatozoa in it.

Although fowl semen can be kept with little success in artificial media, the spermatozoa of the fowl continue to fertilize eggs for two to three weeks after a single mating or artificial insemination. The duration of fertility after coitus was first studied by Barfurth (1896) and since then by numerous investigators (Ivanov, 1924; Crew, 1926; Warren and Kilpatrick, 1929; Walton and Wetham, 1933; Burrows and Quinn, 1938; Nicolaides, 1934; Parker, Mekenzi and Kempster, 1942b; Van Dremmelen, 1945 and 1946). Munro (1938) found spermatozoa remained viable in the ligated vas deference of the cock for 26 to 28 days. The long duration of fertility in the domestic fowl after a single mating, without any apparent spermatotheca or localities of concentrated stored sperm in vivo, and the replacement by fresh sperms from a recent coitus, is still unexplained.

Investigations into the fate of spermatozoa might throw further light on some of the interesting and unexplained phenomena of avian sex physiology.

Spermatozoa, as cells morphologically different from the rest of the body cells, have attracted attention since its discovery as a constituent of the semen by Lieuwenkock in 1676. Earlier workers paid great attention to the morphology of the spermatozoa as a bearer of hereditary characters to perpetuate the species. In recent years research was directed towards correlating various semen characteristics with the fertilizing ability of the spermatozoa while others have mainly endeavoured to prolong the life of mammalian spermatozoa in vitro by affecting changes in their surroundings media and nutritional requirements.

In fact, extension of sperm life is one of the chief aims of dilution which may be brought about by improving the environment of the spermatozoa. Redenz (1928) used glucose successfully for the reduction of electrolytes. Kusnetzowa et. al. (1932) developed a semen diluent containing glucophosphate. Lardy and Phillips (1939) recommended the egg-yolk phosphate diluents. In the fowl the earlier diluents were made from thin egg-white and isotonic saline solutions, (Nikitina, 1932: Griffin 1938). The addition of nutrients (Bernstein, 1933b) to diluents of bull semen did not greatly increase the survival of the sperms. Munro (1938) found detrimental affects of diluents

in fowl as compared with "sperm serum". Swanson and Hermann (1941) reported that the property correlated with fertility was the survival period with vigorous motility. However, these diluents alone or with nutrients does not seem to have prolonged the survival period of spermatozoa in vitro or vivo markedly particularly those of the chicken spermatozoa.

To maintain the fertility power of the bull semen it is important to reduce the initial bacterial population by care in collection and to check bacterial growth by storage at 5 °C. Semen from the cock is collected from the papillae on the wall of the cloaca (Burrows and Quinn, 1937 and 1938b) and is heavily contaminated with bacteria (Grodzwski and Marchlewshi, 1935; Koch and Robilland, 1945; Smith, 1949).

The duration of the period of successful storage of fertile semen is of prime importance in artificial insemination and in the fowl the maintenance of fertilizing power in vivo in the hen's oviduct is particularly important in view of the regular, almost daily, ovulation (Parker et. al., 1942b; Van Dremmelen, 1945b, 1946a). It seems possible that multiplication of bacterial organisms might contribute to the rapid deterioration in vitro of the fertilizing ability of the cock's semen.

With these few facts the object of the present investigation was designed to study:

(1) To determine whether or not the control of bacterial growth in chicken semen by the addition

of antibiotics in semen will result in prolonging the survival period of the fertile spermatozoa in vitro.

(2) To study whether or not there is a complimentary effect or evidence of synergism following the use of antibiotics combinations.

(3) To study the effects, if any, of sex hormones in improving the quality of semen during storage.

(4) To study the effect of thyroxine on the viability and fertilizing capacity of chicken spermatozoa.

(5) To determine the value of some of the diluents in chicken semen.

(6) To determine the effect of some suitable reagents referred to above on the fertilizing ability of chicken semen.

As early as 1917 Ivanov recognized and investigated the possibility of controlling the spread of infection by adding certain chemicals to contaminated semen. In 1940 Shettle, working with human semen, reported encouraging results with sulfanilamide or sulfapyridin. Knodt and Salisbury (1946) were the first to study the feasibility of using certain bacteriostatics to control bacterial growth in bovine semen. He found that 200 to 300 gamma per milliliter of sulfanilamide significantly increased spermatozoal livability, They, however, attributed the beneficial effect of sulfanilamide on fertility to change in metabolism of the spermatozoa rather than to the control of bacterial growth alone.

Phillips and Spitizer (1946) recommended the use of sulfathalidine or sulfasuxidine at the rate of 0.03 per cent to prevent bacterial growth in bull semen. Foote and Salisbury (1948) reported that sulfanilamides have no deleterious effect on spermatogensis of bulls and recommended the use of sodium-sulfa-methazine and carboxy-sulfa-thiazole as antibacterial re-agents to be added to the semen samples.

The recent discovery of antibiotics has aroused considerable interest as to their possible use in bull semen to improve its fertility. Salisbury et. al. (1947) has pointed out that the bacterial growth in bull semen can be inhibited by the addition of sulfanilamide, penicillin, streptomycin and penicillin plus streptomycin without any harmful effects on spermatozoal motility. Almquist (1948-49) has shown that both penicillin and streptomycin increase the fertility rates of certain relatively infertile bulls. He recommended that levels of streptomycin above 100 gamma per milliliter did not significantly decrease sperm livability. Easterbrook et. al. (1950) reported a significant increase in fertility on the addition of 100 gamma per milliliter streptomycin to bull semen. Almquist (1951) reported that penicillin (1000 units per milliliter) and streptomycin (1000 gamma per milliliter) either singly or together are quite valuable to improve fertility of certain low fertile bulls.

Recently workers in the field have focussed attention on some of the newly developed antibiotics including

aureomycin, polymyxin and chloromycetin. Reports by Mayer et. al. (1950), Sykes and Mixner (1950) and Foote and Bratton (1950) indicate that aureomycin inhibits bacterial growth but is relatively toxic to the motility of bull spermatozoa. Polymyxin (Foote and Bratton, 1950) and chloromycetin (Sykes and Mixner, 1950), seems to be less harmful to the spermatozoa than the former antibiotic.

Unfortunately the information available on the use of bacteriostatics in chicken semen is very scanty. Koch and Robbilland (1950) reported that bacterial contamination is the factor most detrimental to preservation of chicken spermatozoa in vitro. Smith (1949) reported that streptomycin checked bacterial growth but did not improve motility or fertility; whereas egg white improved the motility but did not inhibit bacterial growth. Weakley (1950) reported that fertility secured with fowl semen diluted with saline plus polymyxin compared favourably with that obtained with the untreated control semen. The present investigation discusses the effects of the antibiotics - penicillin, terramycin, streptomycin and polymyxin alone, or in combination, on the livability and motility of chicken spermatozoa in vitro.

GENERAL METHOD AND PROCEDURE

The semen used in the preliminary trials was secured from six fully matured New Hampshire cocks about a year old. These cocks were bred at the Maryland University Poultry Farm and were taken from there to the Department of Poultry Husbandry of the University of Maryland and housed there in batteries. Two males were kept to a battery cage. They received feed and water at all times and artificial light twenty-four hours per day.

The cocks had been regularly submitted to induced ejaculation for several weeks preceeding the experiments and thereafter during the period of investigation. The semen was collected from the males by the method described by Burrows and Quinn (1935, 1937) in clean glass test tubes. All glasswares used were thoroughly cleaned and flushed with distilled water and dried in hot air electric incubator before they were used. To avoid temperature shock, dilutions were not made until the semen had reached the temperature of the waterbath used for storing the semen at desired temperature.

Owing to the small volume of the ejaculate emitted by the cock a composite sample was used which was thoroughly mixed with a clean glass rod. The semen was observed daily under the microscope in a hanging drop to determine survival time and motility rated as follows.

Motility Index	State of Spermatozoa
4.	Vigorous progressive motion with swirling.
3.	Progressive motion of the majority, but no swirling.
2.	Majority motile, less than half progressing activity.
1.	Majority stationary with vibrating tails. Few or none actively progressing.
½.	Only a few showing any movement.
0.	Practically none showing any movement.

The dilutions were kept constant at the level of 9 to 1 (i.e. 9 parts of semen plus 1 part of diluent). The control samples were usually diluted with sodium citrate solution (4.75 per cent). The solution of antibiotics were prepared in isotonic sodium citrate solution or as indicated.

The hens used to determine the fertilizing capacity of the semen were maintained in laying batteries and to ensure their infertility were inseminated only after they had laid a series of infertile eggs. Care was excercised to inseminate only the hens that did not have a hard shell egg in the oviduct. Semen was inseminated by the technique described by Burrows and Quinn (1938) using one tenth milliliter per insemination or as indicated. The fertility trials were carried out between October 1952 and March 1953.

Following insemination all the hens were trapnested and the eggs marked with number, date and treatment. The fertility of all the eggs used in these experiments was

obtained by candling after five days of incubation. A record was maintained for each egg laid by each hen from second to fourteen day after insemination.

When a hen produced consecutively five or more infertile eggs she was considered infertile until again inseminated and was free to be used in a new experiment. Hens very rarely produced a fertile egg when the preceeding five eggs have been infertile. However, in order to completely eliminate the chances of carry-over of fertility from one trial to another a period of one week was still allowed between trials.

For purposes of comparison, fertility was calculated as the percentage of fertile eggs produced in the first and second week beginning on the second day following insemination.

R E S U L T S

EFFECT OF ANTIBIOTICS (PENICILLIN, TERRAMYCIN, POLYMYXIN, AND STREPTOMYCIN) ON CHICKEN SEMEN PRESERVATION.

a. Penicillin

The bacteriology of bovine and chicken semen is, indeed, an important factor affecting both the motility and survival period of the spermatozoa. Motility is not as good an index of chicken semen quality as in bovines where it has proved to be a dependable estimation of fertilizing capacity. However, the present study discusses the effect of penicillin used to control the bacterial growth in chicken semen and thereby extending the survival time of spermatozoa.

Since Grodzinski and Marchlewski (1935) and Koch and Robillard (1945) demonstrated that the semen from cocks was highly contaminated with bacteria, Smith (1949) considered that rapid proliferation of bacteria may be responsible for the deterioration in vitro of the fertilizing capacity of the semen. Almquist (1948), (1949), and (1949b) has shown that both penicillin and streptomycin increase the fertility rates of relatively infertile bulls. Myers and Almquist (1951) undertook extensive study of bacteriostatics comparing their effects on spermatozoal livability and control of bacteria in bovine semen.

It may be seen from the table given below that aureomycin brought about a highly significant decrease in bovine spermatozoal livability when compared with the control or penicillin treated samples. Penicillin and streptomycin

single or in combination, maintained motility which was as good as that of the untreated semen.

Table 2 --- showing the comparative toxicities of auriomycin, penicillin and streptomycin upon the livability of bull spermatozoa. (Myers and Almquist, 1945)

Treatment	Percent motile spermatozoa after storage at 4 - 5°C. for days								Bacterial counts on 4 days Million/ml
	0	2	4	6	8	10	12	14	
Control	64	57	47	38	24	17	9	1	39,000
Auriomycin[1]	64	42	15	5	0	-	-	-	30
Penicillin[2]	64	56	45	34	22	14	8	3	460
Streptomycin	64	55	45	31	18	13	8	4	40
Pennicillin plus Streptomycin[3]	64	55	48	38	20	13	8	3	70

1. 1,000 ug per ml.
2. 1,000 units per ml.
3. 1,000 penicillin plus 1,000 ug streptomycin.

The problem of preservation of motility and fertilizing quality of stored diluted chicken semen is by no means solved to date. The aforementioned reports show the progress which has been made so far with antibiotics in bull semen. In regard to chicken semen no work on these lines seems to have been done since the first report by Smith (1949). He used streptomycin at the rate of 50, 100 and 200 gamma per milliliter of fowl semen which have

effectively checked bacterial growth, but did not improve the motility or fertilizing power of spermatozoa in stored diluted semen. In view of these facts and on the consideration of the success obtained by dairymen the present attempt is made to test the effects of some more antibiotics on fowl semen used in routine artificial breeding.

In the initial experiment semen from individual males previously described was collected and stored separately in clean 6 c.c glass tubes. The semen from each male was divided into two equal portions. To one of these series of samples penicillin was added at the rate of 600 units per milliliter. To the other tube which served as a control 0.05 milliliter of isotonic sodium citrate solution was added, so that the rate of dilution in both the tubes remained the same. The six individual males semen samples were stored in a water bath kept at 4.5°C., during one trial, at 10°C. during the second trial, and at 15°C during a third trial. The sperms were observed daily under the microscope in a hanging drop to determine the survival time and motility.

The results are summarized in Table 1. It shows wide individual differences. Further as far as the survival time of spermatozoa, based upon motility, is concerned, a low temperature of 4 - 5°C seems to be the best for storage. Detrimental effect of the environmental temperature is well marked as the temperature is raised to 10°C and 15°C.

Table 1

Survival time of spermatozoa in semen sample from individual males held separately at various temperatures (one half of each sample treated with penicillin).

Trial	No. of male	Sperm survival time in days					
		4-5°C		10°C		15°C	
		Treated	Control	Treated	Control	Treated	Control
I	1	7	7	3	5	3	3
	2	8	6	4	5	3	3
	3	4	3	5	4	3	3
	4	10	9	4	5	3	3
	5	2	2	3	3	3	3
	6	3	3	3	3	3	3
II	1	7	7	5	5	2	3
	2	9	9	4	5	2	3
	3	9	8	6	5	3	3
	4	4	4	5	6	3	4
	5	3	5	5	5	2	3
	6	8	8	4	4	3	3
III	1	5	5	5	6	4	3
	2	6	6	4	5	4	4
	3	6	6	5	6	4	4
	4	7	7	5	5	3	3
	5	6	7	4	4	4	4
	6	5	6	5	5	4	4

These results (Table 1) support the view that a temperature below the hen's body was favourable for the storage of fowl semen (Tshikawa, 1930; Marchlewski, 1935; Munro, 1938b). But in chicken insemination the semen used must be of such quality so as to remain viable over an extended period of time and fertilize several eggs.

Recently Garren and Shaffner (1952) reported that 10° C temperature seems to be the least detrimental storage temperature to fertilizing capacity of undiluted chicken semen. In view of this fact and on the consideration that chicken semen is highly contaminated with bacteria it appeared desirable to use this temperature (10°C) for the study of the effect of antibiotics on the survival time and fertilizing capacity of spermatozoa during storage of chicken semen.

In another experiment the effect of different levels of penicillin were studied. The levels of penicillin used and the results obtained are summarized in Table 2.

In general the results are not consistant. However, there seemed to be a trend obtained in the experimental data which indicated a possible superiority of semen treated with 160 to 800 units of penicillin (Penicillin G-potassium salt) per milliliter over that untreated semen samples. In one group penicillin at the level of 4000 units per milliliter appreciably increased spermatozoal livability. However, concentrations greater than 1000 units are not likely to be tolerated by diluted chicken semen without toxic effects.

