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SOME PHYSIOLOGICAL AND IMMUNOLOGICAL FACTORS AFFECTING THE
FERTILIZING CAPACITY OF TURKEY SPERMATOZOA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
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by


The Ohio State University

1965

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"Some Observations on the Fertility Problems in Turkeys."

FIELDS OF STUDY

MAJOR FIELD: Poultry Science


Studies in Immunology. Professor M. C. Dodd
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INTRODUCTION

In recent years low fertility has become one of the most serious problems of the turkey industry. The trend, often observed, is one of high fertility in the beginning of the breeding season with a rapid drop as the season progresses. Even in high fertility and naturally mated flocks the fertility tends to deteriorate with time. Obviously, the problem can be either with the males, the females, with insemination techniques, or with any combination of these. Thus, it is not only necessary to develop better techniques of handling and storing semen, but also to determine the possible causes of low fertility in turkey hens regardless of the inseminating techniques.

Artificial insemination has become an established practice in the turkey industry. However, at present, this practice is only a substitute for deficient fertility from natural matings. In recent years some progress has been made in this field; van Tienhoven and Steel (1957), van Tienhoven et al. (1958), Harris et al. (1963), Bajpai (1963), and Bajpai and Brown (1963, 1963b, and 1964) have reported on diluents for turkey semen; however, no suitable diluent has been developed. The rapid drop in the fertilizing capacity of turkey semen held in vitro has seriously hampered the best utilization of artificial insemination.
Low temperatures have been successfully employed to preserve the fertilizing capacity of bovine semen, and yet identical or similar techniques have not proved successful with fowl semen. If methods for storing and diluting turkey semen are developed, the potential of providing semen as germ plasm is tremendous, making it possible to disperse the desirable genetic characteristics of individual males or strains of males over many more females than possible with mating methods available at present. It would also be possible for a hatchery to maintain a centralized flock of males. The semen could be collected from the males every two or three days, the semen frozen and dispensed to their supply flocks as needed. This could be done even with unfrozen semen if methods allowing storage up to four hours could be developed.

However, the best possible mating methods cannot correct poor fertility if the defect lies with the female. Hence, it is also necessary to study the female physiology associated with fertilization of the egg. In general the union of an egg and a sperm can be thought to be similar to the antigen-antibody reaction. Often antigens are thought of as substances derived from an infecting organism and antibodies as substances manufactured by the body to attack or neutralize the invader. However, the fact that antibodies can also produce an ailment rather than prevent one should not be overlooked. For example, in combination
with pollen or some other allergy-inducing substances antibodies initiate an allergic reaction.

Studies on the fertilization of eggs from the antigen-antibody point of view have proved useful for some animals (for example, sea urchin, cattle, Japanese quail, and fowl). It may be of equal importance in turkeys.

A program was thus outlined to obtain more facts on the role of sperm antibodies on fertility in turkeys.
REVIEW OF LITERATURE

A new field of research in poultry breeding was opened when Professor Ivanov (1913) artificially inseminated a few hens with semen collected from a sacrificed cock. Payne (1914), Amantea (1922), Timjacov (1935), Serebrovskii and Sokolovskaja (1934), and others allowed natural mating and collected semen by recovering it from the cloaca of the hen immediately after copulation or by interrupting the ejaculates during mating. Research in artificial breeding was noticeably stimulated after the development by Burrows and Quinn (1937 and 1938) of a simple routine method of collecting and inseminating fowl and turkey semen.

Effect of Temperature

Milavanov (1934) reported that rapid cooling or cold shock markedly reduced the motility of fowl spermatozoa. Wales and White (1959) and White and Wales (1960) observed no reduction in motility or increase in dead spermatozoa after subjecting fowl semen to cold shock. deSilva (1964) reported that "there is no significant decrease in the fertilizing power of fowl spermatozoa when the semen is exposed to a rapid fall in temperature at the time of collection." Garren and Shaffner (1952), in studies on the effect of storage between 0°C and 40°C, found that fertilizing
capacity was highest when fowl semen was stored at 10°C. Schindler et al. (1955) observed the effect of temperature of 4°C, 10°C, and 41°C on undiluted and diluted semen in Ringer's solution or whole milk and found that both diluted and undiluted semen retained full fertilizing capacity for four hours at 10°C. Hunsaker et al. (1956) indicated that 15°C appeared to be the optimum temperature for holding fowl semen for two hours, but it could be stored for longer periods without loss of fertilizing capacity. Carter et al. (1957) reported that semen of Large White turkeys gave better fertility when stored at 15°C than at 10°C for very short periods. However, on increasing the period to two hours they observed a sharp decline in fertility at either of the temperatures mentioned above. A number of workers have reported on the storage of fowl spermatozoa at low temperatures. Shaffner et al. (1941) stated that fowl spermatozoa partially dehydrated by the addition of levulose and frozen in test tubes at -79°C showed 30 percent revival of motility on thawing. Polge et al. (1949) reported that spermatozoa in fowl semen diluted with Ringer's solution and frozen by plunging a test tube directly into solid carbon-dioxide and alcohol at -79°C resumed full motility when thawed even after as long as nine months. Shaffner (1942) reported that some fertilizing capacity (25 percent) was shown by spermatozoa revived after one hour of freezing at -79°C; but the embryos died at an early stage (10 to 15 hours). According to Smith and Polge (1950), the addition of glycerol to semen caused a serious reduction in the fertilizing capacity of the spermatozoa and no
fertile eggs were obtained from the hens inseminated with semen containing more than 2 percent glycerol, whether frozen or not. Later it became apparent that the loss in fertility might be due to the rapid removal of glycerol in the hen's oviduct. Therefore, glycerol was removed by dialysis prior to insemination. This technique resulted in some fertility (51 out of a total of 83 hens, Polge, 1951). Allen and Bobr (1955) obtained 73 percent fertility with unfrozen glycerolised semen by placing the glycerolised semen in the uterus of the hen. Clark and Shaffner (1960) obtained 40 percent fertility with intrauterine insemination of semen that had been frozen and the glycerol removed after thawing by dilution and centrifugation. Since, with current techniques the fertilizing capacity of turkey semen drops so rapidly, there is little to be gained by preserving semen by freezing with glycerol and later removing the glycerol. Before such a technique can be utilized, it appears that methods must be available for storing semen in the unfrozen state for several hours without loss of fertilizing capacity. Hence experiments were designed to determine the temperature at which the viability and fertilizing capacity of turkey semen can be best preserved in an unfrozen state.

Diluents

It has long been recognized that, for artificial insemination to be fully exploited, methods for diluting and storing semen are necessary. A number of workers including Ishikawa
Diluents which have been used with limited success in chickens are: normal saline, Ringer's solution, Locke's solution, Tyrode's solution, glycine or egg white added to saline, Ringer's solution with added fructose, human blood serum, bovine blood serum, avian blood serum, fowl seminal fluid, heated pasteurized whole milk, alkyl benzimidazole homologs with phosphate buffer and Lake's solution.

In the majority of the diluents mentioned above, fowl semen can be held only for a short period of time (0 to 1 hour) without loss of fertilizing capacity. In Lake's solution and phosphate buffer with alkyl benzimidazole homologs, fowl semen has been reported to retain fair fertilizing capacity (64 percent and 48 to 60 percent, respectively) after storage for 24 hours and three days. Hobbs and Harris (1963) reported 86 percent fertility with fowl semen diluted 1:2 in a hypertonic carbon
dioxide extender (Δ -1.06°C) after storage for 24 hours. Lake and McIndoe (1959) reported a high concentration (890-1500 mg percent) of free glutamic acid in seminal fluid of cock semen. Small amounts of alanine, glycine, serine, and aspartic acid were also observed by Lake and McIndoe (1959) in fowl seminal plasma. Ahluwalia (1962) reported that, whereas glycine, taurine, histidine, tryptophane and ethanolamine were absent in pooled turkey seminal plasma; arginine (27.66 mg percent), aspartic acid (12.90 mg percent), asparagine (15.31 mg percent), and glutamic acid (956.35 mg percent) were present in significant amounts.

Bajpai and Brown (1963a) studied the effect of milk, Illini Variable Temperature (I.V.T.) diluent, Lake's solution, L-1 diluent (modified Lake's solution), and G-1 diluent (a monosodium glutamate diluent) on the motility, percent bent, and percent dead spermatozoa of turkey semen. They reported that G-1 diluent was the best diluent for maintaining high motility and a low incidence of bent and dead spermatozoa. They also reported that practically no fertility was obtained with I.V.T. (0 percent) and G-1 diluent (12 percent). Wilcox (1960) had reported a marked lowering in fertility of fowl semen on the addition of egg yolk to a phosphate buffer containing antibiotics. Based on Wilcox's (1960) work, Bajpai and Brown (1963a) questioned the validity of incorporating egg yolk in the G-1 diluent. In studies conducted later Bajpai and Brown (1963b) confirmed that the poor fertility obtained by Bajpai and Brown (1963a) with semen diluted in G-1 diluent containing 10 percent egg yolk was a function of egg yolk rather than
the effect of 0-1 diluent itself. In 1963 Harris et al. (1963) in a research note reported that "average fertility for turkey semen stored for six hours in a carbon-dioxide extender, computed on a weekly basis, was 86 percent, 72 percent, 71 percent, and 44 percent for weeks 1 to 4 after a single insemination."

**Spermatozoal Antibodies**

It is rather difficult to give credit to one particular individual for suggesting that fertility or infertility might be related to what we know today as an immune response. However, if we read Darwin's (1898) 'The Descent of Man' with the immunologic theme in mind, a number of references give the impression that he probably considered fertility as an immune response. For example, "No doubt the profligacy of the woman may in part account for their small fertility." Katsh (1939) points out that "if we are to lean towards an immunologic interpretation of Darwin's statement, we could infer that repeated exposure to antigenic material, sperm, had induced infertility. But, it would have to be demonstrated that sperm are antigenic."

In the 1900's Landsteiner (1899), Metchnikoff (1900), and Metalsnikov (1900) almost simultaneously observed that injections of sperm, or testicular extracts into experimental animals resulted in antibody formation (cited from Katsh, 1959). Since then several workers have reported on sperm antigens and spermatozoal antibodies. von Moxter (1900) injected ram spermatozoa into rabbits and obtained antisera which were spermicidal to rat spermatozoa (cited from Katsh, 1959). de Leslie (1901) reported
that male mice became sterile for short intervals (16 to 20 days) after receiving injections of antisperm serum obtained in guinea pigs. Farnum (1901) as cited by Katsh (1959) injected female rabbits intraperitoneally five to eight times with semen or testicular material of dog, bull, or man at intervals of two to six days. He reported that the sera of the treated animals contained precipitins which were specific for each antigen. Pfeiffer (1901) as cited by Katsh (1959) injected rabbits with dried and powdered bull sperm extracts and found that the resulting antiserum reacted strongly with semen solutions and testes extracts and negligibly, if at all, with extracts of other bovine organs. He also reported that it was possible to absorb out all other precipitins in mixtures of antiorgan antisera so that only those specific for semen remained. Strube (1902) also obtained precipitins by injecting rabbits with human semen and testicular extracts (cited from Katsh, 1959).

