EFFECT OF ANTI-EGG-YOLK-DILUENT SERA UPON BOVINE SPERMATOZOA IN EGG YOLK DILUENT

by

Michael L. O'Connor

Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Dairy Science

APPROVED:

R. G. Saacke, Chairman

J. A. Lineweaver

N. R. Krieg

R. G. Cragle

J. M. White

August, 1974
Blacksburg, Virginia

ACKNOWLEDGEMENTS

I welcome this opportunity to offer my deepest appreciation to . As my graduate advisor, provided excellent example, sound advice and constructive criticism. He always encouraged me and other students to think for ourselves, to rely on common sense and strive for improvement. As a friend he continually showed genuine concern, patience and extreme unselfishness. For such intangibles I thank him sincerely.

I extend my appreciation to everyone associated with the Department of Dairy Science and especially for providing such an informal yet professional environment for me to conduct my graduate program.

I would like to extend appreciation to the following individuals:

for his valuable advice and assistance in the technical aspects of this research.

and

for their advice, assistance and their critical evaluation of this research.

My fellow but older graduate students;

and for their help and valuable criticism based on time gained experience.

for her fine work on the graphical illustrations.

for her laboratory assistance.

for her concern, cheerfulness and continual patience in the typing of this manuscript.

Finally, I wish to sincerely thank my parents for allowing me the freedom to pursue my own interests and furthermore, for their encouragement and concern throughout my college career.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In order that artificial breeding may achieve or surpass the conception rates obtained from natural service, many of the semen preservation and breeding techniques must be further perfected. Since it is often difficult to accurately monitor the reproductive status of the female with regard to onset of estrus and subsequent ovulation, in a management situation, the preservation and insemination of a more viable dose of male germ plasm is essential for optimum fertility.

Important to semen preservation is the composition of the diluent. Several acceptable semen diluents have been developed and are currently used in artificial breeding. Such diluents in conjunction with proper handling have resulted in conception approaching that obtained from natural service. In addition to providing an optimum environment for spermatozoa through dilution, cooling and the freeze-thaw process, the ingredients within the diluent must be physiologically and immunologically compatible with tissues of the female reproductive system. This area of immunological incompatibility has been suggested as one cause of subfertility in cattle. The standard semen diluents contain high concentrations of potential antigens such as protein, glycoprotein and lipoproteins. Edwards (1960) demonstrated that egg yolk used in some standard diluents is antigenic. It has recently been shown that cattle possessing uterine titers of antibodies against egg yolk, when inseminated with egg yolk diluted semen, had a significantly lower fertility than animals which had no uterine titers (Griffin et al, 1971). These findings suggested that an immune response to the semen diluent im-

paired fertility. The dimensions of this problem have not yet been determined.

The objectives of this investigation were as follows:

(1) to determine if the antigenicity of egg yolk diluter could be eliminated by interaction with specific antibodies induced against such a diluter.

(2) to examine the effect of immune sera against egg yolk diluter upon the viability of bovine spermatozoa in that diluter.

REVIEW OF RELATED LITERATURE

Criterion used in establishing that an immune response exists.

The study of the effect of the immune response upon reproduction is a relatively new area of research. Numerous reviews (Katsh 1959, Tyler 1961, Katsh and Katsh 1965, Tyler et al 1967, Edwards 1970, Bratanov 1972) have summarized the information concerning immunoreproduction. Antigenic substances and their subsequent effect upon reproductive processes are quite varied. Furthermore, species differences exist in both the type and degree of response to antigens. The major reproductive antigens investigated have been: spermatozoa, seminal plasma, ova, conceptus, female genital fluids, hormones and semen diluents. Each antigen is unique and should be discussed separately. Whether the specific antibodies induced by these antigens interfere with the fertilization process or affect the embryo is still questionable.

The various immunological methods used to measure antibody titer include: agglutination, precipitation, gel diffusion, complement-fixation test, skin allergic test, immunoelectrophoresis, and immunofluorescence. Such tests may be qualitative only or qualitative and quantitative. Classically, sperm immobilization, agglutination and changes in the metabolic characteristics of sperm such as rate of respiration have been the physiological criterion used to study effects of the immune response.

Immobilization is simply the loss of sperm motility. Immobilization is probably caused by interaction of free immobilizing antibody with intact sperm cells (Baum et al, 1961). This antibody requires serum com-

plement to express its fullest activity. However, immobilization is not a specific action limited to serum antibodies. Immobilization is occasionally accompanied by sperm agglutination; nevertheless, agglutination is not a prerequisite to immobilization (Chang, 1947).

Agglutination of spermatozoa can be induced by certain physical and chemical agents and also by immunological reactions. Mann (1964) reviewed the various physico-chemical factors which cause agglutination. These factors such as dilution, washing, cold shock, pH change or certain osmotic conditions generally cause non-specific sperm agglutination. Moyer and Almquist (1962) reported that head to head agglutination occurred in skimmilk diluters containing glucose and sodium citrate. Agglutination increased as sodium citrate concentration decreased from 2.0 to 1.6 percent and diluter pH increased from 6.8 to 7.2. Incorporation of glucose reduced the amount of agglutination. Female genital secretions caused non-specific sperm agglutination. Olds and Van Demark (1957) and Peterson (1965) observed that genital fluids, especially follicular fluid, frequently caused head agglutination of bull spermatozoa. Edwards (1960) reported that washings from the reproductive tract of female rabbits caused head to head agglutination of homologous semen in vitro. Thus there are a variety of physico-chemical agents causing sperm agglutination.

This review will consider the immunological causes of sperm agglutination, specifically sperm agglutinins. Agglutination induced by immune reactions is of a specific nature. Numerous investigators have observed sperm agglutination but have failed to characterize the type

of agglutination whether it was head to head, tail to tail or mixed agglutination. Henle et al (1938) clearly distinguished the difference between head and tail agglutination. They described the distribution of antigens in spermatozoal heads and tails which they separated by sonic vibration and differential centrifugation. Rabbits were immunized against suspensions of whole bull spermatozoa, spermatozoal heads, and spermatozoal tails. When mixed with whole bull spermatozoa in hanging drop preparations, the anti-head sera produced head agglutination, the anti-tail sera produced tail agglutination. The antisera to whole spermatozoa caused mixed agglutination in "networks and strings". When sera prepared against whole spermatozoa were absorbed with spermatozoal heads, tail agglutination resulted; after absorption with spermatozoal tails, head agglutination was observed. Head agglutination was seen in both immune sera and normal sera, whereas tail agglutination could only be produced by the sera of animals immunized against spermatozoa. It should be noted, however, that other antisera, i.e. that produced against seminal plasma, might cause a different type of agglutination.

Alterations in metabolism of spermatozoa when exposed to immune sera is another criterion used to evaluate the effect of the immune response upon reproduction. Matousek (1964) determined that fructolysis in ejaculated bull spermatozoa was inhibited by immune sera produced against seminal and epididymal sperm. Mittal et al (1965) found that spermatozoal death rather than changes in the metabolic rates

caused a reduction in respiration and glycolysis in bull spermatozoa incubated with antisemen serum. Ackerman (1967) demonstrated that the naturally occurring or immune ABO iso-antibodies reduced glycolysis in sperm of humans known to carry AB(H) antigens. Utilizing rabbit spermatozoa, Ackerman (1969) found that in immune sera, glycolysis and respiration were depressed proportionately to serum antibody titer. In oviductal fluid of low titer, glycolysis was not affected, but sperm respiration was depressed. At present, it has not been determined by what mechanism immune sera depresses metabolism.

Current conclusions on the antigenicity of bovine spermatozoa and seminal plasma. Antigenicity of spermatozoa was first observed by Landsteiner (1899) when he demonstrated that the activity of bull spermatozoa was reduced after injection into the peritoneal cavity of guinea-pigs which had previously been subjected to a series of parental injections of bull sperm. Metchnikoff (1899) injected guinea-pigs with semen or testicular tissue of man, bulls, guinea-pigs and rabbits and found that the serum of these guinea-pigs agglutinated and immobilized spermatozoa of these animals in-vitro. Since these initial experiments, the immune reactions in the reproductive tract involving antigens of spermatozoa and seminal plasma have been suspected as one cause of female infertility in both humans and animals. Comprehensive reviews have been compiled on this subject by Katsh, 1959; Tyler, 1961; Edwards, 1970 and Menge, 1970a. It is worthwhile elaborating upon some of the investigations contained within these reviews because they provide pertinent background for this investigation.