Table 2

Effect of Penicillin on the livability of chicken spermatozoa after storage at 4-5° C.

Treatment	Survival time days					
	Trial numbers					
	1	2	3	4	5	6
Controls	8	6	9	8	6	5
1000 units/ml	9	6	9	-	-	-
800 units/ml	8	6	9	-	-	-
400 units/ml	9	3	6	-	-	-
200 units/ml	9	9	12	-	-	-
160 units/ml	8	6	9	-	-	-
6000 units/ml				4	4	4
4000 units/ml				9	8	9
1200 units/ml				7	6	6
800 units/ml				10	8	6
600 units/ml				8	4	7
160 units/ml				9	7	8

The effects of various concentrations of penicillin in diluted bull semen was studied by Almquist (1947) and Almquist, Throp and Knodt (1948). They concluded that concentrations in excess of 1000 units per milliliter brought about significant decrease in spermatozoal viability during long term storage. Foote and Salisbury (1948) reported that 200 units of penicillin proved to be toxic to bull spermatozoa in one of the two lots studied. Hennanx et. al. (1948- cit. by Easterbrooks, 1951) observing one bull which had received a total of 2,400,000 units of penicillin parenterally at the rate of 800,000 units daily for three consecutive days reported marked reduction of semen quality of the bull fifteen hours after the last treatment when compared to a sample taken the day before treatment began.

Further lines of inquiry should be concerned with finding more accurately the optimum level for the treatment of chicken semen or testing combination of this drug with stretomycin, terramycin and polymyxin for evidence of complimentary action in improving the viability, livability and fertilizing capacity of spermatozoa.

b. Terramycin - Terramycin hydrochloride

No work seems to have been done with Terramycin in bull or chicken semen. Easterbrooks (1951) appears to be the first worker who used this drug at levels of 20 gamma per milliliter in bull semen and compared its effect with those of streptomycin by split method device. His findings are

Table 3

Showing the effect of temperature on chicken spermatozoa during storage at 4-5°C and 10°C.

	Survival time in days**							
	4-5°C				10°C			
	Trial numbers				Trial numbers			
Treatment*	1	2	3	4	1	2	3	4
1.	8	8	10	6	5	6	6	8
2.	4	8	9	7	4	4	6	6
3.	6	10	11	9	6	5	8	7

* 1. Untreated semen.

2. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1.

3. Semen treated with terramycin (100 ug/ml of semen) solution at the rate of 9 to 1.

based on the results obtained by treating 1157 and 1153 cows with streptomycin and terramycin respectively. He found an overall difference of 0.1 per cent in favour of the streptomycin treatment which he reported to be statistically non-significant.

In an initial experiment a flat dose of 100 gamma per milliliter of terramycin in sterile distilled water was used to control bacterial growth while studying the effect of 4 - 5°C and 10°C storage temperatures on the livability of chicken spermatozoa.

In this experiment the following 3 groups were included:

1) Pure semen without any diluent.

2) Semen to which isotonic sodium citrate solution was added to make the final dilution to 9 to 1.

3) Semen diluted with terramycin solution (100 ug/0.1 ml.) keeping the final dilution to 9 to 1, and drug concentration to 100 ug/ml. of semen.

The results are presented in Table 3 which indicated that treating the individual samples with terramycin may have enhanced spermatozoal survival.

In other experiments the effects of different levels of terramycin, in two phases, were studied. Following routine collection and examination, each sample of semen was divided into four equal portions. The first three portions received 20, 100, and 200 gammas per milliliter of terramycin respectively while the fourth one received no terramycin to serve as control. This phase of work was replicated 4 times.

In the other phase of the study of terramycin, levels of 40, 100 and 120 gammas per milliliter were used. These treatments were duplicated, one series being stored at 4 - 5°C and the other on at 10°C.

The results of the above experiments are summarized in Tables 4 and 5. In considering the trend in the experimental data which indicated a possible superiority of semen treated with 40 - 100 gamma of terramycin per milliliter over that treated with penicillin at a rate of 160 to 800 units. It may, perhaps, also be mentioned that the drug seems to act better at 10°C than 4 - 5°C.

c. Polymyxin

Polymyxin is a relatively new antibiotic which has become available, and it seems advisable to investigate its bacteriostatic properties relative to semen preservation. The procedure used was the same as previously described for the study of terramycin and the results are summarized in Table 6.

The review of the data in Table 6 indicated that semen mixed with polymyxin survived somewhat longer than untreated semen. The optimum level appears to be between 160 to 400 gamma per milliliter.

d. Streptomycin (sulfate)

Viability of diluted bull semen in the presence of streptomycin was observed by Hennanx et. al. (1948 cit. by Easterbrooks, 1951) and by Sykes and Mixner (1950) to be increased over that observed for untreated samples. Concentrations as high as 300 gamma or more of streptomycin per milliliter did not cause any conspicuous toxic effect.

Table 4

The effect of different levels of terramycin on chicken sperm livability stored at 4-5° C.

Treatment with terramycin	Survival time in days				
	Trial numbers				
	1	2	3	4	5
Saline control	4	9	9	6	5
20 ug/ml	5	9	11	6	6
100 ug/ml	4	11	6	4	9
200 ug/ml	4	9	7	9	5

Table 5

Comparative effect of different levels of terramycin on chicken sperm livability stored at 4-5° C and 10° C.

Treatment with terramycin	Survival time in days									
	4-5° C					10° C				
	Trial numbers									
	1	2	3	4	5	1	2	3	4	5
Control	4	12	13	7	7	5	6	6	8	5
40 ug/ml	4	11	16	6	9	6	8	9	8	9
100 ug/ml	6	10	11	6	7	9	6	9	8	9
120 ug/ml	9	11	17	8	9	5	5	10	7	7

Table 6.

Showing the effect of different levels of polymyxin On the livability of chicken sperm stored at 10-12°C .

	Survival time in days		
	Trial numbers		
	I	II	III
Untreated semen control	4	3.5	4.5
Untreated semen with oil film on top	5	4.5	5
Saline control	4.5	5.5	5
Poly 150 ug/ml	4.5	7	5.5
Poly 150 ug/ml	4.5	6	5
Poly 300 ug/ml	6	6	6
Poly 300 ug/ml	6	6.5	7
Saline control	5	4.5	4.5
Poly 400 ug/ml	6.5	5.5	6
Poly 400 ug/ml	5	5.5	5.5
Poly 600 ug/ml	5	4.5	5
Poly 600 ug/ml	5	5	4.5
Saline control	4	4.5	4
Poly 900 ug/ml	4	5	4.5
Poly 900 ug/ml	5	5.5	5

Almquist, Glantz and Shaffer (1949), however, reported that concentrations greater than 1000 gamma per milliliter decreased viability. Concentrations above 100 gamma per milliliter provided fairly good bacterial control.

When streptomycin (1000 gamma/ml) was added to the semen of a group of relatively infertile bulls, Almquist (1949) reported marked improvement in fertility in comparison to that of the untreated controls. Easterbrooks, Heller, Plastridge and Jungher (1949 and 1950) by adding streptomycin fulfate at the rate of 100 gamma/ml. of diluted bull semen found an increase of 8.7 per cent of fertility over untreated controls.

The only report of the use of streptomycin in chicken semen available is that of Smith (1949) who used the drug in concentrations of 50, 100 and 200 gamma per milliliter. He found that these levels effectively controlled bacterial growth in chicken semen but did not improve the motility or fertilizing power of chicken spermatozoa.

The object of this experiment is to study in detail the possible effect of streptomycin on viability of chicken semen in different concentrations. The levels studied were 150 gamma, 300 gamma, 600 gamma, 700 gamma, 900 gamma per milliliter of semen keeping the dilution as 9 to 1.

The method and procedure is the same as described before except that more control samples were included in order to have better comparison of the results of treated samples. There were three routine control samples diluted

with 4.75 per cent of sodium citrate solution to measure any possible effect of the small amount of dilution. In addition, two untreated controls were also included, one having a film of paraffin oil on the top and the other without any oil. The samples were stored in a water bath maintained at 10 - 12°C temperature. The results obtained are summarized in Table 7.

The data contained in Table 7 show a uniform improvement in sperm viability on addition of various concentrations (150 ug/ml to 1100 ug/ml) of streptomycin to chicken semen. These results indicate that streptomycin is slightly superior to penicillin, terramycin or polymyxin as a bacteriostatic to be added to chicken semen. The optimum level for the treatment of chicken semen with streptomycin in the present experiments appears to be about 600 to 700 gamma per milliliter. The results show that above 700 gamma per milliliter and below 300 gamma per milliliter a decline in sperm livability occured. Further, these results do not agree with the report of Smith (1949) that streptomycin did not improve motility of spermatozoa in stored diluted semen.

e. Comparison of antibiotics and their combinations

The present investigations discuss the comparative effect of terramycin, polymyxin and streptomycin and their combinations with penicillin.

The method and procedure was practically the same as discussed above. In order to minimize the variability known

Table 7.

Showing the effect of different levels of streptomycin on chicken sperm livability stored at 10-12° C.

Treatment	Survival time in days		
	Trial numbers		
	I	II	III
Untreated semen control	5	4	4
Untreated semen with oil film on top	5	4.5	5.5
Saline control	4.5	4.5	5
Streptomycin 150 ug/ml	6	4.5	5
Streptomycin 150 ug/ml	5	4.5	4
Streptomycin 300 ug/ml	4.5	6	5
Streptomycin 300 ug/ml	5	5	4.5
Streptomycin 400 ug/ml	5	7	6
Streptomycin 400 ug/ml	4.5	6.5	6
Saline control	4.5	4.5	5
Streptomycin 600 ug/ml	6	6.5	5
Streptomycin 600 ug/ml	6.5	7	5
Streptomycin 700 ug/ml	6.5	7	6.5
Streptomycin 700 ug/ml	7	6.5	6.5
Saline control	5	4.5	5.5
Streptomycin 900 ug/ml	6	6	5.5
Streptomycin 900 ug/ml	6	6	5.5
Streptomycin 1100 ug/ml	6	6	5.5
Streptomycin 1100 ug/ml	5.5	5.5	5

to exist between ejaculates of the same individual as well as within individuals a composite sample of semen was used. Further, to minimize this variability all comparisons, between treatments and controls of different treatments, were made on a split sample basis. That is to say, that the entire composite semen sample secured from the six New Hampshire males on a single day was divided into the number of portions indicated in Table 8.

Terramycin, Polymyxin and Streptomycin.

Two treatments of 150 gamma and 300 gamma per milliliter for each of these antibiotics are used in duplicates. They were repeated three times, thus making six replicates. The data is summarized accordingly in Table 8, which indicated superiority of semen treated with 300 gamma per milliliter of streptomycin over that of terramycin and polymyxin.

Since each of the antibiotics previously reported have proven to have some beneficial effect, it was decided to conduct a few trials to determine whether or not the antibiotics in combination might prove more effective than any one of them singly.

In these trials, penicillin at the rate of 1000 units/ml. was combined with terramycin, polymyxin and streptomycin at the rate of 150, 300 and 1000 micrograms or units per milliliter. In another trial, streptomycin and polymyxin were combined at the rate of 700 gamma per milliliter. The results secured with these combinations are summarized in Table 9.

Table 8.

Showing the comparative effects of terramycin, polymyxin and streptomycin on chicken sperm livability stored at 10-12° C.

Treatment*	Survival time in days			
	Trial numbers			
	I	II	III	Mean
Saline control	3.5	4	3	3.5
1	5	4	3	3.6
1a	4	4	2	
2	4	4	4	4.3
2a	5	4.5	4.5	
3	4	6	5	5
3a	4.5	5.5	5	
Saline control	3.5	4.5	4	4
4	6	5	5	5.4
4a	7	4.5	5	
5	6	5	5	5.3
5a	6	5.5	4	
6	5	5	7	5.5
6a	5	5	6	

* 1 and 1a Terramycin 150 ug/ml
2 and 2a Ploymyxin 150 ug/ml
3 and 3a Streptomycin 150 ug/ml
4 and 4a Terramycin 300 ug/ml
5 and 5a Polymyxin 300 ug/ml
6 and 6a Streptomycin 300 ug/ml

The trend in experimental data (Table 9) indicates that the addition of terramycin and polynyxin to penicillin had little, if any, effect in increasing the survival time of spermatozoa. Penicillin and streptomycin proved beneficial in prolonging survival time as did the addition of streptomycin and polymyxin.

It is suggested that levels of 150 gamma per milliliter of terramycin and 300 gamma per milliliter of polymyxin or streptomycin with 1000 units of penicillin appears to be fairly good workable levels to preserve chicken spermatozoa livability during short storage periods.

A marked complimentary effect in the control of bacteria was reported by Almquist (1948) and Almquist et. al. (1950) when penicillin and streptomycin were added in combination to dilute bull semen. Doses of 1000 units and 1000 gamma per milliliter of penicillin and streptomycin respectively did not reduce viability of spermatozoa during storage. Using the same concentration of penicillin and streptomycin, Mixner (1949) reported neither increase nor decrease in the fertility of semen from relatively fertile bulls. When streptomycin was compared with a combination of streptomycin plus penicillin, and added to bull semen diluted in egg-yolk citrate and sulfanilamide, Easterbrooks et. al. (1951) reported a trend favouring the addition of streptomycin alone.

In analyzing the data presented in Table 9, no evidence of synergism or complimentary effect is

Table 9.

Showing the comparative effects of antibiotics (penicillin, terramycin, polymyxin and streptomycin) on chicken sperm livability stored at 10-12° C.

Treatment	Survival time in days		
	Trial numbers		
	I	II	III
Saline control	4	4.5	5
Penicillin 1000 units plus			
Terramycin 150 ug/ml	5	4	6
Terramycin 300 ug/ml	4	3.5	5
Terramycin 1000 ug/ml	4	3	4
Saline control	4.5	5	4.5
Penicillin 1000 units plus			
Polymyxin 150 ug/ml	4.5	5.5	4
Polymyxin 300 ug/ml	5	6	5
Polymyxin 1000 ug/ml	4	5	4.5
Saline control	4.5	4.5	5
Penicillin 1000 units plus			
Streptomycin 150 ug/ml	5	5	5.5
Streptomycin 300 ug/ml	5	5.5	4
Streptomycin 1000 ug/ml	6	5.5	4.5
Saline control	4.5	4.5	5
Streptomycin 700 ug/ml plus			
Polymyxin 700 ug/ml	6.5	6	6.5
Polymyxin 700 ug/ml	5.5	5	5.5

manifested. Therefore, the author agreeing with the view of Easterbrook *et. al.*, (1951), favours the addition of streptomycin alone in chicken semen, as preservative during short storage.