Lillie (1912), working with marine forms (sea urchins), proposed that substances of the egg and sperm combine in a kind of antigen-antibody reaction. He found that sea urchin eggs released a substance (when kept in seawater) capable of clumping or agglutinating sea urchin sperms. He named the agglutinating substance released by the sea urchin egg—fertilizin. Loeb (1914), however, questioned Lillie's findings and theories (cited from Katsh, 1959). Since then extensive work has been done on marine forms by Tyler (1956) and his colleagues. It has since been established that fertilizin (Tyler, 1956) is the material
making up the gelatinous coat of the sea urchin egg. When the
eggs stand in sea water, the coat gradually dissolves. Fertilizin
combines with a substance called anti-fertilizin on the surface of
the sperm, and when it does so in solution, the sperm clump
together.

At the same time when Lillie (1912) reported his findings
on marine forms, Kohlbrugge (1912) claimed that, during copulation
in rats, mice, bats, rabbits, and fowl, sperm penetrated the
epithelial lining of the female reproductive structures and were
absorbed there. He illustrated such an instance in the oviduct
of a hen by means of a diagram showing sperm in the connective
tissue layer. Walstein and Ekler (1913) as cited by Katsh (1959)
obtained an Abderhalder reaction for testicular protein after
coitus in rabbits. The authors speculated as to what might have
happened to the millions of sperm liberated into the female tract
during copulation and concluded that the sperm were reabsorbed
during residence in the uterus. Metalmikov and Strelnikov (1912)
placed sperm and testicular grafts with and without enclosure in
colloidin sacs in body tissues and recorded antibody production
(cited by Katsh, 1959).

In 1921, stimulated by the cumulating evidence on sperma-
tozoal antibodies and their role in fertility, an editorial ap-
peared in the Journal of American Medical Association asking:
"If spermatozoa invade the female tissues and cause formation of
specific antibodies which are capable of preventing fertilisation,
may not such a process participate in the problem of sterility?"
May not the traditional sterility of the prostitute depend sometimes on such a process...?" "May not such spermatotoxic substances so modify the sperm or the fertilized egg as to lead to abnormalities of importance in teratology?" Since then subsequent speculation and experimentation has not denied these questions or possibilities.

Vogt (1922) (as cited by Katsh, 1959) theorized that women might become sterile following frequent sexual indulgences. Guyer (1922) prepared antisperm sera by injecting fowl repeatedly with rabbit sperm and found that the sera obtained were not only toxic to rabbit and guinea pig serum in vitro but, also, when injected intravenously at 4- to 5-week intervals into male rabbits, induced partial to complete sterility. McCartney (1923) injected rat or human sperm or testes extract into female rats and observed that sterility of 2 to 22 weeks' duration was induced. Serologic examinations implied that the infertility was due to the presence of spermotoxins in the vaginal and uterine secretions, since these fluids immobilized and agglutinated sperm. Kennedy (1924) reported that (a) both male and female guinea pigs could be sterilized by injections of guinea pig sperm; (b) the sperm-immobilizing capacity of the antibody serum was more potent in the sensitized male than it was in the immunized female; (c) degenerative changes occurred in the testicles of some of the injected males; (d) autologous injections were most effective in inducing sterility in the male. Pomerenske (1928) reported that following the injection of female rabbits with rabbit spermatosoa,
the blood serum and vaginal secretions were toxic to homologous spermatozoa. He also suggested that repeated intravaginal insemination, natural or artificial, may result in formation of antibodies, which subsequently might be found in the blood and vaginal secretions. Fogelson (1926) as cited by Katsh (1959) demonstrated that injections of homologous and heterologous sperm into female rats resulted in sterility of 6 to 29 weeks' duration. Guyer and Pearl (1933) reported a high incidence of sterility in rats injected with homologous testicular material. They also found that female rats injected intraperitoneally with bull spermatozoa had reduced fecundity.

Baskin (1937) as cited by Katsh (1959) was awarded U. S. Patent Number 2,103,240 for a nonspecific spermatoxic vaccine. The object of the invention was "the production from materials obtained from the lower animals, of a determinant for human sperm and semen usable as a vaccine or antigen in vaccination of human females to produce spermatoxic condition in her blood and secretions." Despite the twelve claims made in the patent, it is not recorded in the literature that the material has achieved the goals set forth (Katsh, 1959).

During World War II, Freund et al. (1945) were able to produce immunity to malaria in monkeys by using an adjuvant, which consisted of a mixture of mineral oil, water, lanolin, and killed tubercle bacilli. This opened a new era of research in antibody production. Voisina et al. (1951) recorded testicular damage in guinea pigs injected with homologous testicular
homogenates in adjuvant. Freund et al. (1953 and 1955) established that autologous or homologous testicular material incorporated into adjuvant induced impairment of spermatogenesis in guinea pigs and rats. Voisin and Delaunay (1955) extended and confirmed the findings of Freund and his colleagues that spermatogenesis could be induced by immunologic means.

Isojima et al. (1959) and Katsh (1959a), using spermatozoa or testes extract in adjuvant to immunize female rabbits and guinea pigs, reported a serious impairment in fecundity, with little or no effect on ovulation or number of fertilized ova produced, but a high abortion rate. According to Isojima et al. (1959) and Katsh (1959a), the abortions were due to a delayed type of allergic or anaphylactic response following a possible sensitization of the uterus to spermatozoa. Austin (1957) had already demonstrated that the supernumerary spermatozoa in the uterus of mice are removed by phagocytosis. "The implications of this work in connection with a mechanism whereby the female is rendered sensitive to sperm (Katsh, 1957, 1959, and 1961) are obvious."

In the earlier reports it was usually assumed that antibodies of large molecular size do not pass through the tissues to reach the sites in the reproductive tract where they might impair fertility. This, however, does not explain the drop in fertility obtained by frequent intravaginal inseminations by Pommerenke (1928). However, recent work by McFadden and Smith (1955), Orleans (1955), and Press and Porter (1960), showed that
Small antibody fragments may facilitate passage of antibodies across the membranes of the reproductive tract. Brambell (1954) detected antibodies that can pass through the uterine wall of the pregnant rabbit. Edwards (1960), in contrast to Brambell's (1954) report, failed to find spermatozoal antibodies in the uteri of rabbits following systemic immunization with homologous spermatozoa. But he was able to detect antibodies in the uteri of rabbits if the rabbits were immunized with bull spermatozoa. Parsons and Hyde (1940) had also reported similar responses to heterologous spermatozoa. The question as to whether this knowledge can be applied to larger animals and people has frequently been raised. Tyler's (1961) comprehensive tabulation of results from about 150 reports on immunological investigations using germinal material indicates that the evidence is largely against sensitization of females, by intravaginally introduced spermatozoa. Apparently, it has not happened in appreciable numbers of people or animals. However, this phenomenon cannot be overlooked, because of the observations made by Austin (1959), Kohlbrugge (1912), Parsons and Hyde (1940), Edwards (1960), and Katsh (1957, 1958, 1959, and 1961). On the other hand, failure to detect the presence of antibodies in the reproductive tract by Kiddy et al. (1959), Kerr and Robertson (1953), Pierce (1953), Kerr (1955), and Menge et al. (1962) against homologous spermatozoa and certain organisms leaves the question still unanswered with certainty as to whether antibodies pass from the circulation into the lumen or cellular lining of the reproductive tract.
In many instances antibodies which are produced against a specific antigen or disease-causing agent will also act against other antigens or disease-causing agents. Mudd and Mudd (1929) reported cross reactions between bull and ram spermatozoa and their corresponding antisera. Lewis (1934) reported that testicular tissue reacted with antistrain serum so well that no differentiation between the two tissues was possible. Henle (1938) reported cross reactions between sperm of different species. He reported a strong antigenic resemblance between bull and sheep, but less reactivity between bull and man spermatozoa. Docton et al. (1952) reported that bovine isoimmune sera containing antibodies for bovine erythrocytes also reacted specifically with bovine spermatozoa. They also reported that when antibovine sperm antibodies were produced in sheep, specific lysis of erythrocytes of certain cattle was observed along with the agglutination of bovine sperm. Thus, Docton et al. (1952) theorized that antigens recognizable in bovine erythrocytes have similar or identical counterparts in spermatozoa. Rosenthal (1942) reported that certain strains of *Bacillus coli* caused the sperm of guinea pigs, rats, rabbits, and humans to agglutinate almost instantaneously. He also reported that the active principle was non-filterable and was destroyed by boiling at 100°C. Guyer and Clause (1933) induced temporary or permanent sterility in female rats by injecting nucleoprotein fractions prepared from bull testes (cited by Katsh, 1959).
In recent years spermatozoal antibodies have been reported in cattle by Edwards (1960) and Menge et al. (1962). To date three reports have appeared on the production of spermatozoal antibodies in the domestic hen. McCartney (1923) reported that subcutaneous injections of rooster spermatozoa caused four egg-laying hens to lay infertile eggs from 12 to 67 days, but did not influence the rate of egg production. Lamoreux (1940) conducted studies similar to those of McCartney (1923) and reported that in spite of the fact that the hens showed high titers of sperm antibodies, fertility was not affected by injections of homologous spermatozoa in domestic hens.

Wentworth and Mellen (1964) found that repeated intra-vaginal, intrauterine, and intraperitoneal inseminations produced increasing serum anti-spermatozoa titers with increasing number of inseminations, accompanied with a relative decline in the duration of fertility.

Wentworth and Mellen (1964) used an indirect hemagglutination test to detect the levels of circulating spermatozoal antibodies, while McCartney (1923) and Lamoreux (1960) had used direct agglutination of spermatozoa by antisera for detecting spermatozoal antibodies. Spermatozoal antibodies have not been reported in turkeys so far.
EFFECT OF DIFFERENT TEMPERATURES ON THE METABOLIC ACTIVITY, MORPHOLOGY, AND FERTILIZING CAPACITY OF TURKEY SPERMATOZOA

The rapid drop in the fertilizing capacity of turkey semen held in vitro has seriously hampered the best utilization of artificial insemination. Since the use of low temperature has been successfully employed to preserve the fertilizing capacity of bovine semen, it is logical to test similar procedures on turkey semen. So far, identical or similar techniques have not proved successful with turkey semen.

Experimental and Results

Metabolic activity

A study was conducted to determine the effect of cold shock on the aerobic metabolism of turkey spermatozoa. Four trials were conducted. For each trial a pooled semen sample was collected from a minimum of ten Large White toms (32-38 weeks old). The pooled semen was immediately taken to the laboratory and aliquots of 0.5 ml were transferred into vials held at 0, 5, 15, and 25°C. The semen was held at this temperature for 10 minutes. It was then removed and diluted tenfold with a modified Ringer's solution (948 ml NaCl 0.175 M; 50 ml KCl 0.175 M; 2 ml CaCl₂ 0.175 M; 1 x 10⁶ units Penicillin; 1.0 gm Streptomycin) buffered to a pH of 7.3 with a phosphate buffer (Gale and Brown, 1961). The oxygen consumption was measured by standard Warburg
respirometer techniques (Umbreit et al., 1945). The Warburg bath was held at 38°C and the starting pH of the diluted semen was 7.3. After each trial, sperm concentration was determined by counting spermatozoa by means of a haemocytometer. These counts were made on duplicate samples obtained from each flask at the end of the trial. The oxygen consumption was expressed as microliters of oxygen consumed per billion spermatozoa. In each trial all the treatments were determined in triplicate and measurements were recorded at 30-minute intervals for a total period of 150 minutes. The data were analyzed by means of analysis of variance and estimates of statistical significance were based on P .01.