Antibodies to the semen of many species have been found after iso-immunization of the female with semen or testicular tissue. Kiddy et al (1959) failed to induce infertility in heifers by systemic injections of bull blood. However iso-immunization of heifers via intradermal and intra-uterine routes of immunization with bull semen and homogenized testis combined with Freunds complete adjuvant caused temporary infertility (Menge, 1967). The intradermal route appeared to inhibit fertility more than the intra-uterine injection. A negative relationship existed between serum titer of sperm agglutinins and conception. Results from these two experiments illustrate the antibody-enhancing effect of adjuvant. It can be concluded that, in cattle, chemical adjuvants must be incorporated with the semen or no immunization will occur.

In-vitro treatment of semen with specific antiserum prior to insemination has resulted in fertilization failure or embryo loss in some species. In rabbits, exposure of spermatozoa to cattle antiserum formed against rabbit semen inhibited fertilization and induced embryo mortality (Kiddy et al, 1959). High concentrations of the immune sera caused tail to tail agglutination whereas normal sera induced head to head agglutination. Menge et al (1962b) inseminated heifers with semen treated with homologous antisemen serum and produced an antifertility effect. In a summary of investigations, Menge (1970) reported the effect of antisera against various antigenic materials on the fertilizing ability of rabbit and bull spermatozoa. Only antisera specific against materials that contained spermatozoa, that is, mature testis, semen and epididymal spermatozoa, induced antifertility effects. Such antisera

possessed high titers of sperm agglutinins. However, it must be noted that the presence of sperm agglutinins in antiserum was not entirely responsible for the antifertility effect since antisera against seminal plasma, conceptus and brain tissue induced sperm agglutinins. Furthermore, an antifertility effect on rabbit and bull spermatozoa and presence of sperm agglutinins was found in cervical mucus of heifers immunized via the uterus utilizing adjuvant.

These previous referenced experiments have dealt with iso-immunization of females with semen and the in-vitro exposure of semen to immune sera or genital fluids. Investigations have been conducted in humans and cattle without using experimental immunization to determine if a relationship existed between sperm agglutinating or immobilizing antibodies and fertility. Bratanov and Dikov (1959) reported higher sperm agglutination titers in repeat breeder cows than in normal cows. It was observed that cows that failed to conceive when inseminated with semen from bulls producing a high agglutination titer could conceive when inseminated with semen from males giving a low titer. Similarly, Swanson (1967) reported that blood serum from repeat breeder cows agglutinated and immobilized sperm of bulls to which these cows had been bred previously more often than sperm from bulls they had been bred to for the first time. However, agglutinating antibodies were present in cows of both good and poor breeding efficiency. Swanson concluded that poor breeding efficiency was not entirely caused by circulating sperm agglutinins or immobilizing antibody. Griffin et al (1971) found an extremely low incidence of titers to homologous semen

(2.5%) in cattle suggesting that, under natural conditions, high titers of anti-semen antibodies are not frequently found in the uterus.

Semen is an antigenically complex substance containing spermatozoa and seminal secretions. Some antigens of ejaculated mammalian semen are not components of sperm, but are products of the seminal plasma. Using immunofluorescence, Weil and Rodenburg (1962) postulated that a sperm coating antigen (SCA) was produced in the seminal vesicles and coated the sperm. Hunter and Hafs (1964) found seven antigens in bull spermatozoa, with five antigens being shared with bull seminal plasma. Experiments by Menge and Protzman (1967) clarified which antigens were involved in impaired fertility by specific antibody. They concluded that "intrinsic" sperm antigens and not the sperm-coating antigens, were responsible for induced infertility. In addition, both anti-whole semen sera and anti-seminal plasma sera agglutinated sperm, but only antibody against whole semen affected fertility.

Utilizing papain-digested univalent guinea-pig antibodies against whole rabbit semen as a method of avoiding agglutination, Metz and Anika (1970) found an inhibition in fertility when such antibodies were used to pre-treat the semen before insemination. It may be concluded from this discussion that the infertility effect upon spermatozoa results from blocking essential antigens and not by agglutination per se. Furthermore, antibodies against seminal plasma do not cause an antifertility effect but, if sperm specific antibodies are present in the female, infertility might result, although such a condition is probably rare.

It should be noted, however, that seminal plasma does exhibit anti-

agglutinating properties (Lindahl and Kihlstrom, 1954a). This "male antagglutin" is a non-dialysable conjugated protein component of the seminal plasma of cattle, horses, swine, rabbits and man. Lindahl et al (1956) stated that the protein moiety is attached to an active group composed of a carbohydrate, a sulphuric acid residue and a carboxylic acid residue. Its action is nonspecies-specific. Head to head agglutination of spermatozoa was negated by addition of the seminal plasma component. Female antagglutin is a non-protein constituent found in various female genital tract secretions such as follicular fluid and cervical mucus, however, its action is similar to "male antagglutin" (Lindahl, 1960).

Another important aspect to consider concerning antigenicity of spermatozoa is aspermatogenesis within the male. Suppression of spermatogenesis has been obtained in some species of laboratory animals. Bratanov et al (1964) found sperm auto-immunization in bulls after provoking an inflammation in the region of the testicles or after ligation of the spermatic duct. Evidence indicates that aspermatogenesis is a form of delayed hypersensitivity transferable by means of immunologically competent cells, but not by serum. The auto-antigen resides in the spermatozoa or spermatogenic cells. Katsh and Bishop (1958) describe the histological and physiological effects of induced aspermatogenesis in guinea pigs. Aspermatogenesis is a very rare autoimmune disease, but as an experimental model it does illustrate the antigenicity of spermatozoa.

Antigenicity of Semen Diluents. In artificial insemination, the

female reproductive tract is exposed not only to the components of semen but also those of the semen diluent. Such diluents often contain high concentrations of protein, glycoproteins, lipoproteins, and antibiotics, all of which are potentially antigenic. Edwards (1960) investigated the antigenicity of egg-yolk diluter in rabbits. After intramuscular immunization utilizing adjuvant, antibodies induced against egg yolk, bull seminal plasma and bull spermatozoa were found in both the systemic circulation and reproductive tract of these rabbits. Of more significance were the results obtained after artificial insemination. Repeated artificial insemination of non-pregnant rabbits with bull semen in egg yolk citrate induced serum antibodies against egg yolk, bull seminal plasma and spermatozoa in three of four rabbits, and vaginal antibodies against egg yolk and seminal plasma were present in two of three rabbits. Furthermore, it was noticed that washings from the genital tracts of both untreated and artificially inseminated rabbits induced head agglutination.

Swanson and Hunter (1969) characterized the protein components of egg-yolk using agar-gel electrophoresis and found three major protein zones. When the egg-yolk diluter was reacted with anti-egg yolk citrate immune sera, eight glycoproteins and one glycolipoprotein antigen appeared. In-vitro exposure of yolk citrate diluted homologous spermatozoa with normal sera and egg yolk immune sera failed to affect the percentage of motile spermatozoa. Immune sera formed clumps of egg yolk lipid globules but the cells were not immobilized or agglutinated by such clumps. Fertility experiments indicated that systemically

produced antibodies to egg yolk did not affect fertility of rabbits when mixed with sperm suspended in egg-yolk diluters. However, Hunter and Alsum (1969) reported that repeated artificial insemination of rabbits with egg-yolk citrate minus sperm 2x/week for five weeks produced circulating antibodies against three of the nine potential antigens. When this series of treatments was followed by insemination with egg-yolk citrate diluted semen, fertility was impaired. Uterine strips from these rabbits were suspended in mammalian Ringers solution in a smooth muscle chamber. Upon addition of egg yolk citrate to this solution, a heightened series of uterine contractions were observed in three of seven rabbits. This suggests that repeated insemination with egg yolk induced uterine hypersensitivity resulting in an anaphylactic reaction when challenged with egg yolk in the test solution.