EFFECT OF ANTIBIOTICS (PENICILLIN, TERRAMYCIN, POLYMYXIN, AND STREPTOMYCIN) ON PRESERVATION OF SEMEN DILUTED WITH VARIOUS DILUENTS.

Considerable attention has been paid to the question of diluents for semen, both from the point of view of increasing the volume as well as promoting longivity of spermatozoal life. Likewise conditions of storage have much been extensively investigated and several diluents have been used with success, notably the egg yolk citrate and yolk phosphate pabulum. Even the semen itself has been used after inactivation. But none of these seems to be entirely satisfactory particularly when they are added to chicken semen. In contrast to artificial diluents, the addition of chicken embryo extracts to bull semen is reported to promote motility as compared to that of the untreated semen. Many workers have used such fluids as Ringers and Locke-Ringers which according to Baker (1931) did not maintain high sperm activity over a long period of time and contains quite unnecessary substances.

In the earlier work with fowl semen, diluents were made from thin egg albumen and isotonic saline (Nikitina, 1932; Griffini, 1933). Munro (1938) found detrimental effects of diluents to fowl spermatozoa as compared with sperm serum. In contrast to this, chicken semen was diluted with Ringers solution by Burrows and Trollson (1939) who found that the fertility of hens

inseminated with the semen treated with Ringers at the rate of 1 to 3 exceeded that produced by whole semen. In sperm serum, blood serum, thin egg white, and in shell gland fluid motility of fowl sperm was found to be supported when compared with saline solutions. In Tryode or Ringers solutions, as well as in the fluids from the infundibulum and albumen secreting portion of the oviduct, the sperms were immobilized at 105°F, though the change was reversible on lowering the temperature.

With these facts in view it was thought desirable to test the efficiency of some of the diluents other than the artificial ones, which on addition to chicken semen, would support sperm life and motility during short storage periods without impairing the fertilizing capacity of spermatozoa. The following biologicals were chosen for study in these experiments:

a. Chorio-allantoic fluid (hen's egg).
b. Chicken blood serum.
c. Chicken semen serum (fresh semen with spermatozoa removed).
d. Inactivated chicken semen (semen with spermatozoa removed following inactivation by heat).
e. Pasteurized whole milk.
f. Pasteurized skim milk and whey.
a. Chorio-allantoic fluid

To produce the Chorio-allantoic fluid used as a

diluent, fertile chicken eggs were incubated for about a week after which time the fluid was withdrawn aseptically. The fluid was then centrifuged, allowed to stand for an hour and then the supernatent clear fluid was pipetted out and stored in a refrigerator at 4 - 5°C till it was used.

A series of 6 C.C. test tubes were used and 0.5 C.C. semen was transferred from the large collecting glass tube to each of them by means of a graduated 5 ml. pipette. Embryonated egg fluid was then added to each of these semen samples separately by means of a finely graduated 1 ml. and 0.1 ml. glass pipettes in the descending order 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 C.C.; thus making approximate dilutions of 33, 16, 9, 5, 2, and 1 per cent respectively. The semen and the embryonated fluid in the test tubes was then thoroughly mixed, by gently tilting the tubes up and down several times, and stored at 10 - 12°C in a water bath.

The results, enumerated in Table 10, do not show any consistent trend. Moreover, Chorio-allantoic fluid does not appear to be a satisfactory diluent to having supported the sperm life and motility better than the saline. There is some evidence that Chorio-allantoic fluid is toxic to chicken spermatozoal livability and motility (Table 11).

b. Chicken blood serum

In another experiment the effects of chicken blood

Table 10.

The effect of embryonated egg fluid (chorio-allantoic) on the livability of chicken spermatozoa.

	Survival time in days						
	Trial numbers						
Treatment*	I	II	III	IV	V	VI	VII
1	6	8	9	8	7	6	7
2	9		7	5	6	7	6
3	8		6	6	6	7	7
4	3	9	6	5	8	8	8
5	8	8	6	5	8	7	7
6	7	7	7	5	9	7	6
7	8	7	8	6	10	6	6

* 1 Untreated semen
2 Semen diluted with 33% egg fluid
3 Semen diluted with 16% egg fluid
4 Semen diluted with 9% egg fluid
5 Semen diluted with 5% egg fluid
6 Semen diluted with 2% egg fluid
7 Semen diluted with 1% egg fluid

Table 11

The effect of embryonated egg fluid (chorio-allantoic) on the motility and livability of chicken spermatozoa.

Treatment*	Motility rating in days					Sperm survival time in days
	0	1	2	3	4	
Trial I						
1	4	3-	3-	3	2-	8
2	4	3	3	2-	2	5
3	4	3-	3	3-	2	6
4	4	3-	3	3	2	5
5	4	3-	3	2	2	5
6	4	3	3	3	2-	5
7	4	3	3-	3	2-	6
Trial II						
1	4	3-	3	3	2	7
2	4	3	2-	2-	1	6
3	4	3	2	1-	1	6
4	4	3-	3	2-	2	8
5	4	3-	3	2-	2	8
6	4	3--	3-	2-	2-	9
7	4	3---	3-	3	2-	10

* 1. Untreated semen
2. Semen diluted with 33 per cent egg fluid
3. Semen diluted with 16 per cent egg fluid
4. Semen diluted with 9 per cent egg fluid
5. Semen diluted with 5 per cent egg fluid
6. Semen diluted with 2 per cent egg fluid
7. Semen diluted with 1 per cent egg fluid

serum on the livability and motility of fowl spermatozoa were studied.

The blood was collected from four-week old chicken and stored in a refrigerator at 4 - 5°C for 48 hours. Then the clear serum was drawn out, centrifugated and passed through bacterial filters to eliminate extraneous contamination as far as possible. This material was used as a diluent at the following ratio: 9 parts semen to 1 part diluent, 1 to 1, 1 to 3, 1 to 7 and 1 to 15.

The results are tabulated in Table 12 which show the chicken blood serum has a favourable effect on both livability and motility of chicken spermatozoa as compared with that of the undiluted (pure) semen. Further, all the dilutions used in this experiment have supported sperm life and motility better than undiluted controls; but 1 to 1 and 1 to 3 dilutions seems to have yielded the best results.

c.and d. Fresh and inactivated chicken semen serum

A similar series of parallel experiments were set up to study the comparative effects of two more diluents, inactivated chicken semen serum and chicken sperm serum, compared with chicken blood serum. Semen serum was obtained by centrifuging the semen soon after collection and carefully drawing out the supernatent fluid. With a view to avoid gross bacterial contamination and to have absolutely sperm free fluid it was further passed through a bacterial filter and then stored at 4 - 5°C

Table 12.

The comparative effect of chicken blood serum, inactivated semen serum and fresh semen serum on the livability of chicken spermatozoa stored at 10-12° C.

No. of trials	Diluents	Survival time in days					
			Dilutions				
		Control	9:1	1:1	1:3	1:7	1:15
1	Semen+chicken blood serum	3	5	5.5	5*	5*	4*
2	Semen+chicken blood serum	4	5	5	4*	4*	4*
3	Semen+chicken blood serum	4	5	5.5	5*	4.5*	4*
1	Semen+ #	4	4	3	3*	3*	3*
2	Semen + #	4	4	4	3	3	3
3	Semen+ #	3	4	3	3	2.5	2.5
1	Semen+fresh semen serum	4	5	5	3	3	3
2	Semen+fresh semen serum	4	5	4.5	3	3	3
3	Semen+fresh semen serum	4	4.5	5	3	3	2.5

* Heavy bacterial growth

\# Inactivated chicken semen serum

Control = Untreated semen

before it was used. To obtain inactivated semen serum sample was first heated in a water bath at 60°C for one hour before subjecting it to centrifugation and filteration.

The results enumerated in Table 12 show a distinct superiority of chicken blood serum as a diluent for chicken semen over sperm serum and inactivated shicken semen serum. Fresh semen serum seemed to be better than the inactivated semen serum. Again the dilutions of 1 to 1 and 1 to 3 yielded better results comparatively.

Heavy bacterial contamination was invariably observed in all the samples particularly those having a greater volume of the diluents(1 to 3 to 1 to 15). Consequently in subsequent trials the effect of all these three diluents were checked again on addition of streptomycin and polymyxin, 400 ug. each per milliliter. The results are presented in Table 13. The addition of antibiotics to sperm serum does not appear to have a conspicious effect, in improving the livability of spermatozoa, though there is some indication of improvement in the motility rating. But as far as blood serum is concerned the addition of antibiotics have improved slightly both livability and motility.

e. Pasteurized whole milk

In recent years considerable attention has been paid to the use of whole and skim milk as a diluent for bull semen to be used in artificial breeding of cows.

Table 13

The comparative effect of chicken blood serum, inactivated semen serum and fresh semen serum, containing 400 ug/ml of each of streptomycin and polymyxin on the livability of chicken spermatozoa stored at 10-12°C.

Trials	Diluents*	Survival time in days					
		Dilutions					
		Control	9:1	1:1	1:3	1:7	1:15
1		5	4.5	6.5	5.5	4	3.5
2		4	4	6	6	5.5	5.5
3		3.5	4	5.5	4.5	5	5
1a		4	4	4	4	3	2.5
2a		4	4	4	4	3.5	3.5
3a		3.5	4	3	3.5	2.5	2.5
1b		4.5	4	4	3	2.5	2.5
2b		4.5	5	4	3	2.5	2.5
3b		3.5	3	3	3	2.5	2

* 1 to 3 Semen diluted with chicken blood serum plus antibiotics

1a,2a, 3a Same as above except inactivated chicken semen was used instead of blood serum

1b,2b, 3b Same as above except fresh chicken semen serum was used instead of blood serum

Unfortunately, no specific data or details as to the technique employed are available. A brief summary of the results obtained so far by various Breeder's Associations in the United States during the year 1951 and 1952 is given below. The summary is an extract from the report presented at National A.A.A.B., Springfield, Missouri, during September 1952 by B.W. Kagy, Northern Ohio Breeder's Cooperative Association, Tiffin, Ohio. Ordinarily the milk was boiled, strained and cooled to room temperature. Then penicillin, streptomycin and sulfanilamide added (500 - 1000 units per milliliter).

Summary of results for 1951 - 1952

Sources	Results	
	60-90 days Non-return Milk	Yolk citrate
1. Badger Breeders'-Shawano, Wisconsin	71.20% 69.22%	64.17% 56.95%
2. American Foundation for Study of Genetics, Madison, Wisconsin	59.9 %	75.00%
3. Evergreen Breeder's Association, Chehallis, Washington	68.9 %	66.6 %
4. Northwest Coop. Breeder's, Mt. Vernon, Washington	about 4% increase milk.	

The percentage of fertility obtained from the use of whole homogenised pasteurized milk has invariably been better when compared to that obtained from the use of yolk citrate with the only exception of the American Foundation for the Study of Genetics, Madison (Wisconsin). They obtained about 16 per cent less fertility with milk

than that from the yolk citrate.

It seems that so far milk has not been tried as a diluent for chicken semen. The results of a few experiments on the use of milk as a diluent for chicken semen and its effects on spermatozoal livability and motility are discussed here. Table 14 shows the effects of whole pasteurized milk, when added to chicken semen at different levels, on the livability and motility of sperm in vitro stored at 10 - 12°C.

Though actual estimation of the results was not possible due to very heavy bacterial growth that occurred during the storage of whole pasteurized milk, this diluent seems to have aided in supporting sperm life. The results for the last two dilutions (1 to 7 and 1 to 15) could not properly be evaluated due to heavy bacterial growth.

Table 15 shows the results of a similar series of experiments when antibiotics were added. Though the level of antibiotics used here (Streptomycin and polymyxin. 400 ug/ml. each), seems to be low in view of the actual bacterial contamination of milk, still they appear to have undoubtedly modified the results markedly.

f. Pasteurized skim milk and whey

In contrast to whole milk, skim milk did not produce very good results and the fertility obtained with skim milk was slightly below that from yolk citrate (Kagy, 1952).

Table 14

Pasteurized whole milk as a diluent for semen and its effect on chicken sperm livability in vitro during storage at 10-12°C.

Trials	Diluents*	Survival time in days					
		Dilutions					
	Control	9:1	1:1	1:3	1:7	1:15
1	3.5	4.5	5	4.5	Bact.	Bact.
2	4	4.5	5.5	5	4	3
3	4	4.5	5	4.5	3.5	3

* Semen diluted with pasteurized whole milk

Table 15

Pasteurized whole milk with antibiotics (400 ug/ml streptomycin and polymyxin each) as a diluent of semen and its effect on chicken sperm livability in vitro stored at 10-12°C.

Trials	Diluents*	Survival time in days					
		Dilutions					
	Control	9:1	1:1	1:3	1:7	1:15
1	4	6	6	4.5	3	3
2	4	5.5	5.5	3.5	3	2.5
3	4	5	5.5	4	3	3

* Semen diluted with whole pasteurized milk plus antibiotics

The results obtained with pasteurized skim milk (liquid) in this laboratory when added to chicken semen in various dilutions, alone or with antibiotics, seem to have supported sperm livability and motility in vitro better than the untreated controls during storage at 10 - 12°C. The results are summarized in Tables 16 and 17.

Whey: Whey was prepared by curding hot milk by the addition of sulfuric acid (1 to 3 per cent) drop by drop till coagulation occured. Then strained through several layers of cheese cloth, centrifuged and brought to 7.5 pH. Finally it was passed through a bacterial filter and stored at low temperature (4 - 5°C) till used.

Tables 16 and 17 show the effects of whey, either alone or with antibiotics (400 ug. per milliliter, each of streptomycin and polymyxin) on chicken sperm livability and motility in vitro under conditions of storage at 10 - 12°C when added to chicken semen in different dilutions as a preservative.

The results obtained in Table 16 and 17 are very interesting and encouraging and warrant further investigation.

Table 16

The pasteurised skim milk and whey diluent for semen and its effect on chicken sperm livability in vitro during storage at 10-12°C.

Trials	Diluents*	Survival time in days					
		Dilutions					
		Control	9:1	1:1	1:3	1:7	1:15
1		3	3.5	Bact.	Bact.	Bact.	Bact.
2		3.5	6	5	Bact.	Bact.	Bact.
3		3	4.5	5	Bact.	Bact.	Bact.
4		4	4.5	5	5	4	3.5

* 1 to 3 Semen diluted with pasteurised skim milk

4 Semen diluted with whey

Table 17

The pasteurised skim milk with antibiotics (400 ug/ml of each of streptomycin and polymyxin) as a diluent for semen and its effect on chicken sperm livability in vitro during storage at 10-12°C.