The results obtained are shown in Figure 1. It was observed that the microliters of oxygen consumed by the turkey spermatozoa subjected to a temperature of 15°C for 10 minutes prior to the metabolic trial was significantly greater than the amount of oxygen consumed by spermatozoa subjected to temperatures of 0, 5, and 25°C. Differences between the amounts of oxygen consumed by spermatozoa cold shocked (for 10 minutes prior to the metabolic trial) at temperatures of 5 and 25°C were not significant. The oxygen consumed by the spermatozoa cold shocked at 0°C (for 10 minutes prior to the metabolic trial) was less than the amount of oxygen consumed by spermatozoa shocked at the other three temperatures of 5, 15, and 25°C.
Fig. 1.—Effect of cold shock on the aerobic respiration of turkey spermatozoa.
Fig. 1.—Effect of cold shock on the aerobic respiration of turkey spermatozoa.
A second study was then conducted to determine if the differences in the rate of metabolism following cold shock were related to the number of bent spermatozoa in semen stored at these temperatures. A pooled semen sample was collected from a minimum of 10 Large White toms and was transferred to vials maintained at 0, 5, 10, 15, 20, and 25°C. Dewar flasks were used to maintain vials at the required temperature (Fig. 2). Samples were taken from these vials for morphological studies at intervals of 0, 30, 60, 90, and 120 minutes, and stained with 5 percent nigrosin dissolved in isosmotic glutamate solution (3.0973 gm percent). The smears were prepared on a clean slide by adding a pin drop of semen to a drop of staining solution and spreading it over the slide with a thin glass rod. The slides were then allowed to air dry. The percent bent spermatozoa was determined by counting the number of bent spermatozoa per hundred spermatozoa under oil immersion. Three trials were conducted and each holding temperature was replicated twice in each trial. The data were analyzed by means of analysis of variance and estimates of statistical significance were based on P<.01. The data in percentages were transformed to the arcsine scale for analysis.

The results obtained are shown in Figure 3. At all storage temperatures the percentage of bent spermatozoa increased with the passage of time. However, the incidence of bent spermatozoa in turkey semen subjected to temperatures below 15°C
Fig. 2.--40 x 200 mm dewar flasks for studying the effects of temperature on turkey semen.
Fig. 3.--Effect of cold shock on the incidence of bent spermatozoa in turkey semen.
resulted in significantly higher numbers of bent spermatozoa after 120 minutes of storage. The incidence of bent spermatozoa in turkey semen stored at temperatures of 0, 5, 10; and 15, 20, and 25°C did not vary significantly after 120 minutes of storage. Thus storage at 15°C resulted in the lowest number of bent spermatozoa (Fig. 3) which corresponds with the highest rate of oxygen consumption (Fig. 1). It is interesting to note that the divergence of the lines representing the various treatments (Fig. 3) starts in the opposite direction after 30 minutes of storage, and a significant difference does not become apparent until after 60 minutes of storage.

Fertility

In order to determine whether the above laboratory studies would predict fertilizing capacity of turkey spermatozoa, experiments were designed to study the fertilizing capacity of turkey spermatozoa before and after storage of the semen at different temperatures. The facilities available for fertility studies were best suited for only four temperature treatments. The above laboratory studies indicated that 15°C was least detrimental to turkey spermatozoa, therefore, it was decided to study fertility of semen at 15°C and temperatures above and below 15°C. The temperatures chosen were 10, 15, 20, and 25°C. In all the fertility trials conducted, semen was collected directly into vials maintained at 10, 15, 20, and 25°C. Semen was cooled immediately from the turkey body temperature (41.5°C) to the temperature of
the vial. In all the fertility trials a minimum of five toms (32-44 weeks old) was used for each temperature treatment and the volume of semen inseminated per hen was 0.02 ml. Eggs were set weekly and fertility was checked on the seventh day of incubation. All eggs were separated by candling. Infertile and dead embryos were identified by breaking the eggs. The data obtained were analyzed by means of analysis of variance and estimates of statistical significance were based on P<.01. The purpose of the first fertility trial was to determine if a sudden lowering of temperature from that of the turkey's body temperature would affect the fertilizing capacity of turkey semen and the hatchability of fertile eggs when the semen was inseminated immediately after collection. The semen was collected into vials maintained at 10, 15, 20, and 25°C. Three replicates of 16 virgin hens (Nicholas White, 32 weeks old) in each treatment were inseminated biweekly for 6 weeks.

The data obtained are presented in Table 1. The differences in fertility and hatchability of fertile eggs due to the different treatments were not significant. This indicates that the temperature changes of the magnitude used in this experiment did not damage the fertilizing ability of turkey spermatozoa on immediate insemination.

Since the laboratory studies indicated that temperature shock effects may become evident only after 60 or more minutes of storage, a second experiment was designed to study the effect of storage at the following temperatures (10, 15, 20, and 25°C)
TABLE 1

Effect of collecting turkey spermatozoa at four different temperatures on fertility and hatchability

<table>
<thead>
<tr>
<th>Items</th>
<th>Treatments</th>
<th>10°C</th>
<th>15°C</th>
<th>20°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent fertility¹</td>
<td></td>
<td>62</td>
<td>64</td>
<td>65</td>
<td>68</td>
</tr>
<tr>
<td>Percent hatchability¹</td>
<td></td>
<td>70</td>
<td>77</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>No. of eggs set</td>
<td></td>
<td>1014</td>
<td>1024</td>
<td>995</td>
<td>1008</td>
</tr>
</tbody>
</table>

¹Fertility and hatchability for six weeks.

On fertility and hatchability of fertile eggs. Semen was collected directly into vials maintained at 10, 15, 20, and 25°C and stored for 30 and 60 minutes, thus there were a total of eight treatments. Inseminations were made biweekly. One hundred twelve virgin hens (Ohio randobred, 32 weeks old) were housed in seven pens with 16 hens in each pen. Two hens per pen were placed on each treatment. The data obtained for the first three weeks showed that the differences in fertility and hatchability between the 30- and 60-minute storage periods were not significant. Thus, at the beginning of the fourth week the 60 minutes storage was extended to 120 minutes and inseminations were continued biweekly for eight more weeks. The 30-minute storage treatment remained the same for the entire 12 weeks. The data collected for the four weeks following the change in treatments from 60 to 120 minutes storage were omitted to remove carry-over effect. The data collected for the following 4-week period are presented in Table 2. Storage of turkey semen up to 120 minutes resulted in significantly
TABLE 2
Effect of storing semen at four different temperatures on fertility and hatchability

<table>
<thead>
<tr>
<th>Items</th>
<th>30 Minutes</th>
<th>60 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10°C 15°C 20°C 25°C</td>
<td>10°C 15°C 20°C 25°C</td>
</tr>
<tr>
<td>Trial No. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent fertility</td>
<td>90 93 92 89</td>
<td>89 90 87 88</td>
</tr>
<tr>
<td>Percent hatchability</td>
<td>77 76 71 68</td>
<td>73 75 71 68</td>
</tr>
<tr>
<td>No. of eggs set</td>
<td>241 230 251 223</td>
<td>216 234 226 220</td>
</tr>
<tr>
<td>Trial No. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent fertility</td>
<td>82A 83A 88A 87A</td>
<td>44C 76AB 65B 66B</td>
</tr>
<tr>
<td>Percent hatchability</td>
<td>68 79 81 70</td>
<td>71 68 68 83</td>
</tr>
<tr>
<td>No. of eggs set</td>
<td>211 259 195 228</td>
<td>182 197 165 168</td>
</tr>
</tbody>
</table>

1 Fertility and hatchability for four weeks.
2 Percent fertility values followed by different letters are significantly different at P<.01.
lower fertility at all temperatures except 15°C. At 15°C the average fertility was 83 percent and 76 percent after 30 and 120 minutes of storage (Trial 2, Table 2). This indicates that there was probably some temperature shock involved at this temperature. It is interesting to note that the fertility of turkey semen stored at 15, 25, 20 and 10°C dropped by 7, 21, 23, and 38 percent when the storage period was extended from 30 to 120 minutes (Trial 2, Table 2). Thus, lowering storage temperature from 25 and 20°C to 15°C on storage did not result in a significant drop in fertility, while a further lowering to 10°C resulted in a significant drop in fertility. Hatchability was not affected by the treatments.

Discussion

The endogenous respiration of bull spermatozoa and ram spermatozoa is decreased by 39 and 66 per cent, respectively, when bull and ram spermatozoa are subjected to 0°C for 10 minutes (Mayer, 1955). Results obtained in this investigation with turkey semen subjected to 0°C and 5°C for 10 minutes also indicate that temperatures lower than 15°C result in significant decrease in the rate of metabolic activity of turkey spermatozoa. Temperatures higher than 15°C also seem to have a similar effect on the metabolic activity of turkey spermatozoa (Fig. 1).

Mayer (1955), working with bull and ram spermatozoa, reported that cold shock or temperature shock is dependent on (1) the length of exposure to low temperatures and (2) the actual temperature of exposure. He also reported that the changes induced
by temperature shock are reflected by the changes in rates of anaerobic glycolysis, respiration, and aerobic metabolism. Whatever the mechanism involved, it develops in the short period of 10 minutes. Mayer (1955) also reported that storage changes as reflected by respiration rate of semen stored at 25°C to 30°C are slow but progressive, requiring 24 hours to reduce the respiration rate to the level produced by cold shock. The cold shock technique on ram spermatozoa merely completes the change to a level equivalent to that reached after a 24-hour storage period at 25°C to 30°C. Likewise, the results obtained in this investigation indicate (Figs. 1 and 3 and Tables 1 and 2) that temperature shock occurs in turkey spermatozoa within the 10 minutes of exposure to cold and that it takes approximately 2 hours for differences to show up due to this initial shock in terms of incidence of bent spermatozoa and fertility.

Moore and Mayer (1941) reported that anaerobic glycolysis rate was depressed slightly when bull spermatozoa was shocked at temperatures above 15°C and that at temperatures below 10°C, the rate was depressed markedly. Mayer (1955) reported that at 15°C no change in glycolytic rate was apparent, but as the cold shock temperature was lowered the rate was depressed to a greater extent with the greatest effect occurring at 1°C (62 percent of 25°C to 30°C control). Schindler and Nevo (1962) reported that fowl semen could be kept at room temperature (20°C) in a cone-shaped semen container immersed in a water bath for many hours without losing its vigour. Schindler and Nevo (1962) also reported that
"(1) time until inactivation sets in was inversely related to amount of motility per unit volume of the suspension; (2) the quiescent spermatozoa could be reactivated by simple mixing and on standing became inactive again; (3) in a narrow zone adjacent to the liquid-air interface, the spermatozoa remained motile."

It is thus suggested that in storage studies in this investigation the turkey spermatozoa were almost in an anaerobic condition.