Nedyalkov and Stoyanova (1966) reported that an allergy developed against egg yolk in 80% of the cows failing to conceive after two or more inseminations with glucose-yolk-citrate diluted bull semen. After removal of egg yolk from the diluent, conception improved.

Since anti-egg yolk rabbit sera did not reduce fertility of egg yolk diluted semen when added to the semen before insemination, Swanson and Hunter (1969) proposed that antibodies within the uterus may interfere with fertility. Griffin et al (1971) investigated the relationship between genital tract antibody titers to egg yolk and breeding efficiency in dairy cattle. The frequency of appearance of antibodies to egg yolk in the vagina, uterus and serum increased with the number of services the cows received, higher titers found in animals served four

or more times. Fourteen of twenty-three cows had titers which could not be explained by transport of humoral antibody into the tissues of the reproductive tract, suggesting the possible production of local antibody by the genital tissue after exposure to egg-yolk diluter. Fertility of cows possessing uterine titers to egg yolk was significantly lower than that of cows without such antibodies. From these results, it appears that the presence of yolk antibodies in the cow genital tract adversely affects fertility. In-vitro tests utilizing agglutination and immobilization as criteria were conducted to determine the effect of egg yolk antibodies on semen used in the fertility experiment. Samples with titers to diluent antigens caused mixed agglutination of spermatozoa stored in egg-yolk diluent, but only non-specific head to head agglutination occurred in semen stored in saline. Immobilization of spermatozoa was not evident in either saline or egg-yolk diluted semen. Bratanov et al (1968) reported similar types of agglutination with immune sera from rabbits immunized with bovine semen diluted in egg-yolk. Such observations suggest that egg-yolk antigens alter the sperm cell surface chemistry so that in the presence of specific yolk antibodies the agglutination pattern described above will occur.

Effect of Normal Serum upon Spermatozoa. In addition to the effects produced upon spermatozoa by immune sera and genital fluids containing antibodies as reviewed above, normal blood serum causes non-specific head agglutination and immobilization of spermatozoa (Chang, 1947). Mann (1964) reviewed the early attempts to use blood serum as a diluent for semen of many species.

Chang (1947) has critically evaluated the non-specific effects of sera on spermatozoa. He utilized the degree of sperm immobilization to show the strength of the spermicidal effect of serum in various species. Pure rabbit serum not only immobilized the spermatozoa but actually killed them because, upon resuspension after centrifugation in saline or Tyrodes' solution, immobilization was not reversed. The spermicidal property of serum was lost after storage at room temperature or at 4°C for 4 to 7 days or by heating the fresh serum at 56°C for 5 to 10 min. The spermicidal activity of serum is limited in that it can be exhausted by a certain number of spermatozoa. In addition to storage and heat liability, sodium citrate destroyed the spermicidal factor in the sera. Many similarities existed between this factor and the complement component of serum, however, whether this spermicidal factor is identical with complement is still questionable. Since this factor is so unstable and can be used up by small numbers of sperm, Chang suggested this as the reason why previous investigators failed to notice spermicidal effects on spermatozoa. Agglutination of spermatozoa accompanied immobilization in all the serum preparations investigated, however, agglutination was not implicated as the cause of the spermicidal effects. The agglutination is thought to be a general reaction between spermatozoa and a colloidal substance such as protein.

Various conflicting reports have been made on the thermolability of the agglutinating factor in normal serum. It was observed that the agglutinating action of serum from the cow (Berstein and Lazarev, 1933), turkey (Van Tienhoven and Steel, 1956) and golden hamster (Barros *et al*,

1971) is destroyed by preheating (50-60°C for 30 min to 1 hr). Barros et al (1971) did report, however, that heating serum to 56°C for 1 hr was necessary for serum to induce the acrosome reaction in epididymal spermatozoa. Yanagimachi (1970) observed that preheated (56°C/1 hr) homologous and heterologous serum caused prominent head to head agglutination and subsequent increase in motility of hamster spermatozoa as well as induced the acrosome reaction. He suggested that the albumin fraction of serum is responsible for head to head agglutination. Senger and Saacke (1973) reported that the addition of heat treated (56°C/1 hr) female serum to bull spermatozoa resulted in head to head agglutination which had a beneficial effect on acrosomal maintenance in vitro. Senger (1974) observed similar agglutination when homologous heated serum was added pre-freeze and post-thaw to bull spermatozoa. Agglutinated cells resisted acrosomal deterioration much better than single cells or controls not exposed to serum. Furthermore, agglutinated cells appeared to remain motile for a longer period of time than single cells.

There is no apparent reason for these conflicting results on the heat lability of the agglutinating factor in normal serum. However, using the criterion of motility and acrosomal maintenance, head to head sperm agglutination is not as detrimental as once believed.

EXPERIMENTAL PROCEDURE

The following experiments were designed 1) to determine if the antigenicity of egg yolk diluter could be eliminated by interaction with specific antibodies induced against such a diluter and 2) to examine the effect of immune sera against egg yolk diluter upon the viability of bovine spermatozoa in that diluter.

It was necessary to immunize donor animals and determine the subsequent antibody titer of the serum before the critical experiments could be conducted.

Immunization

Two Holstein virgin heifers were immunized with yolk-citrate-glycerol diluter. Normal sera was obtained from both animals before the immunizations were begun. The composition of the antigenic diluter was as follows:

7 ml glycerol
20 ml fresh egg yolk
73 ml 2.9% sodium citrate
1000 ug dihydro streptomycin sulfate per ml of diluent
1000 units potassium penicillin G per ml of diluent

The first heifer (#728) was immunized via intravenous injection of 2.5 cc of diluter every other day for three weeks. At the end of four weeks, the heifer was bled to determine the antibody titer. The same method and schedule of injections were used to induce an amamnestic response increasing the titer from 1:160 to 1:640.

The second heifer (#736) was immunized subcutaneously at 10 sites (5.0 ml at each site) in the neck and flank areas. A mixture of equal volumes of Freunds complete adjuvant and egg yolk diluter were used to

produce the primary immunization. Again it was necessary to induce an amamnestic response to increase the titer from 1:320 to 1:2560. This was accomplished by reinjecting the heifer with a mixture of equal volumes of incomplete adjuvant and egg yolk diluter at 10 sites (2.5 ml of each site).

Blood was collected via jugular puncture and allowed to clot at room temperature for 2 hrs. Blood samples were centrifuged at 12,100 g for 10 min. Serum was pipetted into 12-ml aliquots and stored at -20°C until use.

Determination of Antibody Titer

Serum samples were tested to determine the antibody titer against egg yolk diluent antigens. An aliquot of each serum sample was thawed and complement inactivated at 56°C for 30 minutes. Since the complement component of mammalian sera when bound in antigen-antibody complex has lytic activity of cellular membranes, it is routinely heat inactivated.

The titer of antibody to egg yolk diluter was determined by the passive hemagglutination test (Boyden, 1951). Passive hemagglutination utilizes sheep erythrocytes as the carrier of the antigen (experimental egg yolk diluter). Sheep erythrocytes are washed three times with physiological saline (0.89% sodium chloride) and suspended in phosphate buffered saline pH 7.2 in a 2.5% concentration. An equal volume of a 1:20,000 dilution of tannic acid was added to the erythrocytes, mixed and allowed to incubate 10 min at 37°C. Tannic acid is used to alter the erythrocyte and reveal new protein receptors to which more antigen

molecules can be attached. The cells were centrifuged at 750 g for 5 min, washed with buffered saline pH 7.2. Seven ml of tanned cells and 7.0 ml of egg yolk diluter were added to 28 ml of buffered saline at pH 6.4. This solution was allowed to stand for 10 min at room temperature. The cells were centrifuged, washed once in 1:100 normal rabbit serum (NRS) and suspended in 7.0 ml of 1:100 NRS. The antigen coated erythrocytes were washed to remove excess (unbound) antigen. NRS was prepared by heating rabbit serum to 56°C for 30 min and exposed for 10 min at room temperature to an equal volume of washed sheep erythrocytes. This suspension was centrifuged, and the supernate diluted 1:100 with saline.