Trials	Diluents*	Survival time in days					
		Dilutions					
		Control	9:1	1:1	1:3	1:7	1:15
1		3	6	6	5	Bact.	Bact.
2		3.5	5	5.5	5	Bact.	Bact.
3		3.5	5	6	4.5	Bact.	Bact.
4		4	5	5.5	6	3.5	3.5

* 1 to 3 Semen diluted with pasteurised skim milk plus antibiotics

4 Semen diluted with whey plus antibiotics

BACTERIOLOGY OF CHICKEN SEMEN BEFORE AND AFTER TREATMENT WITH ANTIBIOTICS

The importance of bacteriological control in the rapidly expanding field of the artificial insemination of cattle has been stressed often by practically all workers in the field, but little attention has been paid to the subject in relation to preservation of chicken semen.

It has been recognized for many years that semen is an excellent medium for the growth and multiplication of bacteria. Probably the first to report such an observation was Spallanzani (1785, cit. by Gunsalus, 1941) who attributed the decrease in fertility of the spermatozoa during storage to putrifection. Roemmele (1927), Kovzysekowsky and Polow (1927) cit. by Gunsalus (1941) and Hammond (1930) reported bacterial growth in semen, but no definite data could be found to indicate whether or not bacteria have a direct detrimental effect on spermatozoa. Kovzyskowsky and Powlow (1927) thought that metabolic products of bacterial activity influenced spermatozoal life. But Gunn (1936) working with ram semen failed to detect any such influence of bacterial metabolites on the survival of spermatozoa stored at low temperature ($4^{o}C$). Salisbury, Willett and Gunsalus (1939) stressed the need for more information concerning the possibility of infections of the female genital tract from the types of bacteria encountered frequently in both fresh and stored semen.

The available information indicates the presence of

several types of bacterial agents in both fresh and stored bull semen. Some of these organisms have been, associated with various pathological conditions of the cow's genital tract. Gilman (1921) reported finding pseudomonas pyocyaneus and unidentified rods, micrococci, streptococci and coliform organisms. This was in agreement with the report by Williams and Kingsbury (1920). Hatziolos (1937, cit. by Gunsalus, Salisbury and Willett, 1941) reported the finding of proteus, coliform, cocci, pseudomonas and other spore forming rods in almost every sample of bull semen examined. Gunsalus, Salisbury and Willett (1941) reported the presence of pseudomonas, coliform, dephtheroids, bacilli, and staphylococci in bull semen. He considered that there was no appreciable germicidal action of semen and found a considerable decrease in fertility in bulls which were affected with pseudomonas pyocyaneus. He further pointed out the importance of reducing the initial bacterial population by care in collection and handling the semen and the necessity of checking growth by storage at low temperatures if the fertilizing power of bull semen is to be maintained.

The spermatozoa of most mammals rapidly lose their fertilizing power within the female genital tract, (Hartman, 1939; Salisbury, 1941; Parker, 1944; Perry, 1945 and Anderson, 1945), whereas the chicken spermatozoa in the hen's oviduct continue to fertilize eggs for two to three weeks (Crew, 1925 - 26; Nicolaides; 1934; Parker, McKenzie and

Kempster, 1942). The fertilizing capacity of the chicken spermatozoa rapidly decreases after storage for 4 hours at room temperature (Barrows and Quinn, 1939a) and fertilized eggs are rarely laid by hens inseminated with semen stored for more than eight hours. Motility affords a good index of fertilizing capacity of mammalian spermatozoa but is not a reliable indication in the case of fowl spermatozoa.

Semen from cocks is collected from the papillae on the wall of the cloaca and is heavily contaminated with bacteria, Grodzinski and Marchlewski (1935); and Koch and Robillard (1945).

The present investigation deals with the common types of bacteria occurring frequently in fresh or stored chicken semen and in samples of semen diluted with isotonic sodium citrate (4.75 per cent) and solutions containing penicillin, terramycin, polymyxin and streptomycin. Parallel observations on the motility of the spermatozoa were also made in order to demonstrate the toxicity of the reagents in vitro. It is not intended, however, to study the full bacterial flora of chicken semen but only the extent to which the control of bacterial growth in chicken semen would be effective in improving semen quality.

Method and Material

A composite sample of semen was used throughout these investigations in the study of total plate counts and the determination of frequently occurring bacterial types in chicken semen. The diluents were prepared in clean glassware. The organism occurring in semen were isolated by the streak and the pour plate technique. At first eugan agar, blood agar and eosin methylene blue agar plates were used and later only eugan agar and eosin methylene blue plates were used. All plates were incubated at 37°C for 48 hours.

A single loopful of diluted or undiluted semen with or without antibiotics was spread across the plates in a streak. Six such streaks were made on each plate. The number of colonies was counted under a colony counter with the aid of a tally. Less than ten colonies were marked by one plus sign (+), between ten and sixty colonies by two plus marks (+ +), over sixty colonies by three plus marks (+ + +) and a heavy confluent growth by four plus marks (+ + + +).

At first the solution of antibiotics were made in isotonic sodium citrate solution but later in sterile distilled water. After addition of the antibiotics or the diluent to the semen samples, the tubes were rotated gently to facilitate mixing and then stored at the desired temperature (10 - 12°C) until expiration of motility.

The types of bacteria present in fresh semen: The sperm masses obtained from the vasa differentia soon after

killing the cock have frequently been reported to be sterile. Of the ten samples of semen collected from living birds none proved to be sterile. The types of bacteria found are listed in their order of predominance; coliform bacilli, staphylococci, hemolytic streptococci, some unidentified spore forming rods and gram positive diplococci (Table 18). All of these types have been previously reported to be present in chicken semen.

Cultures of untreated semen stored at 10 - 12°C for 48 hours yielded equally heavy growth of both coliform bacilli and staphylococci. When these semen samples were stored for more than 48 hours, cultures made from them yielded heavier growth of coliform bacilli than staphylococci. In many samples having enormous bacterial growth the spermatozoa appeared to have disintegrated.

Cultures of samples diluted with isotonic sodium citrate solution showed the same bacterial contamination both qualitatively and quantitatively as the untreated semen after four days storage. But most of the semen samples cultured immediately after dilution had less profuse growth quantitatively than the diluted samples (Table 19). After storage for 96 hours at 10 - 12°c a few samples yielded, on being cultured, less profuse growth of staphylococci than samples from the same specimen without dilution. In most of the cases, better spermatozoal motility was maintained in samples of pure semen than the samples treated with penicillin and isotonic sodium citrate solution.

Table 18

Showing the commonly occurring bacteria in freshly collected semen incubated at 37°C for 48 hours

Trial	Organisms Isolated			
	Colliform bacilli	Staphylo-cocci	Streptococci (Hemolytica)	Other Organisms
1	++	++		
2		++		
3	+++	+		
4	++			G+/Diplococci
5	+++	+	+	
6	++	+		
7	++	++		
8	++++	++	+	
9	++	+++	+	G+/Diplococci
10	++	++		

Table 19.

Bacterial growth in chicken semen (without antibiotics) before and after storage at 10-12° C.

Treatment*	Initial motility	Sperm survival time in (in vitro)	Bacterial growth in chicken semen incubated at 37° C for 48 hours			
			Soon after collection		After 96 hours storage at 10-12° C	
			Coliform bacilli	Staphylococci	Coliform bacilli	Staphylococci
Trial I						
1	3	4	++	++	++++	++++
2	3	3.5	++	++	++++	++++
Trial II						
1	4	3	+	+	+++	+++
2	4	3	+	+	++++	+++
Trial III						
1	4	4	++	++	++++	++++
2	4	4	+++	+++	++++	++++
Trial IV						
1	3	3	+	+	++++	++++
2	3	4	+	+	++++	+++

* 1 Untreated semen.
2 Semen diluted with isotonic citrate solution at the rate of 9 to 1.

However, survival time was extended on the addition of penicillin.

a. Penicillin

The composite sample of freshly collected semen was divided into three equal portions; to one of these a 4.75 per cent solution of sodium citrate solution was added and to the other, 1000 units, of penicillin, keeping the dilution in both the tubes in the ratio of 9 to 1. The third portion was held undiluted. They are then stored at 10 - 12°C. The initial cultures were made soon after collection and dilution and again after 96 hours storage. The results showed that 1000 units of penicillin (Pen. - G - crystalline potassium) did not completely retard the bacterial growth in the sample treated with it, although the growth in this sample was not as profuse as in any of the other samples. Motility ratings were better in the penicillin treated sample where bacterial growth was reduced (Table 20).

b. Terramycin

The composite sample of freshly collected semen was divided into four equal portions. One of these held undiluted and the second one was diluted with 4.75 per cent solution of sodium citrate. The remaining two samples were diluted with solutions containing 150 gamma and 300 gamma per milliliter terramycin. The cultures were made soon after collection and dilution of the semen sample and again after 96 hours storage.

The results are summarized in Table 21, which show

Table 20.

The effect of penicillin in inhibiting bacterial growth in chicken semem stored at 10-12° C and incubated at 37° C for 48 hours.

Treatment *	Initial motility	Sperm survival time in days	Organisms isolated: Soon after collection — Coliform bacilli	Soon after collection — Staphylococci	After 96 hours storage — Coliform bacilli	After 96 hours storage — Staphylococci
First trial						
1	4	3	+	++++	++++	+++
2	4	3	++	++++	++++	++++
3	4	5	+	+++	+++	++
Second trial						
1	4	4	++	++	++++	++++
2	4	4	+++	+++	++++	++++
3	4	5	+	++	+++	+++

* 1. Untreated semen control.

2. Semen treated with isotonic sodium citrate solution at the rate of 9 to 1.

3. Semen treated with penicillin (1000 units/ml of semen) at the rate of 9 to 1.

Table 21.

The effect of terramycin in inhibiting bacterial growth in chicken semen stored at 10-12°C and incubated at 37° C for 48 hours.

Treatment*	Initial motility	Sperm survival time in days (in vitro)	Organisms isolated: Soon after collection: Coliform bacilli	Soon after collection: Staphylococci	After 96 hours storage: Coliform bacilli	After 96 hours storage: Staphylococci
Trial I						
1	3	3	++	++	++++	++++
2	3	3		++	++++	++++
3	3	3			+++	+
4	3	4			++	+
Trial II						
1	3	3	++	++	++++	++++
2	3	3	++	++	++++	++++
3	3	4			+++	++
4	3	5			++	+

* 1 Untreated semen.

2 Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1.

3 Semen treated with terramycin (150 ug/ml of semen) solution at the rate of 9 to 1.

4 Semen treated with terramycin (300 ug/ml of semen) solution at the rate of 9 to 1.

practically the same trend as the samples treated with penicillin except that terramycin appeared to be slightly superior in inhibiting bacterial growth during storage particularly that of staphylococci.

c. Polymyxin

The composite sample of freshly collected semen was divided into four equal portions and treated as deecribed previously with 150 gamma and 300 gamma of polymyxin per milliliter.

The results are summarized in Table 22, showing the same trend as the penicillin treatment. The drug does not seem to be superior to terramycin.

d. Streptomycin

The composite sample of freshly collected semen was divided into four equal portions and treated as described previously, using 150 gamma and 300 gamma of streptomycin per milliliter.

The results are summarized in Table 23. The addition of streptomycin to chicken semen at the rate of 300 gamma per milliliter has supported the motility and extended the survival time of chicken spermatozoa in vitro when compared with either the untreated sample or the one diluted with 4.75 per cent solution of sodium citrate. Further, the reagent appears to be definitely superior as a bacteriostatic to all the antibiotics hitherto used in these experiments. It suppressed the growth of the most common coliform organisms to a greater extent and completely inhibited the growth of staphylococci during a period of

Table 22.

The effect of polymyxin in inhibiting bacterial growth in chicken semen stored at 10-12° C and incubated at 37° C for 48 hours.

Treatment*	Initial motility	Sperm survival time in days (in vitro)	Organisms isolated: Soon after collection: Coliform bacilli	Organisms isolated: Soon after collection: Staphylococci	Organisms isolated: After 96 hours storage: Coliform bacilli	Organisms isolated: After 96 hours storage: Staphylococci
Trial I						
1	3	4	+	+	++++	++++
2	3	3.5	+	+	++++	++++
3	3	4			++++	++++
4	3	6			++	+
Trial II						
1	4	3	++	++	++++	++++
2	4	2.5	++	++	++++	++++
3	4	3			++++	++++
4	4	4.5			++	++

* 1 Untreated semen

2 Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1.

3 Semen treated with polymyxin (150 ug/ml of semen) solution at the rate of 9 to 1.

4 Semen treated with polymyxin (300 ug/ml of semen) solution at the rate of 9 to 1.

Table 23

The effect of streptomycin in inhibiting bacterial growth on chicken semen stored at 10-12°C and incubated at 37°C for 48 hours

Treatment*	Initial Motility	Sperm survival time in days	Organisms isolated: Soon after collection: Coliform bacilli	Soon after collection: Staphylococci	After 96 hours storage: Coliform bacilli	After 96 hours storage: Staphylococci
First trial						
1	3	3	+++	+++	++++	++++
2	3	3	+++	++	++++	++++
3	3	3.5			+++	++
4	3	5			+++	
Second trial						
1	3	3	++	++	++++	++++
2	3	3	+	++	++++	++++
3	3	4			+++	++
4	3	5			+	

* 1. Untreated semen.
2. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1.
3. Semen treated with streptomycin (150 ug/ml of semen) solution at the rate of 9 to 1.
4. Semen treated with streptomycin (300 ug/ml of semen) solution at the rate of 9 to 1.

storage for 96 hours in many cases.

On the consideration of these results it was thought desirable to set up parallel experiments to test further the effectiveness of streptomycin against penicillin and terramycin simultaneously. One thousand units of penicillin were used against 300 gamma of streptomycin.

e. Penicillin and streptomycin

The methods and procedure followed in these experiments were similar to those described before. The results are summarized in Table 24. They again indicate the superiority of streptomycin over that of penicillin, both as a better supporter of spermatozoal livability and motility as well as a good bacteriostatic for the control of bacterial growth in chicken semen during storage at 10 - 12°C.

f. Terramycin and streptomycin

The method and procedure followed in this experiment is the same as described previously except that one control sample (semen diluted with isotonic sodium citrate solution) was used. 150 and 300 gamma per milliliter of terramycin were checked against the same levels of streptomycin.