Mann (1946a, 1946b, 1946c) suggested that although there is no direct evidence available on the use of glucose by poultry spermatozoa it must rely on glucose as the chief source of energy. Ahluwalia (1962) reported high concentrations of glutamic acid in chicken (1178 mgm percent) and turkey 956.35 mgm percent) seminal plasma but practically no sugars in chicken seminal plasma (glucose 3.1 mg percent and inositol 20.4 mg percent). Preliminary investigations conducted at this laboratory showed that turkey semen has a very high transaminase activity. Glutamic acid can be readily converted to α-keto-glutaric acid by either of the two transaminases (glutamic-oxalo-acetic acid transaminase and glutamic-pyruvic acid transaminase) in the presence of oxalo-acetic acid or pyruvic acid. All the above acids, namely oxalo-acetic acid, pyruvic acid and α-keto-glutaric acid are oxidation products in the citric acid cycle. Thus it is postulated that a small amount of sugar is necessary to provide acetyl-coenzyme A for the initiation and maintenance of oxidative processes and the main source of energy is provided by the oxidative utilization of glutamic acid in the citric acid cycle. Hence the decrease in fertilizing
capacity of turkey spermatozoa (21 percent and 23 percent at storage temperatures above 15°C after 120 minutes) is probably due to rapid anaerobic glycolysis, build-up of lactic acid in toxic amounts and exhaustion of the sugar supply necessary for maintaining the oxidative cycle. Gale and Brown (1961) reported that turkey semen is nearly always contaminated with bacteria. Thus it is postulated that at temperatures above 15°C, namely 20°C and 25°C, the bacterial growth is rapid enough to use up the nutrients of the seminal plasma depriving the spermatozoa of the necessary nutrients to maintain the fertilizing capacity of turkey spermatozoa. On the other hand, the decrease in fertilizing capacity of turkey spermatozoa at temperatures below 15°C (38 percent at storage temperature of 10°C) is probably due to irreversible damage to either the glycolytic mechanism or due to irreversible damage to the transaminases or some other substance essential for both the glycolytic and aerobic mechanisms. The drop in fertility (although not significant) at storage temperatures of 15°C after 120 minutes suggests that although there is no damage to the metabolic systems of turkey spermatozoa, there is probably a gradual depletion of sugars and oxygen accompanied with a gradual build-up of lactic acid and carbon dioxide which in turn have some deleterious effect on the fertilizing capacity of turkey spermatozoa. Thus, it appears that diluents which would dilute the toxic substances as well as supply adequate amounts of energy sources such as glucose and glutamic acid may
prove useful in maintaining the fertilizing capacity of turkey spermatozoa after storage at 15°C.

Investigations conducted by Easley et al. (1942), Lasley et al. (1942), Lasley and Bogart (1943), Lasley and Mayer (1944), Mayer and Lasley (1945), Mayer and Lasley (1945), Mayer et al. (1951), Kampschmidt et al. (1953), and Mayer (1955) provide evidence that a factor (or factors) in egg yolk, chick embryo, Mare follicular fluid, lecithin, cephalin, and two lipoprotein complexes—lipovitellin and lipovitellinin—were capable of protecting bull and ram spermatozoa from the effects of temperature or cold shock.

Bajpai and Brown (1963b) showed that 10 and 15 percent egg yolk preserved motility, morphology, and livability of turkey spermatozoa stored at 5°C for 96 hours, but found that 10 percent egg yolk and lecithin (4 mg/ml) were detrimental to the fertilizing capacity of turkey spermatozoa. Thus, if the spermicidal factor (or factors) affecting the turkey spermatozoa could be isolated and/or removed from egg yolk, or if other ways could be found to prevent temperature shock, lower temperatures (i.e., below 15°C) should result in lower rates of metabolism and make longer periods of storage possible.
Lack of information on a diluent containing carbon dioxide as a reversible inhibitor of metabolism of turkey semen induced Bajpai and Brown (1963a) to study the possibilities of using carbon dioxide extenders in storing turkey semen. Bajpai and Brown (1963a) reported that although the diluents (I.V.T. and G-1 diluent) containing carbon dioxide preserved turkey semen adequately in the laboratory both I.V.T. and G-1 diluent markedly inhibited the fertilizing capacity of turkey spermatozoa. However, in a subsequent study Bajpai and Brown (1963b) reported fairly good fertility (63 percent) with G-1 diluent containing no egg yolk and poor fertility (35 percent) with G-1 diluent containing 10 percent egg yolk by volume. In the same year Harris et al. (1963) reported some success with carbon dioxide extenders for diluting turkey semen. It was thus considered desirable to study further the effect of some diluents formulated at this laboratory as well as the effect of carbon dioxide extenders (used by Harris et al., 1963) on the semen characteristics of turkey semen.
Experimental and Results

Effect of adding sodium-bicarbonate to a glutamate diluent on the fertilizing capacity of turkey spermatozoa

Pooled semen samples were collected from 8 to 10 Large White (Nicholas) toms. The semen was diluted 1:1 in isosmotic monosodium glutamate solution (3.0973 gm percent) and in isosmotic solution of monosodium glutamate and sodium bicarbonate (monosodium glutamate 2.034 gm percent and sodium bicarbonate 0.21 gm percent). Sixteen hens (Nicholas) on each treatment were inseminated weekly with 0.02 ml of undiluted and 0.04 ml of diluted semen for 6 weeks. Eggs were set weekly and fertility was checked on the seventh day of incubation. The data collected were analyzed by means of analysis of variance. It was observed that the addition of sodium bicarbonate to the monosodium glutamate diluent had no significant effect (Table 3). Harris et al. (1963) reported that a diluent composed of sodium bicarbonate and citric acid maintained fertility of turkey spermatozoa at a significantly higher level than a diluent composed of sodium bicarbonate and sodium citrate. Both fowl and turkey semen contain large amounts of glutamic acid (Chubb and Cooper, 1962, and Ahluwalia, 1962), instead of citric acid. Hence it was considered desirable to study the effect of diluents composed of glutamic acid, monosodium glutamate and sodium bicarbonate. The purpose of sodium bicarbonate in these diluents was to provide carbon dioxide for inhibiting the metabolic activity of turkey spermatozoa.
TABLE 3

The effect of sodium bicarbonate as a component of monosodium glutamate diluent on the fertilizing capacity of turkey spermatozoa and hatchability of the fertilized eggs

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Percent Fertility Means</th>
<th>Percent Hatchability Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted semen</td>
<td>64</td>
<td>61</td>
</tr>
<tr>
<td>Semen diluted in Na-glutamate(^1) solution</td>
<td>71</td>
<td>61</td>
</tr>
<tr>
<td>Semen diluted in Na-glutamate(^1) and Na-bicarbonate solution</td>
<td>61</td>
<td>66</td>
</tr>
</tbody>
</table>

\(^1\) \(\Delta = -0.65-0.68^\circ\) C.

Effect of carbon dioxide diluents on the fertilizing capacity of turkey spermatozoa

Pooled semen samples were collected into a 15 ml tube held at 15\(^\circ\)C in a dewar flask from a minimum of 10 to 12 Large White Ohio Randombred toms (32 weeks old). Semen samples were pipetted into 5 ml glass ampoules maintained at 15\(^\circ\)C in a vacuum flask. The semen was diluted 1:1 in each of the diluents shown in Table 4. The ampoules were sealed with an oxygen flame and stored at 15\(^\circ\)C for 4 hours before insemination. Likewise, another sample of pooled semen was collected and diluted for insemination immediately after collection. One hundred ninety-two virgin hens (Ohio Randombred, 32 weeks old) were housed in 12 pens with 16 hens in each pen. Two hens per pen were placed on each of the eight treatments. Hens were inseminated with 0.02 ml of undiluted or 0.04 ml of diluted semen biweekly for 6 weeks. The procedures
<table>
<thead>
<tr>
<th></th>
<th>Diluent A</th>
<th>Diluent B</th>
<th>Diluent C</th>
<th>Harris' Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monosodium glutamate</td>
<td>0.960 gms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.075 gms</td>
<td>1.075 gms</td>
<td>1.424 gms</td>
<td></td>
</tr>
<tr>
<td>Sodium citrate</td>
<td></td>
<td></td>
<td></td>
<td>0.610 gms</td>
</tr>
<tr>
<td>Citric acid</td>
<td></td>
<td></td>
<td></td>
<td>1.740 gms</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>1.226 gms</td>
<td>1.226 gms</td>
<td>1.630 gms</td>
<td>2.290 gms</td>
</tr>
<tr>
<td>Distilled water ad</td>
<td>100.000 ml</td>
<td>100.000 ml</td>
<td>100.000 ml</td>
<td>100.000 ml</td>
</tr>
<tr>
<td>Carbon dioxide level</td>
<td>0.642 gms</td>
<td>0.642 gms</td>
<td>0.842 gms</td>
<td>1.200 gms</td>
</tr>
<tr>
<td>(gms/100 ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freezing point depression (Δ)</td>
<td>-0.68°C</td>
<td>-0.90°C</td>
<td>-0.90°C</td>
<td>-0.90°C</td>
</tr>
</tbody>
</table>
adopted for assessing fertility were the same as outlined on page 25. The data obtained were analyzed by means of analysis of variance. The results showed (Table 5) that the fertilizing capacity of undiluted turkey semen and turkey semen diluted in diluents having lower freezing point depression (Δ) of -0.90°C were not different from that of diluents having a higher Δ of -0.68°C. It was also observed that the fertility obtained with undiluted turkey semen and turkey semen diluted in diluents A, B, and C immediately after collection were not different. However, it was observed that the fertilizing capacity of turkey semen diluted in diluents A, B, and C dropped significantly after storage for 4 hours at 15°C. The fertility obtained with undiluted semen stored for 4 hours was significantly different from that of semen stored for 4 hours in diluents A, B, and C. No significant difference was observed in the fertility obtained with semen diluted in diluents A, B, and C after 4 hours of storage at 15°C (Table 5). However, the most significant information conveyed by this trial was that the fertility of undiluted semen after 4 hours of storage at 15°C was not significantly different from that of undiluted semen inseminated immediately after collection. Harris et al. (1963) reported successful storage of turkey semen for 6 hours in carbon dioxide diluents composed of citric acid, sodium bicarbonate and sodium citrate ("average fertility computed on a weekly basis of 86 percent, 72 percent, 71 percent and 44 percent for weeks 1 to 4 after a single insemination") while the carbon dioxide extenders composed of
TABLE 5

Effect of carbon dioxide diluents and freezing point depression on the fertilizing capacity of turkey semen

<table>
<thead>
<tr>
<th>Semen Treatment</th>
<th>(Δ)</th>
<th>0-0.5</th>
<th>4-4.5</th>
<th>0-0.5</th>
<th>4-4.5</th>
<th>0-0.5</th>
<th>4-4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent A</td>
<td>-0.68°C</td>
<td>594</td>
<td>619</td>
<td>77A</td>
<td>41BC</td>
<td>75</td>
<td>80</td>
</tr>
<tr>
<td>Diluent B</td>
<td>-0.90°C</td>
<td>562</td>
<td>554</td>
<td>77A</td>
<td>43BC</td>
<td>71</td>
<td>73</td>
</tr>
<tr>
<td>Diluent C</td>
<td>-0.90°C</td>
<td>635</td>
<td>678</td>
<td>78A</td>
<td>52BC</td>
<td>72</td>
<td>75</td>
</tr>
<tr>
<td>Undiluted</td>
<td></td>
<td>839</td>
<td>530</td>
<td>88A</td>
<td>77A</td>
<td>86</td>
<td>78</td>
</tr>
</tbody>
</table>

*Average percent fertility and hatchability of fertilized eggs of 6 weeks. Fertility Means followed by different letters are significantly different at P<.01.
glutamic acid and sodium bicarbonate; and glutamic acid, sodium bicarbonate plus monosodium glutamate in the above investigation failed to maintain fertility after 4 hours of storage at 15°C. Hatchability was not affected by the various treatments. It was thus considered desirable to test out Harris' diluent in comparison with the diluents employed in this investigation. Semen diluted in Harris' diluent was inseminated in the hens which were being inseminated with semen diluted in diluent A (glutamic acid and sodium bicarbonate Δ -0.68°C). The storage time was extended to 6 hours to provide a closer comparison of results obtained in this investigation with those of Harris et al. (1963). The rest of the procedures followed were the same as described above, with the exception that data obtained during the seventh and eighth week of investigation were omitted to remove any carry-over effects.