Antiserum is prepared and titrated as follows: Antiserum is heated for 30 min at 56°C to inactivate complement and absorbed with an equal volume of sheep erythrocytes. One-half ml aliquots of 1:100 NRS were pipetted to a series of fourteen agglutination tubes (12 x 75 mm). One-half ml of inactivated antiserum was added to the first tube and thoroughly mixed. From this solution, antiserum was titrated to the remaining tubes. After titration, 0.05 ml of antigen coated cells were added to each tube.

The following controls were included: (1) 7.0 ml of saline was substituted for egg yolk and incubated with tanned cells and then added to 0.5 ml 1:100 NRS (with antiserum); (2) 0.5 ml 1:100 NRS (no antiserum) plus 0.05 ml of tanned antigen-coated erythrocytes; (3) 0.5 ml 1:100 NRS (with negative sera) plus 0.05 ml of tanned antigen-coated erythrocytes. Negative serum is normal serum with no titer to egg yolk dilu-

ter. Antiserum titrations were always prepared in triplicate simultaneous serial dilutions so that results were based upon three observations.

Both experimental and control tubes were gently agitated and allowed to stand overnight. Tubes were then graded for agglutination. A positive result consisted of a compact granular agglutination or film of cells at the bottom of the tube. Negative tubes were evidenced by a ring or smooth button of cells at the bottom of the tube.

After titers were determined, the highest titered samples from each heifer were placed in 12-ml aliquots frozen and stored at -20°C until used in subsequent phases of the experiment. Stock quantities of three serum samples stored were as follows: negative serum 28-ml; serum titered 1:640 (from heifer #728), 440 ml; serum titered 1:2560 (from heifer #736), 320 ml.

Neutralization of Egg Yolk Antigens

The immunological technique of equivalent proportions (Burrell and Mascoli, 1970) was used to determine if antigens contained in egg yolk semen diluents could be neutralized with specific antibodies and what quantity of sera was necessary to neutralize the egg yolk diluent.

The procedure used in this technique of equivalent proportions is as follows: varying dilutions (1:1 through 1:256) of the highest titered antiserum (1:2560) were pipetted in 0.5 ml aliquots into a series of serological tubes (12 x 75 mm). To these tubes was added 0.5 ml of egg yolk antigen diluted 1:160. Antigen concentration remained constant and antibody concentration varied with serial dilutions. Again, antiserum titrations were prepared in triplicate simultaneous serial dilutions. Tubes were incubated for 1 hr at 37°C and continually ob-

served for the first appearance of a precipitate. At the end of 1 hr, tubes were refrigerated overnight and precipitates graded the next morning.

The tube exhibiting the maximum precipitate represents the point of equivalent proportion; i.e. the optimum ratio at which both antigen and antibody are fully complexed in the precipitate.

The following three series of tubes served as controls: 1) antiserum titered 1:2560 with no antigen added; 2) negative bovine serum with 0.5 ml of egg yolk antigen (diluted 1:160) added to each tube; 3) physiological saline with 0.5 ml of antigen (diluted 1:160) added to each tube.

Collection and Initial Evaluation of Semen

To conduct the second phase of this study, which was designed to evaluate the effect of immune serum against diluter upon the viability of bovine spermatozoa in diluted semen, semen was collected using an artifical vagina from bulls housed at Virginia Polytechnic Institute Dairy Center. Ejaculates used had the following minimal requirements: initial motility of 50% and sperm concentration greater than 750×10^6 cells per milliliter.

Initial estimations of motility were made by diluting a small amount of semen into a drop of 2.9% sodium citrate solution on a prewarmed microscope slide. Phase contrast microscopy was used to estimate motility to the nearest 10%. Spermatozoan concentrations were determined by diluting raw semen 1:80 with 2.9% sodium citrate solution and measuring light transmittancy using a Bausch and Lomb "Spectronic 20" photoclectric colormeter previously calibrated for bovine semen.

Dilution

Yolk-citrate-glycerol and skimmilk-glycerol were used as semen diluents. Controls consisted of skimmilk diluter with immune and non-immune sera and egg yolk diluter without sera. Since the immunoglobulins present in the immune sera samples were produced against egg yolk diluter, any specific effect these antibodies might have on spermatozoa would be observed in egg yolk treatments only, with non-specific effects of sera being apparent in both diluters. Any differences between diluters over and above the non-serum controls can be interpretated as due to specific antibodies against components of the egg yolk diluter.

The ingredients used in fraction "A" and "B" of the yolk-citrate-glycerol diluent were:

Fraction "A"

20 ml egg yolk
80 ml 2.9% sodium citrate

Fraction "B"

20 ml egg yolk
14 ml glycerol
66 ml 2.9% sodium citrate

The following ingredients were used in fraction "A" and "B" of the skimmilk diluent:

Fraction "A"

100 ml skimmilk

Fraction "B"

86 ml skimmilk
14 ml glycerol

Fraction "B" of both diluents contained potassium penicillin G (1000 units/ml of final diluter) dihydrostreptomycin sulfate (1000 ug/ml of final diluter).

Skimmilk was prepared from raw fresh whole cows milk obtained from bulk tank at VPI dairy center. Milk was centrifuged at 5900 g for 5 min and the butterfat removed. The skimmilk was placed in a covered beaker and immersed into a previously heated water bath and was heated to 85°C for 1 min. Skimmilk was cooled to room temperature and prepared as fraction A and B. Both diluters were prepared the day before semen collection and stored overnight at 5°C as separate fraction in stoppered Erlenmeyer flasks.

Prior to semen collection, diluents were brought to 32°C and semen was diluted at this temperature to a final concentration of 50×10^6 total spermatozoa per ml.

Using the split ejaculate technique, twice the final number of sperm were added to fraction "A" of each diluter and cooled to 5°C in 3-4 hrs. Following cooling, glycerolation was accomplished by pipetting an equal volume of fraction "B" into fraction "A" of the respective diluter in portions of 10, 20, 30 and 40% of the volume at 10-min intervals. Diluted semen was then stored at 5°C overnight in stoppered Erlenmeyer flasks.

Serum aliquots were thawed and complement inactivated at 56°C for 30 min. Serum was stored overnight at 5°C.

Preparation of Treatments

On the first day (Day 1) after semen dilution, the treatments were prepared and coded at 5°C and stored in pre-cooled snap-top polypropy-

lene tubes. Egg yolk and skimmilk diluted semen were divided into aliquots, eight aliquots of egg yolk diluted semen and eight of skimmilk diluted semen. Aliquots were paired into eight treatment groups, two aliquots were used for each treatment. Each aliquot contained 2.7 ml of diluted semen. Four treatments contained egg yolk diluted semen; the remaining four treatments contained skimmilk diluted semen. One series of tubes from each pair were evaluated on Day 1 and the second series 48 hrs later on Day 3.

An outline of the composition of the experimental treatments and controls is presented in Table 1. Immune serum is that produced against yolk-citrate-glycerol diluter as described previously. All treatment components were added to basic diluters in 0.3-ml amounts. With the exception of tubes #1 and #5, all treatment mixtures resulted in extended semen diluted 9:1 with blood serum. In addition to all skimmilk treatments serving as controls, tube #1 and #5 were controls since they received 0.3 ml of fraction "A" of the respective diluter instead of serum.

Evaluation of Treatments

On each observation day (Day 1 and 3), aliquots of each treatment were incubated at 37°C in a water bath. Percent motility, percent intact acrosomes and the percent agglutinated cells were the parameters used to evaluate the treatments. Observations were made at 0, 3, 6 and 9 hr of incubation.

Estimates of percent progressive motility (to the nearest 10%) were accomplished using a Leitz phase contrast microscope equipped with a stage incubator to maintain slide temperature at approximately 37°C.

Intact acrosomes were characterized by the presence of an apical ridge on the anterior portion of the acrosome. The percentage of sperm cells possessing an intact acrosome was determined by making two counts of 100 cells each from an unfixed wet smear utilizing a Zeiss differential interference contrast microscope at 1250 X magnification (Saacke and White, 1970; Saacke and White, 1972). Difference of more than 8% between two counts was reinforced by a third count. The two closest percentages were averaged and the third percentage ignored.