The results obtained are tabulated in Table 25. It seems that 300 gamma of either terramycin or streptomycin are equally effective in preserving spermatozoal livability and motility when added to chicken semen. But streptomycin appears to be slightly better bacteriostatic

Table 24

Comparative Effect of Penicillin and Streptomycin in inhibiting bacterial growth in chicken semen stored at 10-12°C and incubated at 37°C for 48 hours

Treatment *	Initial Motility	Sperm Survival time in days	Organisms isolated: Soon after collection — Coliform bacilli	Soon after collection — Staphylococci	After 96 hours storage — Coliform bacilli	After 96 hours storage — Staphylococci
First Trial						
1	4	3	+	+	++++	++++
2	4	3	++	++	+++	+++
3	4	5	+	+	+++	+++
4	4	6	+	+	+	+
Second Trial						
1	4	4	++	++	++++	++++
2	4	4	++	++	++++	++++
3	4	5	+	++	+++	+++
4	4	7	+	+	+	+

* 1. Untreated semen
2. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1
3. Semen treated with penicillin (1000 units/ml of semen) solution at the rate of 9 to 1
4. Semen treated with Streptomycin (300 ug/ml of semen) solution at the rate of 9 to 1

Table 25

Comparative effects of terramycin and streptomycin in inhibitin bacterial growth in chicken semen stored at 10-12°C and incubated at 37°C for 48 hours

Treatment *	Initial Motility	Sperm Survival time in days	Organisms isolated: Soon after collection — Coliform bacilli	Soon after collection — Staphylococci	After 96 hours storage — Coliform bacilli	After 96 hours storage — Staphylococci
First Trial						
1	3	3	++	++	++++	++++
2	3	3.5			+++	+++
3	3	4			++	++
4	3	5			++	++
5	3	5			+	+
Second Trial						
1	4	3	+	++	+++	+++
2	4	3			++	+
3	4	4			++	++
4	4	5			+	+
5	4	5			+	+

* 1. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1
2. Semen treated with terramycin (150 ug/ml of semen) solution at the rate of 9 to 1
3. Semen treated with streptomycin (150 ug/ml of semen) solution at the rate of 9 to 1
4. Semen treated with terramycin (300 ug/ml of semen) solution at the rate of 9 to 1
5. Semen treated with streptomycin (300 ug/ml of semen) solution at the rate of 9 to 1

for controlling the bacterial growth in chicken semen.

g. Penicillin, terramycin, polymyxin and streptomycin

In this investigation parallel experiments were set up to study the value of penicillin (1000 units/ml of semen), terramycin, polymyxin and streptomycin (300ug/ml of semen , each) as bacteriostatic agents when added to fresh chicken semen under identical conditions.

The results are tabulated in Table 26, which again have borne out the superiority of streptomycin over the other antibiotics used.

With these facts in view a few more trials were conducted to study the efficiency of streptomycin when used singly at various levels in chicken semen. The levels studied in this investigation were 400, 600 and 700 gamma of streptomycin per milliliter. The method and procedure remained the same as followed in previous experiments except that pour plate technique was adopted for total plate counts in place of that of the streak technique. The pour plate technique appears to give more accurate results (Table 27).

A few of the typical series of results obtained in these experiments are summarized in Table 27, the trend of which is in agreement with those obtained in previous experiments. The diluted semen controls have supported the spermatozoal livability for the shortest period (4.5 days) in these series of experiments; and so also was the case with motility rating. On the other hand survival period is extended and motility improved with the addition of streptomycin and

Table 26

Comparative effects of penicillin, terramycin, polymyxin and streptomycin in inhibiting bacterial growth in chicken semen stored at 10-12°C and incubated at 37°C for 48 hours

Treatment *	Initial Motility	Sperm Survival time in days	Organisms isolated: Soon after Collection — Coliform bacilli	Soon after Collection — Staphylococci	After 96 hours storage — Coliform bacilli	After 96 hours storage — Staphylococci
First trial						
1	4	4	++	++	++++	++++
2	4	5			+++	++
3	4	4.5			++	+
4	4	6			++	+
5	4	6			+	+
Second Trial						
1	4	4	++	++	++++	++++
2	4	5			+++	+++
3	4	5			++	+
4	4	5			++	++
5	4	7	+	+	+	+

* 1. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1
2. Semen treated with penicillin (1000 units/ml of semen) solution at the rate of 9 to 1
3. Semen treated with terramycin (300 ug/ml of semen) solution at the rate of 9 to 1
4. Semen treated with polymyxin (300 ug/ml of semen) solution at the rate of 9 to 1
5. Semen treated with streptomycin (300 ug/ml of semen) solution at the rate of 9 to 1

Table 27.

The effect of streptomycin on the survival time of spermatozoa and control of bacterial growth when stored at 10-12° C.

Treatment*	Initial motility	Duration motility in days	Total plate count per c.c. Soon after collection	Total plate count per c.c. After 96 hrs storage at 10-12° C
First trial				
1	4	4.5	500	Too numerous to count
2	4	5		15,200
3	4	6		5,400
4	4	6		3,400
Second trial				
1	4	4.5	560	Too numerous to count
2	4	5		39,000
3	4	5		35,000
4	4	6		21,000

*1 Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1.

2 Semen treated with streptomycin (400 ug/ml of semen) diluted at the rate of 9 to 1.

3 Semen treated with streptomycin (600 ug/ml of semen) diluted at the rate of 9 to 1.

4 Semen treated with streptomycin (700 ug/ml of semen) diluted at the rate of 9 to 1.

the amount of improvement increased as the level of streptomycin is increased from 400 to 700 gamma per milliliter of semen. The bacterial growth in the untreated diluted semen controls was very heavy and confluent and the colonies could not be counted, which was much less profuse in the case of the samples of semen treated with streptomycin. There was a gradual decrease in the number of bacterial colonies as the level of streptomycin increased from 400 to 700 gamma per milliliter of semen; and 700 gamma appears to be the optimum level. Further in all these experiments the sperm motility and survival time in the samples of semen with low bacterial counts held better and longer than that with high counts. The number of bacteria present in these samples of semen seems to be higher than would be expected (as reported for the bull semen). It might be due to the fact that chicken semen is highly contaminated during collection in comparison to semen samples drawn from the bull. Moreover many variables, such as technique of collection, individual handling of birds and sources of general contamination, also exist.

h. Mineral oil as a bacteriostatic

Rubber and metals are the common materials which have a harmful effect when brought in contact with semen during the routine practice of collection of semen. The contact of air and deposits of dirt or salt on the equipment has also been considered harmful to sperm life. On the other hand, dry clean glass and the pure paraffins, e.g. liquid

paraffin and white vasaline (petrolatum) have no such deliterous effects.

The "Cambridge" method of storing semen consists of collecting it in clean sterile hard glass ware and then transferring it to clean, dry, sterile glass tubes or suitable glass containers. The top of the semen in the tubes is covered with a layer of sterile liquid paraffin and finally closing the mouth of the tubes with a stopper or cotton wool plug to keep out dust particles (Walton, 1933). Hammond (1940) considered that semen on contact with air takes up O_2 and gives off Co_2 in the process of respiration. This ordinarily mobilizes the spermatozoa and thereby the duration of their life is shortened.

The purpose of the present investigation is to compare and determine if covering the treated or untreated semen samples with a film of mineral oil would reduce the growth of bacteria, improve the spermatozoal motility or extend their survival period in vitro when stored for short duration (96 hours) at 10 - 12°C.

The results are summarized in Table No. 28 and 29. The data contained in Table No. 28 from six trials did not indicate a marked difference between the treatments particularly the semen with oil and the semen with no oil, though the effect on addition of the antibiotic is quite conspicuous. Likewise the data summarized in Table 29, did not show marked difference both in regard to bacterial growth and motility ratings between the two treatments (semen with oil or without oil).

Table 28

Effect of Mineral Oil when added to chicken semen on the survival time of spermatozoa

Treatment**	Survival time in days						
	Trial Nos.						Average of Trials
	1	2	3	4	5	6	
1.	4.5	4.5	5.5	5	4	4	4.6
2.	4.5	6	5	5	4.5	5.5	5.1
3.	4.5	4.5	5	4.5	4.5	5	4.7
4.	6.5	6.5	7	6	6.5	6.5	6.5
5.	5.5	5.5	6.5	6	6.5	6.5	6.1

* Saline comparable to 9 to 1

** 1. Untreated semen without oil

2. Untreated semen with oil film on top

3. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1

4. Semen treated with streptomycin (700 ug/ml of semen) solution at the rate of 9 to 1

5. Semen treated with streptomycin (700/ug/ml of semen) solution at the rate of 9 to 1 and having a film of oil on top.

Table 29

Bacterial growth in chicken semen during storage at 10-12°C with a film of mineral oil on top of the tube containing semen

Treatment*	Motility rating in days					Sperm Survival Time (Vitro) in days	Total plate counts after 96 hrs storage at 10 - 12°C
	0	1	2	3	4		
First trial							
1.	4	3+	3	3	1+	5	Too numerous to count
2.	4	3	2	2	1	4	Too numerous to count
3.	4	3	2	2	1	4.5	Too numerous to count
4.	4	3	3	3	2+	6	10,000 colonies/cc.
5.	4	3	2	2	1+	6	12,600 colonies/cc
Second Trial							
1.	4	3	3	2	1+	5	Too numerous to count
2.	4	3	3	2	1	4.5	Too numerous to count
3.	4	3	2	2	1+	5	Too numerous to count
4.	4	3	3	3	2	6.5	70,000 bacteria colonies
5.	4	3+	3+	3	2+	6	52,000 bacteria colonies

*1. Untreated semen
2. Untreated semen with oil film on top
3. Semen diluted with isotonic saline solution at the rate of 9 to 1 (no oil)
4. Semen treated with terramycin(300 ug/ml of semen) solution at the rate of 9 to 1.
5. Same as No.4 but with oil film on top.

EFFECT OF ANTIBIOTICS PLUS SEX HORMONES ON CHICKEN SEMEN

In the preceeding pages the commonly occurring bacteria in chicken semen and their influence upon the preserving quality of semen have been discussed. The effect of some of the diluents, from biological sources, on the livability and motility of fowl sperms in vitro stored at 10 - 12°C have also been discussed. The purpose of the present investigation is to explore further the possibility that the sex hormones would likely have a favourable influence on sperm life.

Moore (1928) showed that sperm life in the isolated epididymis of the rat will survive for 30 to 40 days and in the guinea pig for 60-70 days provided one testis is left in tact to add its secretions to the body fluids. When both the testes are removed and the sperm in the epididymis denied egress by ligatures, the length of survival is reduced in the rat from 17 to 18 days and in the guinea pig to 23 days. Consequently Moore concluded that the testis hormone is responsible for the maintenance of sperm life. As a result of this and the additional evidence supplied by further studies -(Moore, 1928; Moore and McGee, 1928) - the "spermatozoon motility test" became a standard test for detecting the presence of testis hormones. But the sperm in the fowl can survive and maintain their functional capabilities in the female tract for a period of 3 to 4 weeks. In view of this fact the

question arises whether their life is in any way supported or prolonged beyond this stage (3 to 4 weeks) in the excretory ducts of the cock by virtue of the presence of functional testes. Because if the testicular secretions create an optimum environment and thus prolong sperm life, one would quite reasonably expect to see this time increased in the male. Munroe (1938) in a carefully designed experiment demonstrated that this did not occur. He thinks that testes hormone is not concerned, as it is in the mammals, with prolonging sperm life. In another experiment, Munroe (1938) while studying the functional changes in fowl sperm mentioned that a male environment is necessary for the attainment of functional maturity. This is possibly due to the presence of testes hormone.

The exact manner in which the testicular secretions produce their sperm preserving effect in mammals is not known. White (1932) has shown that the preserving action of the testes is dependent on the presence of pituitary and believes that the effect is produced either directly by testes hormone or indirectly by the action of this hormone on the epididymal secretions. Whatever may be the precise mechanism governing the maintenance of extended sperm life in excretory ducts of the male or the oviduct of the hen, it is clear that something does occur which create an optimum environment and thus prolong sperm life in these localities without impairing fertilizing power.

The evidence produced so far mainly describe the effect

of testicular hormone on sperm life in the male excretory ducts. The present investigation discusses the effect of sex hormones on the livability of chicken spermatozoa in vitro under conditions of artificial storage at 10-12°C.

A stock solution of both male and female hormones was prepared by dissolving the measured quantities of the synthetic hormones in sesame oil. The male (testosterone propionate) and female (diethylstilbesterol) hormones were added to the sesame oil that was used to cover the semen. One-tenth milliliter of this oil was added to the tubes containing the semen and semen and oil thoroughly mixed. It was felt that even though the oil came to the surface of the semen, some of the hormone will remain in the semen. The results obtained with the use of sex hormones on the livability of chicken spermatozoa in vitro are discussed in the following tables.

In Table 30 the results are summarized regarding the effect of sex hormones on the survival time of spermatozoa in vitro. A review of the data in Table 30 indicated that the sex hormones may have had a beneficial effect. Since the addition of antibiotics had previously been shown to prolong the survival time, it was decided to combine the treatment with antibiotics and sex hormones. The data showing the effect of these treatments are shown in Tables 31 and 32. The data in the following tables (30 - 32) is slightly different than those previously discussed in that the motility here is recorded as the maximum survival time

Table 30

The effects of different levels of male and female hormones on the livability of chicken spermatozoa stored at 10-12° C.

Treatment	Survival time in days		
	Trial number		
	I	II	III
Male hormone			
Saline control with oil film	4	4	5
Semen + hormone (100 ug/ml)	5	5	5
Semen + hormone (100 ug/ml	5	4.5	5
Saline control with oil film	5	4	5
Semen + hormone (200 ug/ml)	6	5	6
Semen + hormone (200 ug/ml)	5	5	7
Saline control with oil film	4	5	6
Semen + hormone (400 ug/ml)	5	5	7
Semen + hormone (400 ug/ml)	4	5	6
Female hormone			
Saline control with oil film	4	5	4
Semen + hormone (40 ug/ml)	4	5	4.5
Semen + hormone (40ug/ml)	4	4.5	5
Saline control with oil film	5	5	4
Semen + hormone (80 ug/ml)	6	5	5
Semen + hormone (80 ug/ml)	5.5	5	4
Saline control with oil film	5	5	5
Semen + hormone (160 ug/ml)	5	5	6
Semen + hormone (160 ug/ml)	4	5	7

Table 31

The effect of male (200 ug/ml) and female (160 ug/ml) hormones on the addition of streptomycin (700 ug/ml) on the livability of chicken spermatozoa stored at 10-12°C.

Treatment*	Survival time in Days		
	Trial Number		
	I	II	III
1.	4	5	5
2.	5	6	6
3.	4.5	5	6
4.	6	9	14
1.	4	5	4
2.	5	4.5	6
3a.	5	5	5
4a	8	14	9

*1. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1
2. Semen treated with streptomycin (700 ug/ml of semen) plus oil film on top.
3. Semen treated with female hormones (160 ug/ml) in oil
3a. Same as 3 but with male hormone instead of female
4. Same as 3 plus antibiotic
4a. Same as 3a plus antibiotic

Table 32

Comparative effect of Penicillin, Polymycin and Streptomycin in the presence of male hormone (200 ug/ml) and female hormone (160 ug/ml) on the livability of chicken spermatozoa stored at 10-12°C

Treatment *	Survival time in days		
	Trial Nos		
	1	11	111
I PENICILLIN 1000 units/ml			
2.	7	4.5	6
3.	8	6.5	8
3a.	8	5	7.5
2.	7	4	6
4.	8	7.5	6.5
4a.	6	6.5	7
II^p POLYMYXIN 300 ug/ml			
1.	5.5		5
2.	6	5	5.5
3.	6	6.5	7
3a.	6	7	6.5
1.	4.5		4.5
2.	5	6	5.5
4.	6.5	7.5	6.5
4a.	6	7	6
III^s STREPTOMYCIN 300 ug/ml			
1.	7	5	6
2.	8	5.5	6
3.	11.5	10	8
3a.	12	10	9
1.	7	5	6
2.	6	5	6
4.	10	8.5	9
4a.	11	9.5	8

*I 1. Whole untreated semen
2. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1 with oil film on top
3 & 3a. Semen treated with antibiotic plus male sex hormone
4 & 4a. Semen treated with antibiotic plus female sex hormone

II^p same as I except polymyxin is substituted for penicillin

III^s same as I except streptomycin is substituted for penicillin.

as any cell showed motility.