The results show (Table 6) a dramatic drop in the over-all fertility obtained with semen diluted in all the diluents in the last four weeks of the investigation. The fertility obtained with semen diluted in Harris' diluent and diluents B and C was significantly lower than the fertility obtained with undiluted semen, both before and after storage. The fertility obtained with fresh undiluted semen was significantly higher than the fertility obtained with undiluted semen stored for 6 hours at 15°C. The fertility obtained with semen diluted in Harris' diluent was not significantly different from the fertility obtained with semen diluted in diluent B composed of glutamic acid sodium bicarbonate and monosodium glutamate at 0-0.5 hours of storage. However, the fertility
**TABLE 6**

Effect of carbon dioxide diluents composed of citric acid and glutamic acid on fertilizing capacity of turkey spermatozoa

<table>
<thead>
<tr>
<th>Semen Treatments</th>
<th>No. of Eggs Set</th>
<th>Percent Fertility (Means*)</th>
<th>Percent Hatchability (Means*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(△)</td>
<td>0-0.5</td>
<td>6-6.5</td>
</tr>
<tr>
<td>Harris' diluent</td>
<td>-0.9°C</td>
<td>410</td>
<td>347</td>
</tr>
<tr>
<td>Diluent B</td>
<td>-0.9°C</td>
<td>414</td>
<td>398</td>
</tr>
<tr>
<td>Diluent C</td>
<td>-0.9°C</td>
<td>416</td>
<td>424</td>
</tr>
<tr>
<td>Undiluted</td>
<td></td>
<td>391</td>
<td>404</td>
</tr>
</tbody>
</table>

*Average percent fertility and hatchability of fertilized eggs of 4 weeks. Fertility means followed by different letters are significantly different at P < .01.
obtained with semen diluted in Harris' diluent at 0-0.5 hours of storage was significantly higher than the fertility obtained with semen diluted in Harris' diluent after 6 hours of storage. No significant difference was observed in the fertility obtained with semen diluted in Harris' diluent, and diluent B and C after 6 hours of storage at 15°C. Hatchability was not affected by the various treatments (Table 6).

The effect of glucose on fertilizing capacity of turkey spermatozoa

While studies with carbon dioxide diluents were being conducted other investigations on non-carbon dioxide diluents were not overlooked.

Wilcox and Shaffner (1960) reported inconsistent fertility with turkey semen diluted (1:4) in a diluent composed of phosphate buffer, antibiotics, and 400 mg percent fructose. Recent investigations conducted in this laboratory (Bajpai and Brown, 1963a and 1963b) show that fertilizing capacity of turkey semen diluted (1:1) in a diluent composed of monosodium glutamate, sodium bicarbonate, potassium chloride, magnesium chloride, and 300 mg percent glucose, was as good as that of undiluted semen. Since monosodium glutamate is a good buffer in itself, an experiment was designed to evaluate the role of glucose as an additive to a turkey semen diluent composed of monosodium glutamate.

Two trials were conducted. Two stock solutions were made: (a) by preparing 3.0973 gm percent of monosodium glutamate solution and (b) by preparing a solution containing 2.7390 gm
percent of monosodium glutamate and 0.300 gm percent glucose. The various diluents were then prepared by mixing solution A and B in the following ratios: 1:0, 1:1, 3:1, 7:1, and 0:1 to give final concentrations of glucose as follows: 0 mg percent, 37.5 mg percent, 75 mg percent, 150 mg percent, and 300 mg percent. In the first trial pooled semen samples were collected from a minimum of eight to ten (32 weeks of age) Large White Nicholas toms. Eight (Nicholas White) virgin hens (32 weeks of age) each were inseminated biweekly with undiluted semen, and semen diluted in each of the diluents. The control hens were inseminated with 0.02 ml of undiluted semen, while the hens receiving the diluted semen (1:1) were inseminated with 0.04 ml of diluted semen. The \( \Delta \) of all the diluents was \(-0.65^\circ C\). The procedures adopted for assessing the fertility were the same as outlined previously on page 25. In the second trial pooled semen samples were collected from a minimum of 8 to 10 Large White (Ohio Randombred) toms (32 weeks of age). Sixteen (Ohio Randombred White) virgin hens each (32 weeks of age) were inseminated biweekly with 0.02 ml of undiluted and 0.04 ml of (1:1) diluted semen. The rest of the procedures were the same as outlined above in Trial No. 1. The data were analyzed by means of analysis of variance.

The results show (Trial 1, Table 7) that the fertility of turkey semen diluted in monosodium glutamate solution containing 300 mg percent glucose was significantly higher than the fertility of semen diluted in monosodium glutamate solution containing no glucose. No significant differences were observed between the
TABLE 7
The effects of four different levels of glucose on the fertilizing capacity of turkey spermatozoa

<table>
<thead>
<tr>
<th>Semen Treatments</th>
<th>Percent Fertility (Means*)</th>
<th>Percent Hatchability (Means*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>Undiluted</td>
<td>73ab</td>
<td>58</td>
</tr>
<tr>
<td>Monosodium glutamate</td>
<td>62b</td>
<td>50</td>
</tr>
<tr>
<td>Monosodium glutamate + 37.5 mg percent glucose</td>
<td>75ab</td>
<td>54</td>
</tr>
<tr>
<td>Monosodium glutamate + 75 mg percent glucose</td>
<td>65ab</td>
<td>55</td>
</tr>
<tr>
<td>Monosodium glutamate + 150 mg percent glucose</td>
<td>65ab</td>
<td>49</td>
</tr>
<tr>
<td>Monosodium glutamate + 300 mg percent glucose</td>
<td>79a</td>
<td>63</td>
</tr>
</tbody>
</table>

*Average percent fertility and hatchability of fertilized eggs of 10 weeks.

Fertility Means followed by different letters are significantly different at P<.01.

fertility of undiluted and diluted semen. The results of Trial 2 (Table 7) do not show a significant difference between the fertility obtained with undiluted semen and semen diluted in sodium glutamate solution with and without various levels of glucose. However, the validity of the results obtained in Trial 2 is questionable because the hens in this trial during the course of investigation contracted Newcastle and the over-all fertility of all the birds for some time was as low as 33 percent.
Effect of reactivating turkey semen after storage in sodium chloride and monosodium glutamate diluents

Investigations reported earlier in the text indicated (1) turkey spermatozoa can be successfully stored for 2 (Table 2) to 4 hours (Table 5) at 15°C without any loss in fertility; (2) that 300 mg percent glucose is beneficial to turkey spermatozoa. Bajpai and Brown (1964) reported that addition of magnesium chloride to isotonic monosodium glutamate solution improved the fertility of turkey semen. Brown (1962) reported that turkey semen could be diluted but not stored in isotonic saline without any appreciable loss in fertility. Temperature studies conducted earlier in the text suggested that the fertilizing capacity of turkey spermatozoa could be restored after storage if the semen was diluted in diluents which would dilute the metabolic end products of glycolytic and oxidative cycles as well as provide a source or sources of energy for the maintenance of the glycolytic and oxidative cycles. Hence it was considered desirable to study the effect of diluting turkey semen after storage at 15°C with diluents composed of (1) sodium chloride, glucose and magnesium chloride; and (2) monosodium glutamate, glucose, and magnesium chloride.

Pooled semen samples were collected from a minimum of 15 to 20 Large White Ohio Randombred toms (32 weeks old). Three equal parts of semen were transferred by pipettes into three different (2-4) vials maintained at 15°C. Semen in vials 2-4 was maintained at 15°C for 4 hours and then the contents of vial 3 were
diluted 1:1 with sodium chloride (A) diluent and the contents of vial 4 were diluted 1:1 with monosodium glutamate (B) diluent. Another sample of pooled semen was collected into vial 1, maintained at 15°C from a minimum of four toms for inseminating immediately after collection. The sodium chloride (A) and monosodium glutamate (B) diluents were made by preparing a solution containing 9.5425 gm percent of sodium chloride, 0.3 gm percent of glucose, and 0.0228 gm percent of magnesium chloride; and a solution containing 2.761 gm percent monosodium glutamate, 0.3 gm percent of glucose, and 0.0228 gm percent of magnesium chloride. Ninety-six virgin hens (Ohio Randombred, 32 weeks old) were housed in 12 pens with 8 hens in each pen. Two hens per pen were placed on each of the following four treatments. In treatment 1 inseminations were made with undiluted semen immediately after collection. In treatment 2 inseminations were made with undiluted semen stored for 4 hours at 15°C. In treatment 3 semen was stored for 4 hours at 15°C after which the inseminations were made with the stored semen diluted 1:1 in sodium chloride diluent (A). In treatment 4 undiluted semen was stored for 4 hours at 15°C after which the inseminations were made with the stored semen diluted 1:1 in monosodium glutamate diluent (B). Hens were inseminated with 0.02 ml of undiluted or 0.04 ml of diluted semen biweekly for 6 weeks. The procedures adopted for assessing fertility were the same as outlined on page 25. The data obtained were analyzed by means of analysis of variance. The results (Table 8) show that there was no significant difference between the fertilizing capacity of
TABLE 8

Effect of diluting stored undiluted semen (for 4 hours at 15°C) in sodium chloride and monosodium glutamate diluents on the fertilizing capacity of turkey spermatozoa

<table>
<thead>
<tr>
<th>Semen Treatments</th>
<th>No. of Eggs Set</th>
<th>Mean Percent Fertility*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inseminated immediately after collection (1)</td>
<td>423</td>
<td>79A</td>
</tr>
<tr>
<td>Inseminated after storage for 4 hours at 15°C (2)</td>
<td>485</td>
<td>66AB</td>
</tr>
<tr>
<td>Stored for 4 hours at 15°C and diluted 1:1 in sodium chloride diluent, prior to insemination (3)</td>
<td>455</td>
<td>10D</td>
</tr>
<tr>
<td>Stored for 4 hours at 15°C and diluted 1:1 in monosodium glutamate diluent prior to insemination (4)</td>
<td>432</td>
<td>46C</td>
</tr>
</tbody>
</table>

* Mean Percent Fertility of 6 weeks.

( ) Treatments 1 to 4.

Fertility Means followed by different letters are significantly different at P<.01.