Head to head agglutination occurred in all treatments exposed to serum. Percent agglutination was determined from differential counts of agglutinated and single cells in microscope fields of the differential interference contrast microscope (Senger, 1973). Two counts of 100 cells each were made prior to counts for percent intact acrosomes at 1250 X magnification. The same smear was used for both percent agglutination and percent intact acrosomes. The same evaluations were made on Day 3 of storage.

Table 1. Outline of the Composition of the Experimental Treatments and Controls.

TUBE NO.	BASIC DILUTER WITH SEMEN	TREATMENT COMPOSITION
1	2.7 ml Egg Yolk	0.3 ml Fraction "A" Egg Yolk (Control)
2	2.7 ml Egg Yolk	0.3 ml Negative Serum*
3	2.7 ml Egg Yolk	0.3 ml Immune Serum (titered 1:640)
4	2.7 ml Egg Yolk	0.3 ml Immune Serum (titered 1:2560)
5	2.7 ml Skimmilk	0.3 ml Fraction "A" Skimmilk (Control)
6	2.7 ml Skimmilk	0.3 ml Negative Serum*
7	2.7 ml Skimmilk	0.3 ml Immune Serum (titered 1:640)
8	2.7 ml Skimmilk	0.3 ml Immune Serum (titered 1:2560)

* Negative Serum had no titer to egg yolk diluter.

RESULTS

The passive hemagglutination test detected serum antibody titers of 1:640 and 1:2560 in the two donor heifers. Triplicate simultaneous serial dilution trials resulted in the same titer determination for each serum sample. No hemagglutination appeared in the controls.

The results of the equivalent proportion test are presented in Table 2. The maximum precipitate occurred in the tube containing egg yolk diluent antigen (diluted 1:160) and antiserum titered 1:2560 (diluted 1:2 with saline). Such a combination results in a ratio of 1:80 antigen to antibody. Therefore 80 volumes of antiserum (titered 1:2560) are required to neutralize 1 volume of 20% egg yolk semen diluent. Triplicate simultaneous serial dilutions confirmed these results. Control dilutions were negative as anticipated.

The second phase of this experiment examining the effects of immune sera upon the viability of spermatozoa diluted in egg yolk revealed the following results. The overall general effects that the independent variables had upon the seven ejaculates as determined by percent motility, percent intact acrosomes and percent agglutination are presented in Table 3. The detrimental effect of hours of incubation (hours) at 37°C and the inferiority of skimmilk diluter to egg yolk were anticipated. Split ejaculates evaluated on day 3 after 48 hr storage at 5°C were inferior to those evaluated on day 1 immediately following the addition of serum, as determined by percent motility and percent intact acrosomes. Percent agglutination increased on day 3. With regard to percent motility and percent intact acrosomes, data in

Table 2. Results of Equivalent Proportions Test.

I. Antiserum Tests:

0.5 ml of serially diluted antiserum (titered 1:2560) + 0.5 ml of Egg Yolk Diluent antigen diluted 1:160

	Antiserum Serial Dilution									
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
A.	+++	+++	+++	±	----	----	----	-----	-----	-----
B.	+++	+++	+++	±	----	----	----	-----	-----	-----
C.	+++	+++	+++	±	----	----	----	-----	-----	-----

II. Control Tests:

A. Negative Serum:

0.5 ml of serially diluted negative serum + 0.5 ml of Egg Yolk Diluent antigen diluted 1:160

--- --- --- --- ---- ---- ---- ----- ----- -----

B. Antiserum-Saline:

0.5 ml of serially diluted antiserum (titered 1:2560) + 0.5 ml of saline (no antigen)

--- --- --- --- ---- ---- ---- ----- ----- -----

C. Saline-Antigen:

0.5 ml of physiological saline + 0.5 ml of Egg Yolk Diluent antigen diluted 1:160

No precipitation or flocculation.

Table 3. Overall Summary of the Evaluation of Spermatozoa (mean of 7 ejaculates).

Fixed Effects	Parameters Measured		
	Percent Motility	Percent Intact Acrosomes	Percent Agglutination
Days[a]			
1	34.64	82.91	54.79
3	26.87	78.09	58.39
Diluters[b]			
Egg Yolk	34.14	85.43	61.74
Skimmilk	29.38	75.57	51.43
Hours[c]			
0	53.13	88.27	53.55
3	41.25	83.11	65.01
6	22.23	77.87	56.42
9	6.43	72.74	51.37
Treatments[d]			
Control	23.31	77.98	-
1	33.39	81.08	48.31
2	32.77	81.17	63.68
3	33.57	81.76	57.77

[a] percentages represent $\bar{X}$ of 0, 3, 6 and 9 hr of incubation at 37°C of both diluters and all treatments for each day of evaluation.

[b] percentages represent $\bar{X}$ of 0, 3, 6 and 9 hr of incubation at 37°C of both days and all treatments for each diluter.

[c] percentages represent $\bar{X}$ of both days, both diluters and all treatments for each hour of incubation at 37°C.

[d] percentages represent $\bar{X}$ of 0, 3, 6 and 9 hr of incubation at 37°C of both days and both diluters for each treatment.

Table 3 suggests that serum treatments were more beneficial to spermatozoa than the control treatment. Head to head agglutination did not occur in control treatments. There were significant differences ($P < .05$) between all serum treatments in percent agglutination. It should be noted that due to the agglutination effect it was difficult to accurately estimate the percent progressive motility.

The analysis of variance is presented in Table 4 and describes the sources and degree of variation, for both, intact acrosomes and percent agglutination. Treatments were a significant source of variation ($P < .01$) for both variables. However, there was no significant difference, as tested by Duncan's Multiple Range Test, between treatments due to antibody titer of the serum. All serum treatments (serum titered 1:2560, 1:640 and negative sera) were significantly more beneficial to spermatozoa in maintenance of the acrosomal cap than the control treatment; although not quantitated, agglutinated samples also exhibited greater duration of motility than did non-agglutinated samples. Ejaculates, hours of incubation, diluter and the day of evaluation were also significant sources of variation for both percent intact acrosomes and agglutination. First order interactions which were highly significant ($P < .01$) included ejaculate x hour, ejaculate x diluter, day x diluter and hour x diluter for acrosomal maintenance. Ejaculate x day interaction was significant ($P < .05$). For percent agglutination, only diluter x day and diluter x hour were highly significant ($P < .01$). An overall correlation coefficient (r) of 0.463 was present between percent intact acrosomes and percent agglutination.

Table 4. Analysis of Variance for Percent Intact Acrosomes and Percent Agglutination (mean of all hours of incubation, all diluters, both days of evaluation).

Source of Variation	Degrees of Freedom	Mean Square	
		Percent Intact Acrosomes	Percent Agglutination
Ejaculate	6	977.808**	1660.478**
Treatment	3	326.169**	6732.658**
Hours of incubation (0, 3, 6 and 9)	3	5020.185**	3006.433**
Days (1 and 3)	1	2597.789**	1087.560*
Diluters (Skimmilk, egg yolk)	1	10884.257**	8943.518**
Ejaculate x Hour	18	112.710**	234.237
Ejaculate x Diluter	6	216.002**	259.263
Ejaculate x Day	6	98.582*	267.152
Day x Diluter	1	943.661**	3253.185**
Hour x Diluter	3	708.899**	2401.923**
Treatment x Diluter	3	28.797	55.228
Residual	348	35.775	244.556

** significant at $P < .01$
* significant at $P < .05$

The same major sources of variation were significant for percent intact acrosomes and percent agglutination in analysis of variance for individual days (day 1 and 3) computed separately (Tables 1 and 2, Appendix). However the following first order interactions became significant for percent agglutination: ejaculate x hour on both day 1 and day 3, ejaculate x treatment and ejaculate x diluter on day 3. For intact acrosomes there was no ejaculate x hour interaction on day 1 as there was on day 3 and days 1 and 3 combined. The correlation coefficients between percent intact acrosomes and percent agglutination were 0.118 and 0.732 for day 1 and 3 respectively.