In Table 32 the effect of male and female sex hormones combined with different antibiotics is shown. Most favourable results were secured when the semen was treated with sex hormones and antibiotics in comparison with other treatments involved.

THE EFFECT OF ANTIBIOTICS PLUS THYROXINE ON CHICKEN SEMEN

At least three organs are associated with reproduction - the pituitary, testes and thyroid. The pituitary is generally accepted as a regulator of sex organs; but testes are easily affected by a change in temperature. The thyroid may be involved in regulating reproduction since the thyroid activity varies according to season and may thereby affect the functioning of other organs. Clinically, thyroid therapy is effective in some cases of human sterility. The effect of thyroxine on the reproductive system of mammals and birds has been studied by numerous investigators during the last two decades. It is generally believed that thyroxine acts synergistically with the gonadotrpins in increasing gonadal activity. Thyroidectomy is usually followed by involution of sex organs with reduced fertility or sterility. Evans and Simpson (1930), Van Harm (1933), Smelsor (1934) and Zalasky and Wells (1937) considered that the thyroid effect on fertility is through the pituitary. They found that pituitaries from rats fed with thyroid or treated with thyroxine possessed an increased gonad stimulating potency and pituitaries from hypothyroid rats were of lower potency.

Berliner and Warbutton (1947) observed distinct seasonal variations in sperm production in rams. The semen quality was lowest during the summer, most probably associated with the decline in thyroid hormone secretion in

hot weather. Rams treated with thyroxine showed improved semen quality. Thyroidectomy in rams results in poor semen quality and decreased sperm production which is corrected by thyroprotien administration and thyroxine injections. Reineke (1946) obtained improvement in 10 out of 14 bulls with poor breeding performance after thyroxine adminstration. Speelman (1945) reported loss of libido in bulls following thyroidectomy which was restored following treatment with thyroid therapy. Selye (1947) observed that cretinism and myxedema adversely affect reproduction and thyroidectomy causes involution of the sex organs in human beings. Wheeler and Andrews (1943) reported a significant change in chicken semen volume associated with seasons; the greatest volumes being produced from November to March. Astwood, Bissel and Hughes (1944) reported that thiouracil in the ration at the level of 0.5 per cent retarded growth and development of wattles, comb and spurs of growing chicken. Shaffner and Andrews (1948) reported that thiouracil feeding reduced semen volume in some cases; initial motility, sperm survival time and fertility reduced in all cases. Shaffner, (1948) found that by feeding high levels of thyroprotien (10 g/100 lbs. of feed) reduced per cent fertility, initial motility and survival time.

Walton and Edwards (1948) and Ely, Herman and Winchester (1942) have shown that the rate of oxygen consumption of spermatozoa may be related to fertility.

Davis, DeCosta and Hastings (1934) and Canzanelli, Gyild and Repport (1939) demonstrated that thyroxine increased the oxygen consumption of tissues in vitro. Schultze and Davis (1948) considered that addition of proper amounts of DL - Thyroxine to bovine semen appears to exert an influence on sperm metabolism. Schultze and Davis (1949) reported an initial increase in fertility with the addition of thyroxine to bovine semen of average or above average fertility. Further the increase in the fertility on the addition of thyroxine to bovine semen could be maintained for a 4-day storage period. Schultze and Davis (1947) reported that when thyroxine was added to the semen at the rate of 0.3, 0.7, 1.0 and 4.0 micrograms per 10 milliliters of bull semen, the oxygen consumption was increased by 5, 9, 4.6 and 4.0 per cent. respectively above that of the controls. While a high dosage of 80.0 micrograms per 10 milliliters decreased oxygen consumption to 92 per cent of that of the controls.

The purpose of this experiment is to determine the effect of thyroxine on the livability in vitro and the fertilizing capacity of chicken spermatozoa stored at 10 - 12°C.

A stock solution of thyroxine was made by weighing 20 mg of thyroxine and dissolving it slowly and completely in distilled water by the addition of 0.1 N sodium hydroxide drop by drop. When completely dissolved the

solution was made up to 100 milliliter volume. The thyroxine solution added to the semen was prepared from this stock solution so that each 0.1 milliliter of the final diluted solution contain 5 ug, 50 ug, 100 ug, and 200 ug of thyroxine. All thyroxine solutions were stored at 4-5°C until used during the week. Every week fresh solutions were prepared, stored and used accrodingly. The results obtained are presented in Tables 33 - 35.

Table 33 contains the results obtained with the use of different levels of thyroxine (5 ug, 50 ug, 100 ug, and 200 ug per milliliter), in chicken semen on the livability of spermatozoa in vitro stored at 10 - 12°C. All these levels of thyroxine, which apparently are excessively high, have supported sperm life better than the untreated controls. It is probably due to inhibition of oxygen consumption as indicated in the experimental data obtained by Schultze and Davis (1947) referred to above.

In another trial a similar series of experiments were set up to which antibiotics (streptomycin 700 ug per milliliter) was added. The data in Table 34 shows the results of the use of different levels of thyroxine when used alone and with streptomycin (700 ug/ml) in parallel experiments. These results indicate that streptomycin had supplimented the effects of thyroxine by extending the survival time of chicken spermatozoa in vitro stored at 10 - 12°C.

In another series of experiments the influence of

Table 33

The effect of different levels of thyroxine on the livability of chicken spermatozoa in vitro stored at 10-12°C

Treatment**	Survival time in days		
	Trial Nos		
	I	II	III
1*	4.5	4.5	4.5
2.	5.5	5.5	4.5
2a.	5	4.5	5
1.	4.5	4.0	5
3.	6	4.5	5
3a.	5.5	5.5	5
1.	4.5	4.5	4.5
4.	7.5	6.5	5
4a.	7.5	6.5	5.5
1.	5	6	6
5.	5.5	6	6
5a	5.5	6	6

** 1. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1

2 & 2a Semen treated with thyroxine (5 ug/ml of semen) solution at the rate of 9 to 1.

3 & 3a Semen treated as above except 50 ug of thyroxine per ml of semen were used this time.

4 & 4a Semen treated as above except 100 ug/ml of semen of thyroxine used.

5 & 5a Semen treated as above except 200 ug/ml of thyroxin used.

1* The saline controls were made slightly alkalined by adding a few drops of NAOH sol. (1%) equal to that required to dissolve thyroxine in distilled water as indicated in the procedure.

Table 34

The effect of different levels of thyroxine with streptomycin (700 ug/ml) on the livability of chicken spermatozoa in vitro stored at 10-12°C.

Treatment**	Survival Time in Days Trial Nos. I	II	III
1*	4.5	4	4.5
2	5	4.5	5
3	5.5	6	6
4	6	5.5	5
5	7.5	6.5	5.5
1	5	5	5.5
6	5.5	6	5
7	6.5	7.5	8
1	5	6	6
8	5.5	6	6
9	7.5	8	8.5

*1. The saline control were made slightly alkalined by adding a few drops of NAOH sol. (1%) equal to that required to dissolve thyroxine in distilled water as indicated in the procedure.

**1. Semen diluted with isotonic sodium citrate solution at the rate of 9 to 1
2. Semen treated with thyroxine (5 ug/ml)
3. Semen treated with thyroxine (5 ug/ml) plus strepto.
4. Semen treated with thyroxine (50ug/ml)
5. Semen treated with thyroxine (50ug/ml) plus strepto.
6. Semen treated with thyroxine(100ug/ml)
7. Semen treated with thyroxine(100ug/ml) plus strepto.
8. Semen treated with thyroxine(200ug/ml)
9. Semen treated with thyroxine(200ug/ml) plus strepto.

thyroxine and sex hormones with and without antibiotics have been studied. The results are summarized in Table 35 and are compared with a series of three controls.

In the first group the effect of thyroxine (200 ug/ml) with or without antibiotics is compared with untreated semen and it was found that the addition of thyroxine and antibiotics improved survival time over that of the other treatments. In the next group the combined effects of thyroxine and sex hormones on sperm liv ability was studied without the addition of antibiotics. This combination has, no doubt, yielded satisfactory results in comparison to controls, but fell short of the thyroxine and antibiotic combination. In view of this fact the same experiment was repeated (that is, thyroxine and sex hormones group) with the addition of antibiotics and the results so obtained were striking. Further the effect of male and female hormones together plus thyroxine and antibiotics was studied. The results obtained are comparable with those of the preceeding groups and it is now clear that thyroxine enhanced the survival time of spermatozoa in vitro.

A combination of thyroxine and sex hormones (male and female) has also quite a favourable effect on chicken sperm life in vitro which is complimented by antibiotics. There is no clear indication of a supplementary effect of sex hormones when used together (both male and female) with thyroxine and antibiotics (Table 35).

Table 35

The effect of Thyroxine (200 ug/ml) in the presence of sex hormones (male 200 ug/ml, female 160 ug/ml) and Streptomycine (700 ug/ml) on the livability of chicken spermatozoa stored at 10-12°C.

Treatment**	Survival Time in Days		
	Trial Nos.		
	I	II	III
1*	4.5	6	5
2	5	5.5	6
3	5	6	6
4	5.5	6	6
4a	7.5	8.5	9.5
5	6	7	6
6	6	7	6
1	6	6	6
7	9	9.5	11
8	9	9	9.5
9	9	10	9.5
9a	9.5	9.5	8

**1. Untreated semen
2. Semen diluted with isotonic sodium citrate solution with oil film on top
3. Semen diluted with isotonic sodium citrate solution
4 & 4a Semen treated with thyroxine (200 ug/ml)
5. Semen treated with thyroxine (200 ug/ml) plus male sex hormone (200 ug/ml)
6. Semen treated with thyroxine (200 ug/ml) plus female sex hormone (160 ug/ml)
7. Semen treated with thyroxine (200 ug/ml) plus male hormone (200 ug/ml) plus strepto (700 ug/ml)
8. Semen treated with thyroxine (200 ug/ml) plus female sex hormone (160 ug/ml) plus strepto (700 ug/ml)
9 & 9a Semen treated with thyroxine plus male and female hormones plus streptomycin.

* Saline control were made slightly alkalined by adding a few drops of NAOH sol. (1%) equal to that required to dissolve Thyroxine in distilled water as indicated in the procedure.

THE EFFECT OF ANTIBIOTICS, SOME DILUENTS, THYROXINE, OR SEX HORMONES ON THE FERTILIZING CAPACITY OF CHICKEN SEMEN

Our knowledge of fertilization is far less advanced in mammals and birds than in lower forms due to the difference of physiological experimentation in vitro. A single ejaculate of semen contains hundreds of millions of spermatozoa, but only a very small fraction of this number is necessary for fertilization. Their anatomical structure under the electron microscope as well as their metabolism have been extensively studied in recent years. It is known that the fertilizing capacity of spermatozoa is lost much earlier than the motility.

Reproduction in mammals has recently been reviewed by Catchpole (1949), Asdill (1950), and Chang and Pincus (1951). Bonadonna (1948) (cited by Asdill, 1950) has reviewed the extent and technique of artificial insemination in Europe. Swanson (1949) has compared the effects of varying the composition of egg Yolk-citrate buffer upon sperm motility, while Foote and Salisbury (1948), and Almquist, Glantz and Shaffer (1949) observed the effects of a variety of antibiotics upon semen. Salisbury and Bratton (1948) found the practical limits of dilution of bull semen for maximal fertility by artificial insemination to be 1 to 100. When 300 mg. of sulfanilamide was added per 100 c.c. of semen the dilution rate could be increased to 1 to 400. Schultze and Davis (1948) found that 7 mg. per cent of thyroxine increased

the oxygen consumption of spermatozoa if the concentration is not lower than 800,000 spermatozoa per cubic milliliter. They also reported (Schultze and Davis, 1949) an increase of 6 per cent in the fertility of bull semen when thyroxine was added at this level. Schultze and Davis (1948) found that under field conditions, fertility of bull semen declined 4.6 per cent with each day of storage.

a. Fertility of semen treated with streptomycin

The effects of penicillin, terramycin, polymyxin, streptomycin, and their combination on the motility and livability of chicken spermatozoa in vitro have been studied and reported before under the heading "Antibiotics" (Tables 1 to 9). Since it was not possible within the scope of this work to further test the comparative values of each of these reagents, streptomycin was selected on the basis of the results obtained in vitro, to determine its effect on the fertilizing capacity of chicken spermatozoa in vivo.

Consequently the composite semen sample after collection was divided into three equal portions and a control group of birds was inseminated immediately with one of these semen samples. Adding 900 micrograms of streptomycin per milliliter to one of the remaining two tubes, both the tubes then stored for eight hours in a water bath at 10 - 12°C. After 8 hours storage they were inseminated separately into two another groups of hens. The experiment was repeated four times.

The results are summarized in Table 36 and 37. When the semen was stored for 8 hours at 10 - 12°C the fertility of the hens did not usually extend beyond the first 7 days and in one case when the semen was inseminated immediately after collection, some hens remained fertile for 14 days. There is no appreciable difference between the fertility level of the control group and that of the groups treated with 900 micrograms per milliliter of streptomycin. Although the improvement brought about on the addition of 900 micrograms per milliliter of streptomycin on the sperm motility and livability in vitro (Table 7) was quite conspicuous.

In another experiment the effects of different levels (100, 200, 500 and 1000 gamma per milliliter) of streptomycin on the fertilizing capacity of chicken semen were studied (Table 37). The fertility obtained on the addition of streptomycin to fresh chicken semen which was inseminated immediately after collection in this experiment are much better than the previous one (Table 36), but unfortunately the fertility produced by the whole semen in the control groups is so poor that a real comparison or a satisfactory evaluation of the effects of streptomycin is difficult to be made on the basis of these results. However, a review of the data in tables 36 and 37 indicated that streptomycin is toxic to the fertilizing capacity of chicken spermatozoa under storage.

Table 36

The Effect of Streptomycin (900 ug/ml) on the fertilizing capacity of Chicken semen stored at 10-12°C for eight hours prior to insemination.