Undiluted semen inseminated immediately after collection (1) and undiluted semen inseminated after 4 hours of storage at 15°C (2). A significant drop in fertility (Table 9) was observed when undiluted semen stored for 4 hours at 15°C was inseminated after diluting it in monosodium glutamate (4) and sodium chloride (3) diluents (Table 8). Stored undiluted semen (4 hours at 15°C) when diluted in monosodium glutamate diluent (4) on insemination gave significantly higher fertility than stored undiluted semen (4 hours at 15°C) diluted in sodium chloride diluent (3). The latter results seem to suggest that monosodium glutamate is probably utilized by turkey spermatozoa in presence of glucose.
TABLE 9

Composition of diluents for the study of inactivated and adsorbed egg yolk and serum (added to monosodium glutamate) on the fertility of turkey semen

<table>
<thead>
<tr>
<th></th>
<th>Diluent 1</th>
<th>Diluent 2</th>
<th>Diluent 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monosodium glutamate</td>
<td>2.7610 gms</td>
<td>2.7610 gms</td>
<td>2.7610 gms</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>0.0228 gms</td>
<td>0.0228 gms</td>
<td>0.0228 gms</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.3000 gms</td>
<td>0.3000 gms</td>
<td>0.3000 gms</td>
</tr>
<tr>
<td>Distilled water ad</td>
<td>100.0000 ml</td>
<td>100.0000 ml</td>
<td>100.0000 ml</td>
</tr>
<tr>
<td>Inactivated and adsorbed egg yolk(^1,2)</td>
<td>10 percent by volume</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inactivated and adsorbed serum(^1,2)</td>
<td>10 percent by volume</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Both egg yolk and serum were inactivated by incubating at 56°C for 2 hours.

\(^2\) Both egg yolk and serum were incubated twice with turkey semen for 4 hours each at room temperature. One part of egg yolk or serum was incubated with one-half parts of turkey semen each time.
and magnesium chloride. However, the fact that the fertility of undiluted stored semen is significantly higher than semen diluted after storage, suggests that dilution of turkey spermatozoa after storage is probably of little practical value.

**Effect of inactivated plus adsorbed egg yolk and serum added to monosodium glutamate on the fertility of turkey semen**

Philips (1939) reported a beneficial effect of egg yolk upon the livability of bull spermatozoa during storage. Since then the yolk of fresh eggs (chickens) has been incorporated as an essential constituent in all popular bull semen diluents. But Bajpai and Brown (1963b) reported fairly good fertility (63 percent) with G-1 diluent containing no egg yolk and poor fertility (35 percent) with G-1 diluent containing 10 percent egg yolk by volume. However, Bajpai and Brown (1963b) also reported that 10 and 15 percent egg yolk preserved motility, morphology, and livability of turkey spermatozoa at 5°C for 96 hours. They thus theorized that if the spermicidal factor (or factors) could be isolated and/or removed from the egg yolk, turkey spermatozoa could be stored in yolk diluents without any appreciable loss in fertility for longer periods at lower temperatures. While conducting studies on spermatozoa antibodies it was observed that turkey sera showing positive titers against turkey spermatozoa could be inactivated by incubating it for 2 hours at 56°C. It was also observed that nonspecific antibodies against turkey spermatozoa in normal rabbit sera were successfully removed by
incubating the sera twice for 4 hours each with turkey spermatozoa. Lillie (1912) reported that sea urchin eggs released an agglutinating substance, fertilizin, which was capable of agglutinating sea urchin spermatozoa. It is possible that turkey egg yolk also has such factor or factors.

Tyler (1956) reported that sea-urchin egg fertilizin combines with a substance called anti-fertilizin on the surface of the sea urchin sperm, causing them to clump in solution. Thus if the factor in the egg yolk is of the nature of an antibody it can also be inactivated and adsorbed by the same procedures which remove antibodies against spermatozoa in serum. Hence it was considered desirable to study the protective effect of both inactivated plus adsorbed egg yolk and serum on the fertilizing capacity of turkey spermatozoa.

Bajpai and Brown (1964) reported that addition of magnesium chloride to isotonic monosodium glutamate solution improved the fertility of turkey semen, while investigations conducted earlier indicated that 300 mg percent glucose is beneficial to turkey spermatozoa. Thus an experiment was designed to study the effect of diluting and storing turkey semen in monosodium glutamate diluent, and monosodium glutamate diluent containing inactivated plus adsorbed egg yolk, and inactivated plus adsorbed serum on the fertilizing capacity of turkey spermatozoa.

Pooled semen samples were collected into a 15 ml tube held at 15°C in a dewar flask from a minimum of 15 to 20 Large White Ohio Randombred toms (32 weeks old). Five equal parts of
semen were transferred by pipettes into five different (2-5) vials maintained at 15°C. Semen in vial 2 was maintained at 15°C for 4 hours before insemination. Semen in vials 4, 5, and 6 were diluted 1:1 within 10 minutes of collection with monosodium glutamate diluent (1), serum glutamate diluent (2), and yolk glutamate diluent (3), shown in Table 9. Vials 3 to 6 were then transferred to a 15°C water bath and cooled gradually over an hour to 8°C. At the end of 3 hours of storage at 8°C the vials 3 to 6 were again transferred to dewar flasks maintained at 15°C. The purpose for gradually cooling the undiluted semen in vial 2 from 15°C to 8°C over an hour and then storing at 8°C for 3 hours was to find out whether gradual cooling would avoid temperature shock. On the other hand the reason for gradually cooling the semen diluted in glutamate diluents and serum and yolk-glutamate diluents was to investigate whether inactivated and adsorbed serum and egg yolk can prevent temperature shock if there was some damage due to temperature shock. Another sample of pooled semen was collected into vial 1 (at 15°C) for inseminating immediately after collection from a minimum of four toms. One hundred forty-four virgin hens (Ohio Randombred, 32 weeks old) were housed in 12 pens with 12 hens in each pen. Two hens per pen were placed on each of the following six treatments. In treatment A inseminations were made with undiluted semen immediately after collection from a minimum of four toms. In treatment B inseminations were made with undiluted semen stored for 4 hours at 15°C. In treatment C undiluted semen was cooled from 15°C over an hour to 8°C. The cooled semen was
then stored at 8°C for 3 hours and warmed to 15°C before inseminations. In treatment D semen was diluted 1:1 in monosodium glutamate diluent (1) at 15°C and was inseminated after treating the diluted semen as in treatment C. In treatment E semen was diluted 1:1 in serum-glutamate diluent (2) and was inseminated after treating the diluted semen as in treatment C. In treatment F semen was diluted 1:1 in yolk-glutamate diluent (3) and was inseminated after treating the diluted semen as in treatment C. Hens were inseminated with 0.02 ml of undiluted or 0.04 ml of diluted semen biweekly for 6 weeks. The procedures adopted for assessing fertility were the same as outlined on page 25. The data obtained were analyzed by means of analysis of variance.

The results (Table 10) show that the fertilizing capacity of undiluted spermatozoa inseminated immediately after collection (A) was significantly higher than the fertilizing capacity of spermatozoa in semen treatments C, D, E, and F, where undiluted or diluted semen was cooled to and stored at 8°C for 3 hours. No significant difference was observed in the fertility of undiluted semen inseminated immediately after collection (A) and the fertilizing capacity of undiluted semen stored for 4 hours at 15°C (B). However, the fertility of undiluted semen (C) or semen diluted in monosodium glutamate diluent (D) or serum-glutamate diluent (E) gradually cooled from 15°C to 8°C (over an hour) and stored for 3 hours at 8°C was significantly lower than the fertility of undiluted semen stored for 4 hours at 15°C. There was no significant difference between the fertility of undiluted semen (B) stored for
TABLE 10

Effect of inactivated and adsorbed egg yolk and serum (added to monosodium glutamate) on the fertilizing capacity of turkey spermatozoa

<table>
<thead>
<tr>
<th>Semen Treatments</th>
<th>No. of Eggs Set</th>
<th>Mean Percent Fertility*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inseminated immediately after collection (A)</td>
<td>423</td>
<td>79A</td>
</tr>
<tr>
<td>Inseminated after storage for 4 hours at 15°C (B)</td>
<td>485</td>
<td>66AB</td>
</tr>
<tr>
<td>Collected at 15°C, cooled over an hour to 8°C, stored at 8°C for 3 hours, and inseminated at 15°C (C)</td>
<td>484</td>
<td>47D</td>
</tr>
<tr>
<td>Collected at 15°C, diluted 1:1 in monosodium glutamate diluent and treated as in treatment C (D)</td>
<td>374</td>
<td>43D</td>
</tr>
<tr>
<td>Collected at 15°C, diluted 1:1 in serum-glutamate diluent and treated as in treatment C (E)</td>
<td>428</td>
<td>51CD</td>
</tr>
<tr>
<td>Collected at 15°C, diluted 1:1 in yolk-glutamate diluent and treated as in treatment C (F)</td>
<td>362</td>
<td>62BC</td>
</tr>
</tbody>
</table>

* Mean Percent Fertility of 6 weeks.

( ) Treatments

Fertility means followed by different letters are significantly different at P<.01.

4 hours at 15°C and the fertility of semen diluted in yolk glutamate diluent (F) gradually cooled from 15°C to 8°C (over an hour) and stored for 3 hours at 8°C. The fertility obtained with semen stored in serum-glutamate diluent (E) did not vary significantly from the fertility obtained with semen stored in yolk glutamate diluent (F). The results (Table 10) indicate that gradual lowering of temperature from 15°C to 8°C does not prevent the damage to the glycolytic and oxidative mechanism in turkey spermatozoa.
spermatozoa. The data (Table 10) also show that whereas mono-
sodium glutamate and serum-glutamate diluents do not have a pro-
tective action against temperature shock, yolk-glutamate diluent
prevents temperature shock.

Discussion

Fertility obtained with undiluted semen (64 percent), with
semen diluted in monosodium glutamate solution (71 percent), and
with semen diluted in glutamate bicarbonate solution (61 percent)
suggested that the fertilizing capacity of turkey spermatozoa was
not affected by bicarbonate (Table 3) and hence it could be used
as a source to provide carbon dioxide for carbon dioxide diluents.
Studies conducted with carbon dioxide diluents composed of gluta-
mate and glutamic acid (Table 5) suggested that storage of turkey
semen in carbon dioxide diluents would result in a significant
loss in fertility. Harris et al. (1963) reported successful
storage of turkey semen for 6 hours in carbon dioxide diluents
composed of citric acid, sodium bicarbonate, and sodium citrate.
However, the results obtained in the present investigation with
semen diluted in Harris' diluent (Table 6) show that the ferti-
lizing capacity of turkey spermatozoa is significantly decreased
both before and after storage. There was a definite indication
in Trial 1 that 300 mg percent of glucose had a beneficial effect
on the fertility of turkey semen. Although in Trial 2 the differ-
ences in fertility obtained with different levels of glucose (37.5
mg percent, 75 mg percent, 150 mg percent, and 300 mg percent)
were not significant, the mean percent fertility of semen diluted in 300 mg percent glucose glutamate diluent was 5 percent higher than the fertility obtained with undiluted semen (Table 7). Schindler and Nevo (1962) reported "that the motility of fowl and bull spermatozoa is aerobic obligatory in the absence of a reducing sugar in the medium." Thus it is suggested that addition of a reducing sugar such as glucose in amounts of 300 mg percent to a turkey semen diluent should improve the fertilizing capacity of turkey spermatozoa. The temperature studies reported earlier indicated that turkey semen could be stored without any significant loss in fertility for 2 hours at 15°C. The data presented in Table 5, Table 8, and Table 10 suggest that it is possible to store undiluted semen (turkey) without any significant loss in fertility up to 4 to 4.5 hours.