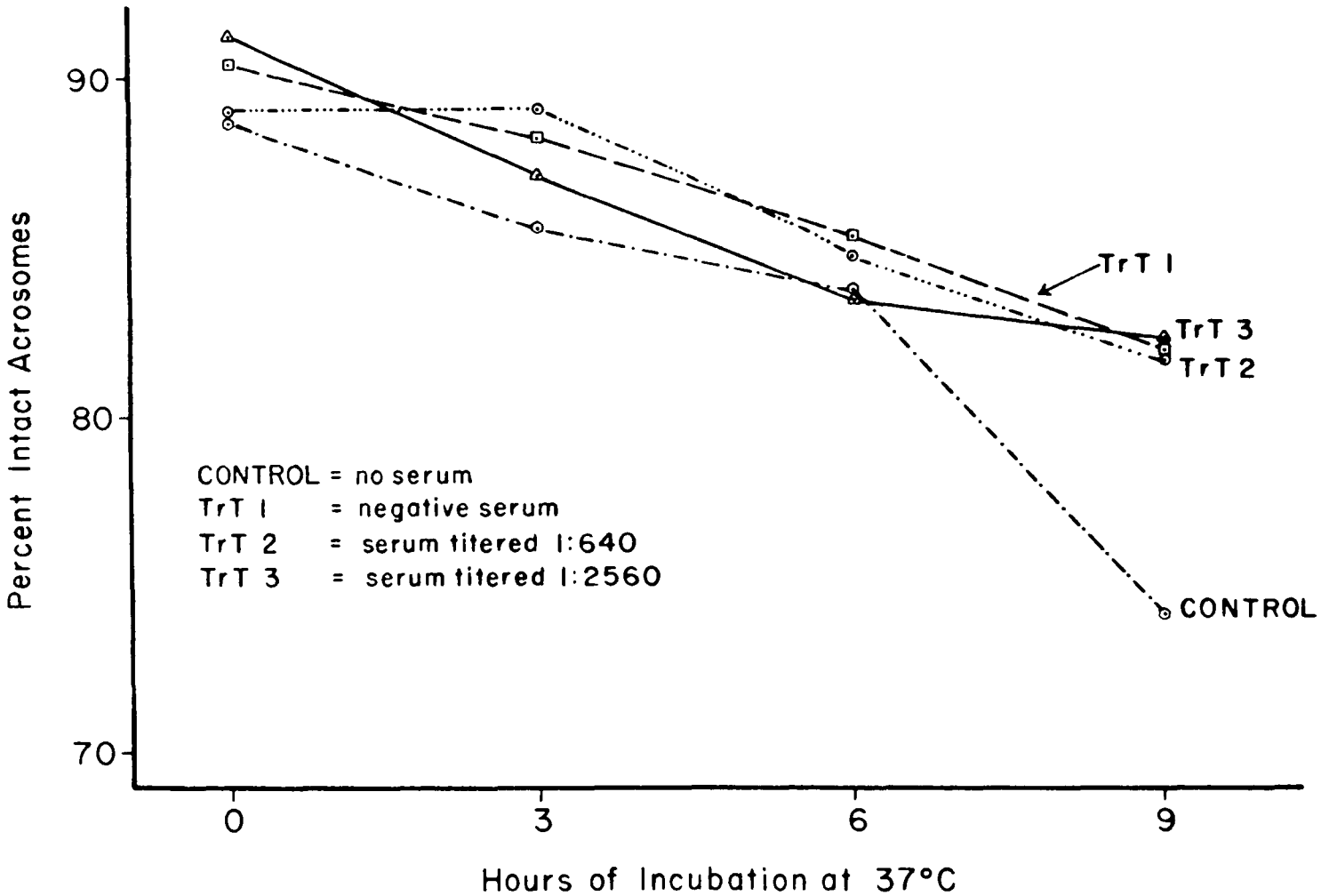
Of critical importance is the comparison of treatment means for percent intact acrosomes and percent agglutination between diluters over the nine-hr incubation period. The analysis of variance presented in Table 4 shows that there was no significant treatment x diluter interaction. Spermatozoa whether diluted in egg yolk or skimmilk responded to the treatments in a similar manner. Although, as noted previously, egg yolk was the superior diluter. Hourly means for percent intact acrosomes for all treatments within diluters are presented in Table 5. These means are graphically presented for each diluter separately in Text-figures 1 and 2. Significant differences ($P < .05$) between treatments within diluters are indicated in Table 5. For both diluters all serum treatments were significantly different ($P < .05$) from the control at 9 hr and for the overall mean of all hours of incubation. In skimmilk at 6 hr, treatment 2 and 3 had a significantly greater percent intact acrosomes than the control. In addition, treat-

Table 5. Comparison of Treatment Means between Diluters for Percent Intact Acrosomes at 0, 3, 6 and 9 hr of Incubation Days 1 and 3 combined. (Mean of 7 ejaculates)

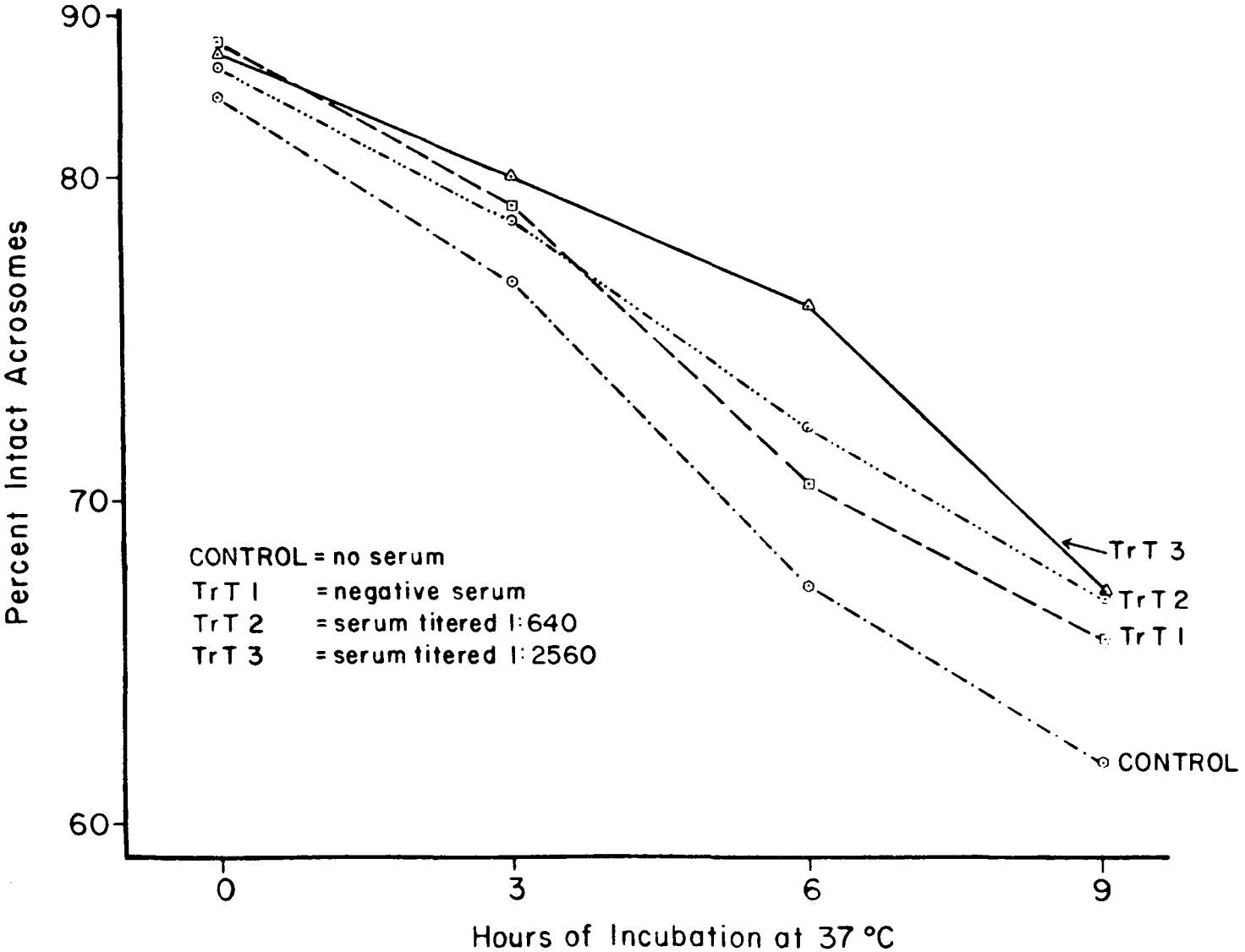
Treatment	Diluter	Hour of Incubation at 37° C 0	3	6	9	$\bar{X}$
Control	Egg Yolk	88.92	85.60	83.46	74.10^{b}	82.95^{b}
	Skimmilk	85.53	76.85	67.42^{c}	61.89^{b}	72.90^{b}
1- Negative Serum	Egg Yolk	90.39	88.32	85.42	81.96^{a}	86.75^{a}
	Skimmilk	87.39	79.10	70.35^{bc}	65.71^{a}	75.65^{a}
2- Serum titered 1:640	Egg Yolk	89.03	89.10	84.78	81.75^{a}	86.15^{a}
	Skimmilk	86.78	78.71	72.14^{ab}	67.07^{a}	76.15^{a}
3- Serum titered 1:2560	Egg Yolk	91.17	87.12	83.35	82.28^{a}	85.95^{a}
	Skimmilk	86.96	80.03	76.00^{a}	67.10^{a}	77.50^{a}