Trials	Treatment*	No. of hens inseminated	No. hens fertile	Percentage of eggs fertile: Eggs of fertile hens**	Eggs of all hens inseminated: 1st wk.	2nd Wk.	Sperm Survival Time (Vitro) in days
1	1	5	3	50	29.1	-	3
	2	5	1	20	5	-	3
	3	5	1	25	4	-	4
II	1	5	3	61.3	36	-	4.5
	2	4	2	40	15	-	5.5
	3	4	1	75	17.6	-	6.5
III	1	5	3	53.3	33.3	26.6	3.5
	2	5	2	63.6	30.4	0	3.5
	3	4	3	29.4	28.5	0	5
IV	1	4	3	45.4	33.3	0	4.5
	2	4	3	26.2	20	0	4
	3	5	2	42.8	15	0	6

*1. Untreated semen inseminated immediately after collection
2. Untreated semen stored for 8 hours prior to insemination
3. Semen treated with Streptomycin, 900 ug/ml, and stored for eight hours prior to insemination.

** Number of hens that laid at least one fertile egg (first week period) following insemination.

Table 37

The effect of different levels of streptomycin (100, 200, 500 and 1000 ug/ml of semen) on the fertility of chicken semen inseminated immediately after collection

Trials	Treatment*	No. Hens inseminated	No. Hens fertile	Percentage of eggs fertile: Eggs of fertile hens**	Eggs of all hens inseminated: 1st wk.	2nd wk.	Sperm Survival Time (Vitro) in days
I	1	5	1	66.6	12.5	0	3.5
	2	6	4	47.0	30.8	16.6	4
	3	6	1	100.0	18.5	8.3	4.5
II	1	5	1	8.0	8.0	0	4
	2a	7	7	41.4	41.4	16.6	5.5
	3a	5	3	70.0	39.0	25	0

* 1. Untreated semen inseminated immediately after collection
2. Semen treated with streptomycin (100 ug/ml) and inseminated immediately
2a. Semen treated with streptomycin (200 ug/ml) and inseminated immediately
3. Semen treated with streptomycin (500 ug/ml) and inseminated immediately
3a. Semen treated with streptomycin (1000ug/ml) and inseminated immediately

b. Fertility of semen diluted with whole milk and Whey

In this study whole milk (pasteurized and homogenized) was used as diluent for chicken semen both alone and with antibiotics (streptomycin 500 ug/ml), without storage. The semen after collection was divided in five vials, treated as indicated below, and inseminated immediately after collection.

Out of the five aforesaid groups one served as control and to the other whey was added in the ratio of 1 to 1. To the remaining three tubes whole milk, milk containing streptomycin, and milk with streptomycin plus male sex-hormones in the ratio of 1 to 1 was added. All the samples after treatment were inseminated immediately.

Table 38 presents the data regarding the fertility produced by the addition of milk and whey to chicken semen at the rate of 1 to 1 and inseminated immediately after collection and dilution.

The fertility produced from semen to which whole milk was added was somewhat better than from untreated semen. It is interesting to note that fertility dropped abruptly on the addition of streptomycin (500 ug/ml) to whole milk. The cause of the drop is unexplainable at the present.

Strangely enough the hens inseminated with semen treated with whey (without antibiotics) did not lay a single fertile egg, although the motility rating and survival time of spermatozoa in vitro was much better than

Table 38

The effect of whole pasteurized milk and whey alone or with streptomycin (500 ug/ml of semen) on the fertilizing capacity of chicken semen inseminated immediately after collection.

Treatment*	No. of hens insemi-nated	No. hens fer-tile	Percentage of eggs fertile: Eggs of fer-tile hens**	Eggs of all hens insemi-nated: 1st wk.	2nd wk.	Sperm Survi-val Time (Vitro) in days
1. Whole Semen	6	3	100.0	39.1	25	5
2. Semen + Milk	6	3	71.4	38.4	50	5
3. Semen + Milk Strepto	6	2	25.0	10.0	25	5.5
4. Semen + Milk + Strepto + Male Hormone	6	4	68.4	39.2	25	6.5
5. Semen + Whey (no anti-biotics)	2	0	0	0	0	7

*1. Untreated semen

2. Semen diluted with whole pasteurised milk at the rate of 1:1

3. Semen diluted with whole pasteurised milk containing Streptomycin (500 ug/ml) at the rate of 1 to 1

4. Semen diluted with whole pasteurised milk containing Streptomycin and male sex hormone, at the rate of 1 to 1

5. Semen diluted with whey (no antibiotics) at the rate of 1 to 1

the others (7 days against 5.5 days).

In another experiment the semen after collection was divided into 5 portions in 6 c.c. clean glass tubes and one of these samples was inseminated into a group of hens immediately. While the other samples of the semen were treated as detailed below and stored in a water bath at 10 - 12°C for 8 hours before used for insemination.

1. Whole semen inseminated immediately - control.
2. Whole semen inseminated after 8 hour storage - control.
3. Semen and whole milk (without antibiotics) inseminated after 8 hours storage.
4. Semen and whole milk plus streptomycin (500 ug/ml) inseminated after 8 hours storage.
5. Semen and whey plus streptomycin (500 ug/ml) inseminated after 8 hours storage.

The data regarding the effect of milk and whey alone or with streptomycin on the fertility of spermatozoa when added to chicken semen after 8 hour storage at 10 - 12°C is presented in Table 39.

All these treatments after 8 hours storage prior to insemination have yielded failry high levels of fertility when compared with that secured from whole semen inseminated after 8 hours storage.

c. Fertility of semen diluted with chicken blood serum and treated with thyroxine

In the preceding sections under the heads of "diluents"

Table 39

The effect of whole pasteurized milk and whey alone and with antibiotics (streptomycin and polymyxin each 400 ug/ml) on the fertilizing capacity of chicken semen stored for 8 hours at 10 - 12°C

Trials	Treatment*	No. hens inseminated	No. hens fertile	Percentage of eggs fertile: Eggs of fertile hens**	Eggs of all hens inseminated: 1st wk.	2nd wk.	Sperm survival (vitro) time in days
I	1	4	3	45.4	33.0	0	4.5
	2	4	3	26.2	20.0	0	4
	3	5	3	71.0	38.0	20	5.5
	4	2	1	80.0	57.0	-	6
	5	5	2	100.0	33.3	28.5	7
II	1	5	3	100.0	40.0	18.0	5
	2	5	1	40.0	9.5	0	4.5
	3.	4	3	60.0	40.0	13.2	5
	4.	5	3	71.4	50.0	15.0	6
	5	5	2	22.2	11.0	20.0	7

* 1. Untreated semen inseminated immediately after collection.
2. Untreated semen stored for 8 hours prior to insemination.
3. Whole pasteurized milk (without antibiotics) stored for 8 hours prior to insemination.
4. Whole pasteurized milk plus antibiotics stored for 8 hours prior to insemination.
5. Whey plus antibiotics stored for 8 hours prior to insemination.

** Number of hens that laid at least one fertile egg (1st week period) following insemination.

and "hormones" the beneficial effect of chicken blood serum (Table 12, 13) and thyroxine (Tables 33 and 34) on the motility and livability of chicken spermatozoa in vitro have been discussed. Being encouraged with these results it was considered desirable to investigate how far their conserving effect, if any, on the fertilizing capacity of chicken semen would be maintained in vivo. With this end in view a few experiments were performed to test the fertilizing quality of the semen treated with 900 micrograms per milliliter of streptomycin and 50 micrograms per milliliter of thyroxine and stored for 8 hours at 10 - 12°C prior to insemination. Parallel experiments were also set up to study the effect of chicken blood serum with 900 micrograms per milliliter of streptomycin added to chicken semen as a diluent in the ratio of 1 to 1.

Table 40 shows that neither thyroxine or blood serum appear to be suitable for treating or diluting chicken semen. Though the percentage of fertility produced by the whole semen particularly the one used immediately after collection is below the expected level, the females in the other two groups treated with thyroxine or blood serum did not lay even a single fertile egg in either of the trials. It may be mentioned that the treated semen samples have had better motility rating and extended survival time in comparison to control groups in vitro.

d. Fertility of semen treated with sex hormones

The beneficial effects of sex hormones on the livability and motility of spermatozoa when added to

Table 40

The effect of thyroxine and chicken blood serum on the fertilizing capacity of chicken semen stored for 8 hours at 10-12°C prior to insemination.

Trials	Treatment*	No. of hens inseminated	No. hens fertile	Percentage of eggs fertile: Eggs of fertile hens**	Eggs of all hens inseminated: 1st wk.	2nd wk.	Sperm Survival Time (Vitro) in days
I	1	5	3	50	29.1	-	3
	2	5	1	20	5.0	-	3
	3	5	0	0	0	-	5
	4	3	0	0	0	-	7
II	1	5	3	61.3	36.0	-	4.5
	2	4	2	40	15.0	-	5.5
	3	5	0	0	0	-	6.5
	4	4	0	0	0	-	6

*1. Untreated semen inseminated immediately after collection

2. Untreated semen stored for 8 hours prior to insemination

3. Semen treated with Thyroxine (50 ug/ml) plus Streptomycin (900 ug/ml) and stored for 8 hours at 10-12°C prior to insemination

4. Semen diluted with blood serum (1:1) plus Streptomycin (900 ug/ml of semen) and stored for 8 hours prior to insemination

** Number of hens that laid at least one fertile egg (1st week period) following insemination

chicken semen, alone or with antibiotics, have already been discussed (Tables 30, 31 and 32). The purpose of the present investigation is to determine how far the conserving influence of androgen and estrogen would go to maintain in vivo the fertilizing capacity of chicken semen under conditions of short storage.

The male sex hormone (Testosterone propionate); The investigation was designed to test the fertilizing capacity of chicken semen treated with androgen plus streptomycin with or without storage compared to that from untreated semen inseminated immediately after collection as well as inseminated after eight hours storage at 10 - 12°C. The trials were repeated four times and the results are summarized in Table 41.

The level of fertility secured on the addition of testosterone propionate at the rate of 160 ug/ml of semen is higher than that obtained from the control group inseminated with the whole semen after 8 hours storage. It also compared favourably with the fertility from whole semen inseminated immediately after collection. Further the fertility level, when calculated on the basis of hens that laid at least one fertile egg following insemination, is better than either of the groups referred to above in these experiments. The fertility produced by whole semen stored for 8 hours was the lowest.

The female sex hormone (Diethylstilbesterol): Similar experiments as for the male sex hormone were conducted and the same procedure was followed in studying the

Table 41

The effect of male sex hormone (testosterone, propionate, 160 ug/ml semen) with streptomycin (500 ug/ml semen), on the fertilizing capacity of chicken semen stored for 8 hours at 10-12°C.

Trials	Treatment*	No. hens inseminated	No. hens fertile	Percentage of eggs fertile			Sperm survival (vitro) time in days
				Eggs of fertile hens**	Eggs of all hens inseminated		
					1st wk.	2nd wk.	
I	1	5	3	53.3	33.3	26.6	3.5
	2	5	2	63.6	30.4	0	3.5
	3	5	3	43.0	27.2	0	6
II	1	4	3	45.4	33.3		4.5
	2	4	3	26.2	20.0		4
	3	5	3	77.0	43.9		6
III	1	7	5	74.0	53.1	27.0	6
	2	5	1	40.0	9.5	0	6
	3	5	3	54.5	30.0	12.5	8
IV	1	6	3	100	39.1	25	5
	2	5	2	62.5	20.0	0	6
	3a	6	4	68.4	39.2	25	6.5

* 1. Untreated semen inseminated immediately after collection.
2. Untreated semen stored for 8 hours prior to insemination.
3. Semen treated with male sex hormone plus streptomycin and stored for 8 hours prior to insemination,
3a. As 3, but no storage.

** Number of hens that laid at least one fertile egg during the first week period following insemination.

fertilizing quality of semen treated with 160 micrograms of diethylstilbesterol plus streptomycin (500 ug/ml). The trials were repeated four times each and the results are presented in Table 42.

The review of the data in Table 42 indicated the superiority of the sample treated with diethylstilbesterol plus streptomycin over that of whole semen. A higher level of fertility was secured when the semen was treated with female sex hormone than when untreated.

COMPARATIVE EFFECT OF ANTIBIOTICS, SEX HORMONES AND MILK

In these experiments the comparative conserving effect of streptomycin (500 ug/ml.), female sex hormone (diethylstilbesterol, 160 ug/ml. plus streptomycin, 500 ug/ml.), and male sex hormone (testosterone propionate, 160 ug/ml. plus streptomycin, 500 ug/ml.) on the fertilizing quality of semen separately treated with either of these materials were studied. The whole investigation was designed to consist of five groups as follows:

1. Whole semen inseminated immediately .... Control.
2. Whole semen inseminated after 8 hours storage ..... Control.
3. Semen plus streptomycin (500 ug/ml.) after 8 hours storage.
4. Semen plus diethylstilbesterol (160 ug/ml.) plus streptomycin (500 ug/ml.) after 8 hours storage.
5. Semen plus testosterone propionate (160 ug/ml.) plus streptomycin (500 ug/ml.) 8 hours storage.

Table 42

The effect of female sex hormone (diethylstilbesterol, 160 ug/ml semen) with streptomycin (500 ug/ml semen) on the fertilizing capacity of chicken semen stored for 8 hours at 10-12°C.

Trials	Treatment*	No. hens inseminated	No. hens fertile	Percentage of eggs fertile: Eggs of fertile hens**	Eggs of all hens inseminated: 1st wk.	2nd wk.	Sperm survival (vitro) time in days
I	1	5	3	50.0	29.0		3
	2	5	1	20.0	5.0		3
	3	5	2	33.3	13.6		4.5
II	1	5	3	61.3	36.0		4.5
	2	4	2	40.0	15.0		5.5
	3.	5	4	64.3	56.25		7.5
III	1	5	3	53.3	33.3	26.6	3.5
	2	5	2	63.6	30.4	0	3.5
	3	5	4	53.0	38.5	30.7	6
IV	1	4	3	45.4	33.3		4.5
	2	4	3	26.2	20.0		4
	3	3	2	71.4	41.3		6.5
V	1	7	5	74.0	53.1	27.0	6
	2	5	1	40.0	9.5	0	6
	3	5	2	55.5	27.7	0	7.5

* 1. Untreated semen inseminated immediately after collection.
2. Untreated semen stored for 8 hours prior to insemination.
3. Semen treated with female sex hormone plus streptomycin and stored for 8 hours prior to insemination.

** Number of hens that laid at least one fertile egg during the period of first week following insemination.

The results are presented in Table 43. Unfortunately the level of fertility in the immediate control group did not reach the expected level. It has, no doubt, made the accurate estimation of comparative effects of various treatments, difficult. However, the hens inseminated with whole semen stored for 8 hours prior to insemination produced the lowest level of fertility (18 per cent on the average), whereas the group of birds inseminated with the semen diluted with milk plus sex hormones and antibiotics produced a high level of fertility (i.e., 40 and 56 per cent; male and female hormones respectively -- Table 43) even though the treated semen was diluted at the rate of 1 to 1 and stored for 8 hours prior to insemination. In general the results for the samples of semen treated with sex hormones plus antibiotics appear to be better when compared with other groups. The addition of streptomycin to chicken semen did not produce the desired quality of fertility particularly after storage (Tables 37 and 43). Further the survival time of chicken spermatozoa in vitro is the shortest for the whole semen control groups and the longest for the semen samples treated with antibiotics plus sex hormones.