There was no significant difference between the fertility (66 percent) obtained with undiluted semen stored for 4 hours at 15°C and the fertility (62 percent) obtained with semen gradually cooled over an hour from 15°C to 8°C and then stored for 3 hours at 8°C in egg-yolk glutamate diluent. On the other hand, there was a significant difference between the fertility (66 percent) obtained with semen stored for 4 hours at 15°C and the fertility (47 percent) obtained with semen gradually cooled over an hour from 15°C to 8°C and stored for 3 hours at 8°C in glutamate diluent (Table 10). Thus it appears that inactivated and adsorbed egg-yolk in the egg-yolk glutamate diluent had a protective action on turkey spermatozoa. From the data presented in Table 8, it is
also evident that dilution of turkey semen after storage for 4 hours at 15°C in either of the glutamate or sodium chloride diluents results in a loss in fertility. The latter results (Table 8) suggest that some unknown factor or factors are also needed besides the sources of energy such as glucose and glutamic acid to prevent loss in fertilizing capacity of the turkey spermatozoa. However, as inactivated and adsorbed egg yolk seems to preserve fertility fairly well even at temperatures below 15°C (8°C), it is likely that the unknown factors necessary for preservation of the fertilizing capacity of turkey semen are present in the egg yolk. On the other hand as turkey semen was used to adsorb the egg yolk it is possible that the seminal plasma which remains behind as supernatant provides the unknown factors while egg yolk performs the protective function. Based on these results it seems logical to suggest that glutamate diluents consisting of glucose (300 mg percent), magnesium chloride (22.8 mg percent), and inactivated plus adsorbed egg yolk should prove useful in storing turkey spermatozoa for longer periods at temperatures below 15°C.
SPERMATOZOAL ANTIBODIES AND THEIR RELATIVE ROLE IN FERTILITY IN TURKEYS

It is well known that the fertility of turkey hens declines after a few weeks. Domestication, intensive selection, and use of rigorous artificial insemination programs could be jointly responsible for this drop in fertility. Although the cause cannot be pinpointed, previous reports by McCartney (1923), Lamoreux (1940), and Wentworth and Mellen (1964) in fowl suggest that this could be due to a build-up of high serum anti-spermatozoa titers. Thus, experiments were designed to study spermatozoal antibodies in turkey hens, and if they are produced to study their effect on fertility.

Experimental and Results

Direct agglutination

Thirty-two virgin (Large White) hens (28 weeks old) were housed in cages and were brought into production by providing artificial illumination for 16 hours per 24-hour day. Blood was obtained from the wing vein (vena humera profunda) on November 27, December 4, and December 12, 1962. Sera obtained from these blood samples were frozen for conducting direct agglutination tests (described by Kibrick et al., 1952, and Menge et al., 1962). Each hen was then inseminated with 0.02 ml of turkey semen. The eggs collected were set and checked for fertility after 7 days of
incubation. This was done to provide an estimate of fertility before the birds were subjected to four different treatments. Thus, each group of eight hens acted as its own control. Birds in Groups A, C, and D were inseminated biweekly with 0.02 ml of semen. Birds in Group B were inseminated 5 days a week with 0.02 ml of semen. Group C was injected intramuscular (I/M) with 0.02 ml of semen on alternate days nine times and then twice a week, for the duration of the experiment. Group D was injected with a crude nuclear extract of 0.02 ml semen on alternate days nine times and then twice a week for the duration of the experiment.

The crude nuclear extract was prepared as follows: Fresh turkey semen was suspended in a 2.2 M sucrose solution and was homogenized in a McSham Erway homogenizer. Each volume of semen was suspended in 15 volumes of sucrose solution. The homogenate was then centrifuged at approximately 36 to 38,000 R.C.F.'s in a centrifuge. The supernatant was drained off through a cheesecloth, and the residue was suspended in 0.175 M saline for purposes of injection.

Blood was collected once a week from the wing vein of the hens for the duration of the treatment period. Sera obtained were frozen and tested for sperm agglutinins by the modified gelatin-agglutination test (Menge et al., 1962). Three-tenths ml of serially diluted sera were added to 0.3 ml of a 5 percent gelatin solution in normal saline containing approximately 30 million/ml washed turkey spermatozoa. The spermatozoa were
washed twice with 15 volumes of normal saline to each original volume of turkey semen. The sera-sperm mixture was incubated in a water bath at 37°C for one hour, and then examined microscopically for agglutination of spermatozoa. Eggs from each hen were set weekly and checked for fertility as described earlier on page 25.

The data are presented in Table 11. None of the treatments rendered a hen completely infertile. Fertility was poor (70 percent) in Group A hens which served as controls. Hens in Group B which were inseminated 5 days a week with turkey semen laid an average of 94 percent fertile eggs. Percent fertile eggs laid by Group C (injected I/M with semen) dropped from 90 to 64 percent. The percent fertile eggs laid by Group D (injected I/M with nuclear extract of semen) declined from 95 to 42 percent.

The sera obtained before and after the treatment period showed the same amount of agglutination (1:32 to 1:64). Thus it is obvious that either turkey hens do not produce sperm agglutinins or the method of testing for the sperm agglutinins is inadequate. On the other hand, it is also probable that the antigen-antibody complex formed is not strong enough to keep the actively motile spermatozoa agglutinated. Kibrick et al. (1952) who originally designed the agglutination test used by Menge et al. (1962), added gelatin to increase the viscosity of 5 percent medium. Assuming the percent gelatin was probably not enough to inhibit the motility of turkey spermatozoa, the concentration of gelatin was
# TABLE 11

**Effect of repeated inseminations with turkey semen and I/M injections of turkey semen and nuclear extract of turkey spermatozoa on fertility in turkeys**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Percent Fertility in Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hens inseminated biweekly (A)</td>
<td>0* 72 76 72 82 77 56 78 67 72</td>
</tr>
<tr>
<td>Hens inseminated 5 days a week (B)</td>
<td>95 93 96 100 89 94 97 89 94</td>
</tr>
<tr>
<td>Hens injected I/M with turkey semen (C)</td>
<td>90 80 67 87 85 79 71 64 78</td>
</tr>
<tr>
<td>Hens injected I/M with nuclear extract of turkey semen (D)</td>
<td>95 70 92 79 67 79 54 42 72</td>
</tr>
</tbody>
</table>

*Week before treatment.*
increased to 10 percent. Increasing the concentration of gelatin to 10 percent did not increase sperm agglutination. However, the drop in percent fertile eggs laid by the hens injected with semen (Group C) and nuclear extract (Group D) of semen seemed to suggest the possibility that the lowering of fertility may be due to a phenomenon induced by injection of semen and nuclear extract of semen. Taking this into consideration attempts were made to develop a more reliable procedure for detecting circulating sperm antibodies in the turkey hens.

**Indirect agglutination**

Boyden (1951) and Fisher (1952) have reported adsorption of antigens to tannic-acid-treated erythrocytes and subsequent hemagglutination upon mixing the erythrocytes with specific antiserum. Since then the hemagglutination technique has been used with several viral and protein systems (Benedict and O'Brien, 1958; Carmichael and Sarkai, 1958; McKenna et al., 1958; Ross and Ginsberg, 1958; Scott et al., 1957; Stavitsky and Aruguilla, 1955; Garabedien and Syverton, 1960; and Brown et al., 1961). Since spermatozoa have been reported to be highly antigenic by several workers, the possibility of employing the hemagglutination technique for determining spermatozoal antibodies was considered logical. While work was being conducted at this laboratory, Ann Southam (1963) reported the successful use of hemagglutination technique for detecting human spermatozoal antibodies. Likewise, Wentworth and Mellen (1963) used the technique for detecting circulating antibodies to fowl spermatozoa in domestic hens. A
modified version of the methods described both by Wentworth and Mellen (1964) and Ann Southam (1963) was developed to study spermatozoal antibodies in the serum of turkey hens.

Blood samples were obtained from non-immunized turkey hens and actively immunized (with turkey spermatozoa) turkey hens. The serum was separated and stored in a frozen state.

Pooled semen samples were collected from a minimum of 10 to 12 Large White toms (Ohio Randombred) and kept frozen until use.

The frozen spermatozoa were thawed and suspended in two volumes of phosphate buffered saline at a pH of 7.2, containing 10 mg per liter of Tetrasodium-Ethylene-Diamino-Tetra-Acetate (EDTA). The suspended cells were homogenized for several minutes in a chilled glass McSham Erway homogenizer immersed in an ice-water bath. The suspension was then centrifuged for 10 minutes at 2,000 revolutions per minute in a clinical centrifuge and the supernatant fluid collected. The precipitate was again extracted as above and the sediment was discarded. The pooled supernates were then diluted with buffered saline at a pH of 6.4 to obtain a final dilution 1:200 spermatozoa antigen.

Sheep blood preserved in Alsevar's solution was obtained from Colorado Serum Company. It was not used when it was more than 10 days old. The cells were separated by gentle centrifuging, washed twice in normal saline and once in buffered saline at a pH of 7.2. For preparing tannic acid treated sheep erythrocytes, one volume of packed sheep red blood cells was mixed with
20 volumes of 1:20,000 tannic acid. This mixture was incubated at 37° for 10 minutes, centrifuged and washed twice with buffered saline at a pH of 7.2 and once with 0.15 M saline solution. Another portion of the washed packed cells was used for adsorbing the test sera and the remainder was suspended in 0.15 M saline solution to make a 2.5 percent solution of sheep red blood cells.

One volume of 2.5 percent tanned sheep red blood cells and two volumes of the test antigen (turkey semen extract) were mixed and incubated at room temperature for 30 minutes. The antigen coated sheep red blood cells were then washed twice with 0.15 M saline containing 0.5 percent normal rabbit serum (NRS) which had been inactivated (at 56°C for 30 minutes) and adsorbed previously with an equal volume of tanned and packed sheep red blood cells (SRBC). The tanned and sensitized sheep red blood cells were finally resuspended in NRS saline to make a concentration of 2.5 percent. These sensitized cells were stored at 5°C for subsequent use. Sensitized cells older than 5 to 7 days were discarded. The sensitized cells were washed in NRS every time before subsequent tests.

All test sera including the immune sera were inactivated at 56°C for 30 minutes. One part was tested as such and the other part was adsorbed twice with equal volumes of tanned and packed sheep erythrocytes for 4 hours each at room temperature. Normal rabbit sera were always used as negative controls and for making the saline diluents.
The hemagglutination test was conducted in the following manner: To 0.5 ml of serially diluted test sera were added a drop (0.05 ml) of tanned sensitized sheep red blood cells suspension. Serum controls contained tanned but non-sensitized 2.5 percent sheep red blood cells. Each test included inactivated and adsorbed normal rabbit serum as a negative control. In addition, the following controls were used: (1) test sera plus tannic acid coated erythrocytes in buffered saline; (2) diluent plus tannic acid coated erythrocytes in buffered saline, and (3) diluent plus antigen sensitized sheep red blood cells. The tubes containing the tests were thoroughly shaken and kept overnight in the refrigerator at 5°C and read the following morning. The tubes were again shaken and left at room temperatures for approximately 3 to 4 hours and read again. Stavitsky and Arguilla's (1955) criteria were used for reading the intensity of the reaction. All tests were discarded if inactivated and adsorbed normal rabbit serum showed a positive reaction or if the saline controls for the adsorbed test sera showed any reaction.