Different superscripts by hr indicate significant differences between treatments within diluter as tested by Duncan's Multiple Range Test ($P < .05$).

Text-figure 1. Effect of treatments on acrosomal retention in semen diluted in egg yolk. (mean of day 1 and 3 combined)


90
80
70
Percent Intact Acrosomes
0
3
6
9
Hours of Incubation at 37°C
CONTROL = no serum
TrT 1 = negative serum
TrT 2 = serum titered 1:640
TrT 3 = serum titered 1:2560
TrT 1
TrT 3
TrT 2
CONTROL

Text-figure 2. Effect of treatments on acrosomal retention in semen diluted in skimmilk. (means of day 1 and 3 combined)


90
80
70
60
Percent Intact Acrosomes
0
3
6
9
Hours of Incubation at 37 °C
CONTROL = no serum
TrT 1 = negative serum
TrT 2 = serum titered 1:640
TrT 3 = serum titered 1:2560
TrT 3
TrT 2
TrT 1
CONTROL

ment 3 was superior to treatment 1.

To inspect the treatment effects more closely, it is necessary to examine the results of each day of evaluation with the day effect removed. Analysis of variance computed separately for each day indicates that there was no significant treatment x diluter interaction on either day 1 or day 3. Results of evaluations on day 1 for percent intact acrosomes for treatments within diluters are presented in Table 3 of the appendix. There were no differences between treatments diluted in egg yolk at any hour of incubation. However, in skimmilk at 6 hr there were a variety of differences between treatments. At 9 hr and for the overall mean of all hours, all serum treatments were significantly ($P < .05$) better than the control.

On day 3 (Appendix, Table 4) in skimmilk at 6 hr treatments 2 and 3 were significantly higher in percent intact acrosomes than treatment 1 or the control. But in contrast to day 1, it was in the egg yolk diluter where the serum treatments were significantly better than the control at 9 hr and for the overall mean of all hours.

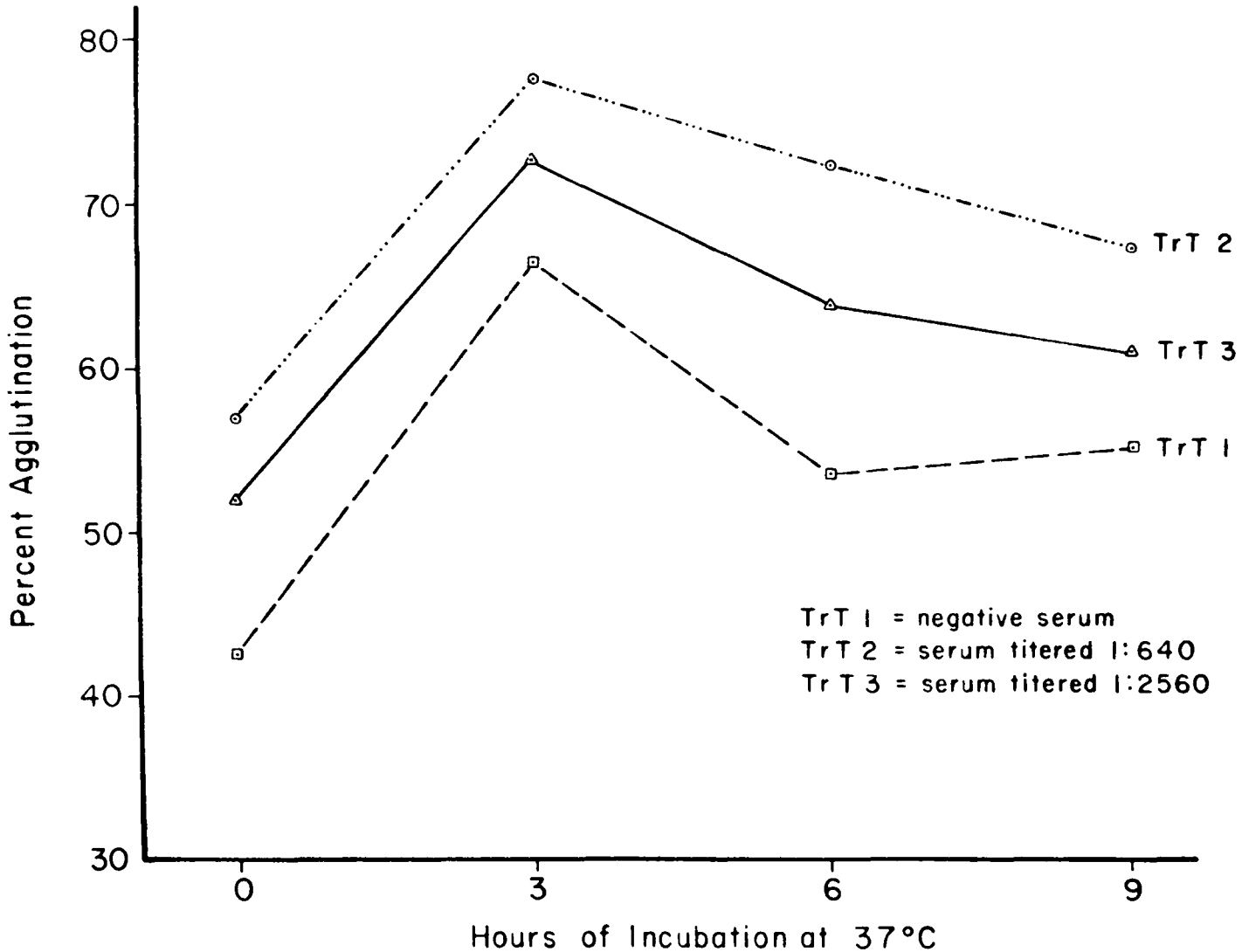
Results of evaluations of percent agglutination across all hours of incubation on days 1 and 3 combined are presented in Table 6. Text-figures 3 and 4 graphically illustrate the means for percent agglutination for each hour of evaluation. Percent agglutination was considerably higher in egg-yolk diluted samples. There was no significant variation due to treatment x diluter interaction. However, there were significant differences ($P < .05$) between treatments within both diluters. The ranking of the serum treatments were the same in each diluter.