Table 43

Comparative effect of streptomycin (500 ug/ml. semen) female sex hormone (diethylstilbesterol, 160 ug/ml + streptomycin; 500 ug/ml. semen) and male sex hormone (testosterone propionate, 160 ug/ml + strepto. 500 ug/ml semen) on the fertilizing capacity of chicken semen stored for 8 hours at 10-12°C

Trials	Treatment*	No. of hens inseminated	No. hens fertile	Percentage of eggs fertile: Eggs of fertile hens**	Eggs of all hens inseminated: 1st wk.	2nd wk.	Sperm Survival Time (Vitro) in days
I	1	5	3	53.3	33.3	26	3.5
	2	5	2	63.6	24.4	0	3.5
	3	4	3	29.4	28.5	0	5
	4	5	4	53.0	38.5	30.7	6
	5	5	3	43.0	27.2	0	6
II	1	7	5	74.0	53.1	27.0	6
	2	5	1	40.0	9.5	0	6
	3	4	2	20.0	12.5	0	7.5
	4	5	2	55.5	22.7	0	7.5
	5	5	3	54.5	30.0	12.5	8
III	1	5	3	61.3	36.0		4.5
	2	4	3	26.2	20.0		4
	3	4	1	75.0	17.6		6.5
	4a	5	4	64.3	56.25		6
	5a	6	4	68.4	39.2	25	6.5

* 1. Untreated semen inseminated immediately after collection
2. Untreated semen stored for 8 hours prior to insemination
3. Semen treated with Streptomycin (500 ug/ml) and stored for 8 hours prior to insemination
4. Semen treated with female sex hormone (160 ug/ml) plus streptomycin and stored for 8 hours prior to insemination
4a. Semen + female sex hormone + Strepto in milk (1:1) and stored for 8 hours prior to insemination
5. Semen + male hormone + Strepto and stored for 8 hours prior to insemination
5a. Semen + male hormone + Strepto in milk (1:1) and stored for eight hours prior to insemination

The underlying purpose of the present investigation involved the study of antibiotics and the extent to which they would be beneficial in improving the quality of semen by controlling the bacterial growth in chicken semen. Such an attack in respect to bovine semen has been successful. The main difficulty experienced in the course of this study was the paucity of literature on these lines about chicken semen. Our knowledge of the optimal environmental conditions that normally exist in the male and female genital tracts of the fowl, where the spermatozoa remain viable for longest time is still incomplete.

However, before considering the effects of antibiotics on chicken semen quality, it may not be out of place to mention a few of the theories postulated in respect to the stimulating or inhibiting influences on spermatozoa:

1. The theory that bacteria produce compounds which unduly stimulated the spermatozoa thus decreasing the length of time that they can be stored (Edmonson, et al., 1949)

2. The lysis of spermatozoa by bacterial toxins e.g. Streptococci (Jordon and Burroughs, 1947).

3. The presence of beneficial bacteria: Either the immediate by-products of the living bacteria or the bacterial utilization of harmful sperm by-products causing the beneficial effects. Moreover, bacteria may produce enzymes, which are either beneficial

in sperm metabolism or act on harmful waste products.

In the present study all the bacterial types previously reported have been found except Streptococcus fecalis and diptheroids. No specific reason for their absence can be assigned except that it may be partly accidental and partly due to the care in the collection and handling of semen, as fecal contamination is usually the common source of these organisms. In contrast to the findings reported by Smith (1949), the growth of Bact. coli in the treated semen during storage was usually found more profuse than Staphylococci which may be due to the fact that Bact. coli is more resistant to adverse environmental conditions. The concentration of the antibiotic used in the present study may not have been strong enough to entirely suppress the growth of bacteria during storage. It may also be due to some extent, to long storage of the streptomycin used (about 2 years old) which might have caused loss in potency; or the semen used by Smith (1949) contained quantitatively less bacteria than the one used in the present study.

In the study of total bacterial counts no definite relation could be found to exist between the total bacterial population and the storage time of semen in vitro. Since different bacteria have varied effects(Edmondson,et al., 1949) upon the stored semen, these findings are not surprising as a large number of living beneficial bacteria might be

present in the diluted semen with extended storage time.

In reviewing the data involved in the present study, a harmful effect of high bacterial count on chicken semen is manifest and has borne out the fact that the control of bacterial growth in diluted chicken semen with antibiotics invariably improved motility and extended survival time of spermatozoa in vitro over that of the untreated semen, although how such a beneficial effect is brought about is not well explained. The data obtained supports the view of Edmondson, et al., (1949) and Jordon and Burroughs' (1947).

In contrast to the results regarding the use of antibiotics reported by most of the workers in the dairy field no evidence of complementary effects or synergism was found. This is in partial agreement with some of the reports appearing recently. Mixner (1949) using a combination of penicillin (1000 units/ml) and streptomycin (1000 ug/ml) reported neither increase nor decrease in the fertility of semen from relatively fertile bulls. He suggested that a level of 1000 gamma per milliliter of streptomycin may not be as favourable as a lower level; or streptomycin alone may be more effective than a combination of penicillin and streptomycin. Almquist (1950) using sulfanilamide, penicillin, and streptomycin singly and in combination did not show a marked improvement in favour of the combinations, and reported that the effectiveness of these diluters was dependent upon the relative

fertility levels of these bulls. Bratton and Foote (1950), Foote and Bratton (1950) and Easterbrook (1951) reported a trend favouring the addition of streptomycin alone. The data secured in the present study is in conformity with the findings reported above, except that levels from 300 to 700 gamma of streptomycin were found more suitable than 1000 gamma per milliliter with chicken semen. The levels of streptomycin (50,100 and 200 gamma per milliliter) used by Smith (1949) are lower than those used either by the present author or the workers in the dairy field. It might be due to different variables under which the experiments were performed as well as the quantitative presence of the bacterial organisms in the samples of semen used.

In view of the beneficial effects of streptomycin on stored diluted chicken semen secured in vitro the plan of study was extended to finally checking its effects on the fertilizing capacity of chicken semen. The fertility secured from the semen treated with different levels of streptomycin (100,200,500 and 1000 gamma/ml) and inseminated immediately after treatment was higher than that obtained from the untreated semen controls. But when streptomycin-treated semen was stored for eight hours prior to insemination the level of fertility obtained was not satisfactory,which is not surprising. The favourable results obtained in these trials with the semen treated with streptomycin and inseminated immediately after treatment over that stored for eight hours prior to insemination, may be explained on the

basis that the time elapsed between the collection of the semen, its treatment with streptomycin and insemination was so short that it would not allow the antibiotic to act adversely in the semen. The drop in the fertility level of the stored treated semen may be accounted for by the toxic effect of streptomycin during storage on the fertilizing capacity of spermatozoa, which was not visible when the treated semen was inseminated immediately after collection and treatment. The discrepancy in the fertility levels between the treated and untreated semen inseminated immediately after collection and treatment cannot satisfactorily be explained, as the difference in the semen samples was very negligible. Probably it may be the case that the treated semen did not deteriorate due to the presence of streptomycin having an inhibiting influence on the bacterial growth, whereas the untreated semen had not been under such an influence. However, the influence of streptomycin on the semen used in these trials cannot well be established, because the level of fertility obtained from the untreated semen controls was below the expected level. Moreover as the present study is mainly directed to determining the improvement in the fertilizing capacity of chicken semen by controlling bacterial growth with antibiotics the beneficial effect, if any, produced in these trials cannot be considered of great value.

The extension of sperm life in artificial media without impairing its fertilizing power is a complex problem involving several factors. Regardless of the fact that antibiotics improved motility and enhanced livability of chicken spermatozoa in vitro, semen treated with streptomycin and inseminated after eight hours storage at 10-12°C did not improve fertility. Spermatozoa have two outstanding biological characteristics, motility and fertility, which are not closely correlated. Spermatozoa have a metabolism of their own which is maintained by a number of intracellular catalysts(cytochrome, certain enzymes and coenzymes). As antibiotics favourably influenced sperm motility and enhanced their life in vitro but not their fertilizing capacity,it was considered desirable to extend the study of antibiotics in semen diluted with some natural diluents. In contrast to the earlier works, with chicken semen where such diluents were used,the present study is mostly confined to determining the effects of antibiotics in the semen diluted with them.

Out of several biological products used and referred to earlier,milk was found to be the most suitable. It improved the semen quality both in vitro and in vivo, a result which might be expected, because of its nutritive value and consistency.But as to why the blood serum,in contrast to milk, by the same analogy,has an impairing effect on the fertilizing quality of semen is not understood. It is, probably due to the fact that during the process of

separating serum from blood some of the factors necessary for sperm life and its fertilizing capacity were lost. Similar seems to be the case with whole and skim milk, which, when used to dilute bovine semen revealed the fact that whole milk increased while skim milk decreased the percentage fertility over that of the egg yolk citrate diluter. Likewise the fertility secured in the present study from the semen diluted with whole milk was of higher level than that obtained from the semen diluted with whey. In regard to the effects of antibiotics it is interesting to note that the addition of streptomycin to whole milk used to dilute chicken semen at the rate of 1 to 1, produced a fairly good level of fertility in comparison to the whole milk alone.

In the final phase of the study the effects of some of the hormones combined with antibiotics are considered. In contrast to the reports of workers in the dairy field (Schultze and Davis, 1949) it is surprising to note that not a single fertile egg was laid by hens inseminated with semen treated with thyroxine combined with antibiotics, regardless of the fact that this treatment proved to be very favourable to supporting sperm life in vitro. In fact thyroxine was used in the present study in higher concentrations than those used by Schultze and Davis (1949) but it did not show any sign of toxcity in vitro. The favourable effect of Thyroxine in semen in vitro may be due

to immobilization of spermatozoa by inhibiting oxygen consumption as suggested by Schultze and Davis (1949). But strangely enough no difference in motility rating was observed over those of other treatments. Then complete loss of fertility on the addition of thyroxine to chicken semen is the other question, which, in the light of the present information, is unexplainable, particularly, when thyroxine has been shown to increase fertility of bovine semen. However, such an occurrence can be explained with the assumption that, in contrast to mammalian spermatozoa, the avian spermatozoa is very peculiar in that its fertilizing capacity is highly sensitive and labile to environmental changes; and that motility and fertility are not closely related.

Finally, the treatment of semen with sex hormones combined with antibiotics has been found to be most favourable in improving the quality of chicken semen both in vitro and in vivo. Though the fertilities produced by semen treated with either of the male or female hormones separately, cannot clearly be discreminated from each other, there is the trend favouring the female sex hormone. These findings are somewhat in contrast to those reported by Smith (1949) that streptomycin did not improve motility and viablity of spermatozoa in vitro, and Munroe (1938) who reported that sex hormones have no conserving effect on sperm life in vitro or in vivo yet the male hormone environment is necessary

for the attainment of functional maturity. As sex hormones invariably improved semen quality in vitro as well as in vivo the author sharing the view of Moore (1928) and Moore and McGee (1928) concludes that sex hormones are concerned in the maintenance of semen quality.

The exact manner in which sex hormones exercise their preserving influence on the spermatozoa is not clearly understood. Whatever may be the precise nature of this mechanism governing the maintenance of extended sperm life in the excretory ducts of the male or the oviduct of the female, something is present to prolong sperm life in these localities without impairing their fertilizing power. The evidence brought forth from the data involved in the present study has shown, contrary to the view of White (1932), that the sex hormones have the potential of enhancing sperm life without impairing their fertilizing capacity, quite independent of the presence of the pituitary or the epididymal secretions both in vitro and in vivo.

SUMMARY

Studies were conducted to determine the effects of antibiotics (penicillin, terramycin, polymyxin, and streptomycin) upon the in vitro survival, control of bacterial growth, and fertilizing capacity of whole chicken semen and semen diluted with various diluents or treated with some hormones.

The duration of spermatozoal motility when semen was stored at 10-12°C was increased by controlling bacterial growth with antibiotics. Of the antibiotics used in this study, streptomycin proved to be superior over penicillin, terramycin, and polymyxin in improving motility and extending livability of spermatozoa in vitro. No evidence of complementary effect or synergism was found following the use of these antibiotics in combinations over that of streptomycin used singly in concentrations of 300 to 700 gamma per milliliter of semen. Regardless of the favourable effects on spermatozoal livability in vitro, streptomycin impaired fertilizing capacity of spermatozoa in vivo during short storage at 10-12°C.

All the bacterial types reported previously in chicken semen with the exception of streptococcus fecalis and diphtheroids were found in the present study. In the study of total bacterial counts no close correlation was observed between bacterial population and survival time of spermatozoa.

Addition of thyroxine in the concentration of 5, 50, 100 and 200 gamma per milliliter of chicken semen prolonged sperm life in vitro, but impaired its fertilizing capacity in vivo (50 ug/ml) Semen treated with either of the sex hormones combined with streptomycin had a very favourable effect on sperm livability in vitro and conserved its fertilizing capacity during short storage at 10-12° in vivo.

After storage for eight hours at 10-12°C the semen treated with a combination of antibiotic and sex hormones and diluted with whole milk produced better fertility than that of the untreated semen.

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VITA

Shahid Husain Qureshi

Animal Husbandry Department, Utter Paradesh, Lucknow, India

Doctor of Philosophy, 1953

Date of birth: April, 1909

Place of birth: Bijnore, India

Secondary Education: Muslim University, Aligarh, India

Collegiate Institutions attended	Date	Degree	Date of Degree
Muslim University, Aligarh	Aug.,1922	F.Sc.	April, 1926
Bihar and Orissa Veterinary College	April, 1930	G.Vsc.	April, 1933
University of Maryland	July, 1949	M.S.	Aug., 1950
University of Maryland	July, 1949	Ph.D	June, 1953

Publications:

Some observations on *Amoebotaenia sphenoides* (Railt.)

The Bionomics of the Fowl Tick, *Argas persicus*

The incidence of Helminthiasis in fowls in U.P., India

Electron-microscopic study of Newcastle Disease Virus passaged through Turtles

Three Annual Reports of the Assistant Disease Investigation Officer (Poultry), U.P., India

Positions held:

Veterinary Assistant Surgeon, Civil Veterinary Department, U.P., India

Assistant Disease Investigation Officer (Poultry), Civil Veterinary Department, U.P., India

Disease Control Officer (Poultry), Animal Husbandry Department, U.P., India

Graduate Assistant, Livestock Sanitary Laboratory, University of Maryland