For immunizing the turkey hens, pooled semen samples were collected from Large White turkey toms (Ohio Randombred) and homogenized with an equal volume of Freund's adjuvant. Five Large White turkey hens (Ohio Randombred) which were in production (laying eggs) were injected subcutaneously weekly with homogenized turkey semen in Freund's adjuvant for 4 weeks. Two similar control turkey hens were injected only with Freund's adjuvant in a similar manner. An initial dose of 2 ml of turkey semen was injected in
each turkey hen followed by subsequent doses of 0.5 ml of turkey semen per hen per week. Thus, a total of 3.5 ml of turkey semen was injected in each of the five birds.

In all the tests conducted, the unadsorbed test sera always reacted with tannic acid coated erythrocytes and the hemagglutination titers of the unadsorbed test sera were always higher than the hemagglutination titers of adsorbed test sera (Table 12). The adsorbed test sera collected before immunization from immunized birds did not show any hemagglutination while the unadsorbed test sera showed hemagglutination titers from 1:6 to 1:24. The adsorbed test sera obtained from immunized birds did not show any hemagglutination in the two weeks following the initial injection of turkey semen, while the unadsorbed test sera obtained from the same hens showed hemagglutination titers of 1:2 to 1:64. Hemagglutination titers from 1:4 to 1:48 were observed in the adsorbed test sera obtained from immunized birds in the third week, while hemagglutination titers from 1:32 to 1:128 were observed in the unadsorbed test sera obtained from the same birds. In the fourth week following the initial immunizing injection of turkey semen the adsorbed test sera collected from the immunized hens showed hemagglutination titers from 1:48 to 1:256, while the unadsorbed test sera showed hemagglutination titers from 1:256 to 1:1024 (Table 12). The test sera hemagglutination titers in the non-immunized hens remained the same throughout the treatment period. The adsorbed test sera obtained before and after injection of
TABLE 12
Hemagglutination titers of sera collected from non-immunized hens and hens immunized with turkey semen

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Before Insemination</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immunized</td>
<td>1:24</td>
<td>1:8</td>
<td>1:32</td>
<td>1:8</td>
<td>1:32</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Immunized</td>
<td>1</td>
<td>1:6</td>
<td>-</td>
<td>1:16</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1:32</td>
<td>-</td>
<td>1:32</td>
<td>-</td>
<td>1:64</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>1:2</td>
<td>-</td>
<td>1:6</td>
</tr>
<tr>
<td>4</td>
<td>1:24</td>
<td>-</td>
<td>1:16</td>
<td>-</td>
<td>1:32</td>
</tr>
<tr>
<td>5</td>
<td>1:6</td>
<td>-</td>
<td>1:16</td>
<td>-</td>
<td>1:32</td>
</tr>
</tbody>
</table>

Hemagglutination titers are averages of duplicate sera samples.

Unadsorbed test sera titers. (The unadsorbed test sera always gave agglutination with tannic acid coated erythrocytes.)

Sera samples adsorbed twice with equal volumes of tanned and packed sheep erythrocytes for 4 hours each at room temperature.
Freund's adjuvant in non-immunized hen 1 showed hemagglutination titer of 1:8 while the unadsorbed test sera showed hemagglutination titers from 1:24 to 1:32. The test sera obtained from non-immunized hen 2 did not show any hemagglutination for the entire duration of the investigation (Table 12). The results obtained with unadsorbed test sera suggest that non-specific antibodies against sheep erythrocytes are present in turkey sera, while the data obtained on the adsorbed test sera suggest that turkey hens are capable of producing circulating turkey sperm antibodies.

Discussion

Sera obtained from turkey hens immunized with turkey semen and nuclear extracts of turkey semen did not show any change in agglutination when the sera was tested for sperm agglutinins by the direct agglutination test. However, the drop in percent fertile eggs laid by the hens injected with turkey semen (26 percent group C) and nuclear extract of turkey semen (53 percent group D) suggested the possibility that the lowering of fertility may be due to a phenomenon induced by the injection of turkey semen and nuclear extract of turkey semen (Table 11). In other words the results seemed to suggest that the direct agglutination test was probably not sensitive enough to detect spermatozoal antibodies in the turkey hens. Since hemagglutination technique has been used in the past with several viral and protein systems it was considered desirable to develop a hemagglutination test for detecting spermatozoal circulating antibodies in turkey hens.
While work was being conducted in this laboratory, Wentworth and Mellen (1963) using the hemagglutination technique reported highly significant correlations between serum titer and duration of fertility in White Plymouth Rock hens insemination repeatedly with fowl semen. The data presented in Table 12 show that unadsorbed test sera obtained from non-immunized and immunized birds not only showed high hemagglutination titers with antigen sensitized sheep erythrocytes but also with tannic acid coated sheep erythrocytes. The results thus indicated that turkey sera have non-specific antibodies capable of agglutinating sheep erythrocytes. Wentworth and Mellen (1964) also reported that the hemagglutination test used by them "probably detects non-specific antibodies" along with the spermatozoal antibodies. Southam (1963) adsorbed human sera with equal volumes of packed and tanned sheep erythrocytes and reported successful use of hemagglutination technique for detecting spermatozoal antibodies (human) without the interference of non-specific antibodies. Results in Table 12 show that hemagglutination titers for circulating turkey sperm antibodies in turkey adsorbed sera can be detected by the hemagglutination technique. The hemagglutination titers of turkey sperm antibodies in the adsorbed test sera of the actively immunized turkey hens suggests that the process of appearance of the antibodies to be detected in appreciable amounts is a slow one. In this investigation taking approximately three weeks. The present investigation also indicates that turkey hens are capable of producing circulating turkey sperm antibodies.
Considering the amount of semen (3.5 ml) injected to produce circulating antibodies against turkey spermatozoa, the possibility of using the hemagglutination test to detect sperm antibodies in turkey hens receiving an insemination dose of 0.02 ml per week or two weeks seems questionable. However, the fact that one of the non-immunized hens (1) (which was in production at the time of the investigation as well as fertile) gave hemagglutination titer of 1:8 before and after the injection of Freund's adjuvant suggests that spermatozoal antibodies in the sera of hens in production receiving normal insemination doses can be detected.

Thus further studies on adsorbed test sera obtained from low fertility and high fertility hens are advocated before the present hemagglutination test can be used to interpret whether circulating turkey spermatozoal antibodies cause lowering of fertility in turkey hens.
SUMMARY AND CONCLUSIONS

In the recent years low fertility has become one of the most serious problems of the turkey industry. The factor or factors affecting the fertility of turkey semen could be associated either with the males, the females, with the insemination techniques, or with any combination of these. Thus, it was considered that attempts should be made, not only to develop better techniques of handling and storing semen, but also to determine the possible causes of low fertility in turkey hens regardless of inseminating procedures. Hence, some of the many physiological and immunological factors affecting the fertilizing capacity of turkey spermatozoa were studied:

The following investigations were conducted:

1. Effect of different temperatures on metabolic activity, morphology, and fertilizing capacity of turkey spermatozoa.

2. Effect of turkey semen diluents on the fertilizing capacity of turkey semen were explored by the following procedures:
   a. Addition of sodium bicarbonate to a glutamate diluent.
   b. Carbon dioxide diluents.
   c. Diluents containing glucose.
   d. Reactivation in diluents containing sodium chloride, and monosodium glutamate.
e. Diluents containing inactivated and adsorbed egg yolk and serum.


The temperature studies indicated that the optimum temperature for collection and storage of turkey semen for periods longer than 60 minutes was 15°C. It was observed that the fertility of semen stored at 15°C dropped by only 6 percent (Table 2) over a period of 120 minutes. This statistically insignificant drop is probably due to a gradual depletion of energy sources and oxygen in the seminal plasma accompanied with a gradual accumulation of lactic acid and carbon dioxide which, in turn, have some deleterious effect on the fertilizing capacity of turkey spermatozoa. Thus, it is suggested that diluents which would dilute the toxic substances as well as supply adequate amounts of energy sources such as glucose and glutamic acid may prove useful in maintaining the fertilizing capacity of turkey spermatozoa during storage at 15°C.

The decrease in fertilizing capacity of turkey spermatozoa at storage temperatures above 15°C is probably due to rapid anaerobic glycolysis, build-up of lactic acid in toxic amounts, exhaustion of the sugar supply necessary for maintaining the oxidative cycle and/or rapid bacterial growth which in turn interferes with the sperm metabolism. Thus, it is suggested that diluents which would inhibit the metabolic rate as well as control the growth of bacteria at temperatures above 15°C may prove useful in storing turkey semen at room temperatures.
The decrease in fertilizing capacity of turkey spermatozoa at temperatures below 15°C is probably due to irreversible damage to either the glycolytic mechanisms or the enzymatic systems (transaminases) or some other substance essential for both the glycolytic and aerobic mechanisms. Thus, it is suggested that if ways can be found to prevent temperature shock, lower temperatures should result in a lower rate of metabolism and make longer periods of storage possible.

It was also found that the metabolic processes of turkey spermatozoa are damaged within 10 minutes of exposure to temperatures below 15°C and that it requires a storage period of at least 120 minutes to show significant differences in the number of bent spermatozoa.

Of the types of diluents which were studied it was observed that diluents composed of glutamate, glucose, magnesium chloride, and inactivated and adsorbed egg yolk may prove useful in storing semen for longer periods of time at temperatures lower than 15°C. The fertilizing capacity (66 percent) of undiluted turkey spermatozoa stored for 4 hours at 15°C was not statistically different from the 62 percent obtained from semen stored in yolk-glutamate diluent at 8°C for 3 hours after being gradually cooled from 15°C to 8°C over an hour. Regardless of the treatment used, the decline in fertility of undiluted semen was not statistically significant over a storage period of 4 to 4.5 hours at 15°C (Table 5, Table 8, and Table 10).
The fertilizing capacity of turkey spermatozoa was not affected by adding sodium bicarbonate to a monosodium glutamate solution (isosmotic) and hence it was used as a source of carbon dioxide in diluents. Results obtained with carbon dioxide diluents indicated that it was not possible to store turkey semen in carbon dioxide diluents without a significant loss in the fertilizing capacity of turkey spermatozoa (Table 5 and Table 6).

Addition of glucose (300 mg percent) to monosodium glutamate solution (isosmotic) had a beneficial effect on fertility of turkey semen. However, it was found that when stored semen (for 4 hours at 15°C) was diluted in diluents (isosmotic) composed of sodium chloride, glucose and magnesium chloride, and monosodium glutamate, glucose and magnesium chloride, the diluents failed to maintain the fertilizing capacity. Thus it is suggested that some unknown factor or factors is/are needed besides the sources of energy supplied by the sodium chloride and glutamate diluents to prevent a loss in the fertility of turkey semen after storage.

Intramuscular injections of turkey semen and nuclear extracts of turkey semen in turkey hens brought about a drop of 26 and 53 percent fertility over a period of 7 weeks. However, the direct agglutination test on the sera of these birds failed to show any circulating spermatozoal antibodies. As the direct agglutination test showed the same amount of agglutination before and after the treatment period, it seems that the antigen-antibody complex formed in the test is not strong enough to keep the actively motile spermatozoa agglutinated. An indirect reliable
hemagglutination test was developed for detecting circulating turkey spermatozoal antibodies in the sera of turkey hens. Results indicate that sperm antibodies can be produced in turkey hens by active immunization with turkey semen. Hence, it is suggested that sera from low fertility and high fertility flocks of turkeys should be tested to determine whether any relationship exists between circulating sperm antibodies and fertility in turkey hens.
BIBLIOGRAPHY