Table 6. Comparison of Treatment Means between Diluters for Percent Agglutination at 0, 3, 6 and 9 hr of Incubation Days 1 and 3 combined. (Mean of 7 ejaculates)

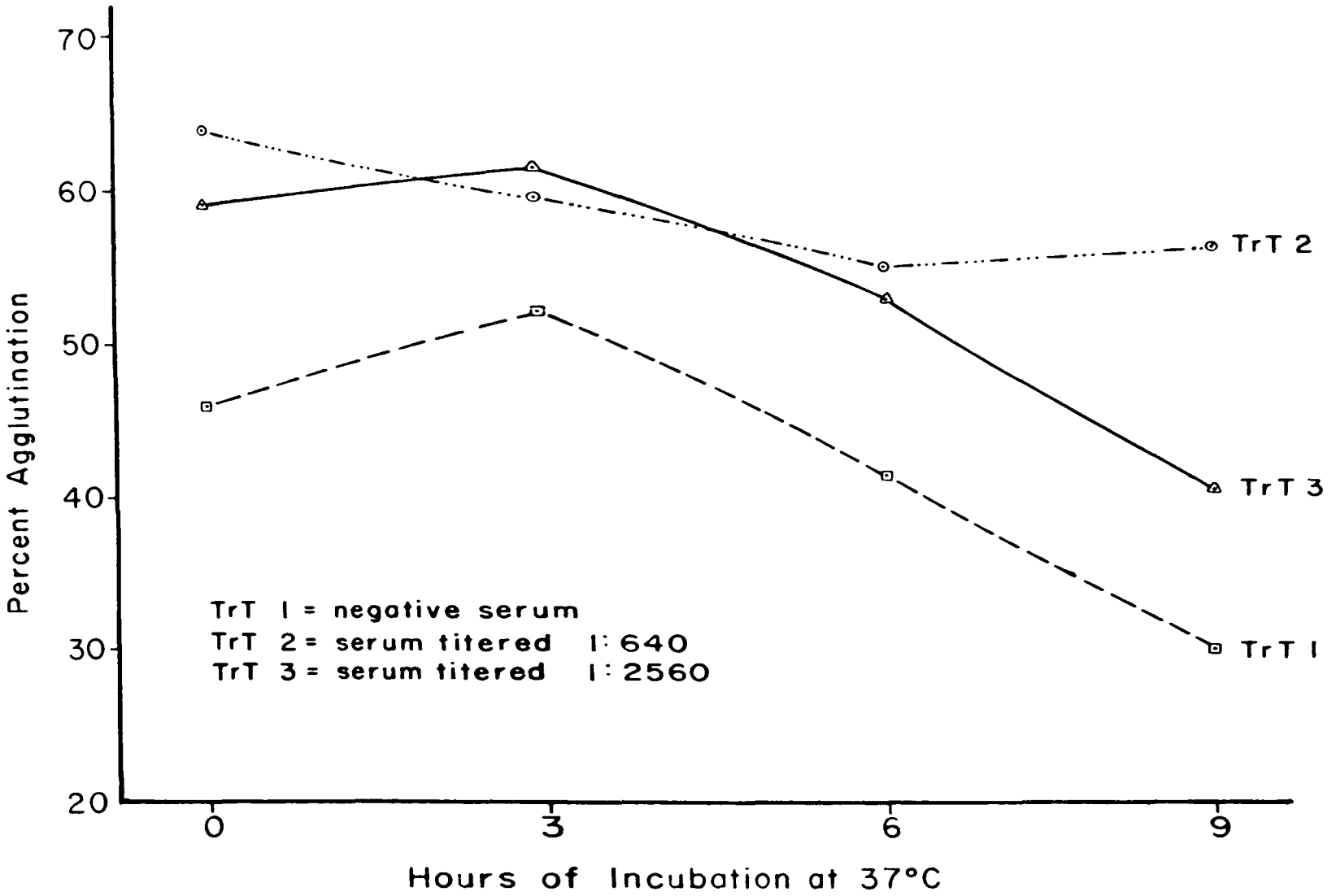
Treatment	Diluter	Hour of Incubation at 37°C 0	3	6	9	$\bar{X}$
1- Negative Serum	Egg Yolk	42.46^{b}	66.36	53.46^{b}	54.69	54.24^{c}
	Skimmilk	46.00^{b}	52.18	41.29^{b}	30.04^{b}	42.38^{b}
2- Serum titered 1:640	Egg Yolk	57.96^{a}	77.64	72.25^{a}	66.78	68.66^{a}
	Skimmilk	64.04^{a}	59.67	54.96^{a}	56.11^{a}	58.70^{a}
3- Serum titered 1:2560	Egg Yolk	52.10^{ab}	72.92	63.89^{ab}	60.39	62.33^{b}
	Skimmilk	58.71^{a}	61.25	52.64^{ab}	40.21^{b}	53.20^{a}

Different superscripts by hr indicate significant differences between treatments within diluter as tested by Duncan's Multiple Range Test ($P < .05$).

Text-figure 3. Effect of serum treatments on percent agglutination in semen diluted in egg yolk. (means of days 1 and 3 combined)


80
70
60
50
40
30
Percent Agglutination
0
3
6
9
Hours of Incubation at 37°C
TrT 2
TrT 3
TrT 1
TrT 1 = negative serum
TrT 2 = serum titered 1:640
Tr T 3 = serum titered 1:2560

Text-figure 4. Effect of serum treatments on percent agglutination in semen diluted in skimmilk. (means of days 1 and 3 combined)


70
60
50
40
30
20
Percent Agglutination
TrT 2
TrT 3
TrT 1
TrT 1 = negative serum
TrT 2 = serum titered 1:640
TrT 3 = serum titered 1:2560
0
3
6
9
Hours of Incubation at 37°C

Serum used in treatment 2 induced the highest percent agglutination while that used in treatment 3 was intermediate and treatment 1 contained the serum inducing the least agglutination.

Tables 5 and 6 of the appendix compare treatment means between diluters for percent agglutination for each hour of incubation on days 1 and 3 respectively. Serum treatments ranked in the same order on both days. There was no treatment x diluter interaction on either day. The overall incubation means for the egg yolk treatments evaluated on day 1 were lower than the same treatments evaluated on day 3. However, skim-milk treatments were higher in percent agglutination on day 1 than on day 3 for all treatments. On day 1 in both diluters, the 0 hr evaluation for agglutination was considerable lower than the 3, 6 or 9 hr evaluation.

DISCUSSION

The antigenic potency of egg-yolk semen diluter was described by Swanson and Hunter (1969) in rabbits. Electrophoresis revealed three major protein zones in egg yolk citrate. Analysis by agar-gel double diffusion revealed that upon interaction of egg-yolk antigens with immune serum, eight glycoproteins and one glycolipoprotein appeared. The high titers obtained (1:640 and 1:2560) in the present experiment reflect the strong antigenicity of egg yolk in cattle. The large volume of immune serum (80 volumes) required to neutralize one volume of 20% egg-yolk diluent further illustrates the antigenic potency of egg yolk. It should be noted from the results of the equivalent proportion test (Table 2) that the maximum precipitate or point of equivalent proportion occurred in three tubes (antiserum diluted 1:1, 1:2, and 1:4). Dilution 1:2 was chosen as the optimum ratio because it contained more antibody than the tube containing antibody diluted 1:4, such a concentration of immune serum would insure neutralization of all egg-yolk diluent antigens.

However the results illustrate how impractical it would be to neutralize egg yolk antigens by the addition of immune sera titered 1:2560. Such a quantity of 80 volumes of serum would greatly modify the composition of the basic diluent. An alternative would be to use sera containing an extremely high titer of immunoglobulins against egg yolk. Possibly the gamma globulin portion of the serum could be concentrated before adding it to the diluter.

There were three criterion used to evaluate the effect that immune

serum had upon the viability of spermatozoa, percent motility, percent intact acrosomes and percent head to head agglutination. Acrosomal cap retention (percent intact acrosomes) has been shown to be the single most accurate laboratory test of semen quality (Saacke and White, 1972).

In this present study evaluation of the treatments for the percent intact acrosomes revealed no significant differences between treatments due to the titer of specific antibodies to egg-yolk diluent. These critical results indicate that there are no detrimental effects upon bovine spermatozoa exposed to anti-egg-yolk serum. Although the analysis of variance (Table 4) revealed significant variation due to treatments further analysis by Duncan's Multiple Range Test showed that such variation existed between the non-serum control and all serum treatments regardless of antibody titer.

There was no treatment x diluter interaction indicating that the treatments responded the same whether diluted and incubated in skimmilk or egg yolk. If the antibodies were harmful to spermatozoa, such effects would appear in the egg-yolk treatments only. Non-specific effects of serum were apparent in the skimmilk samples. Consequently skimmilk diluted samples received substantial benefit from serum. This beneficial effect of serum addition was shown by Senger (1974) in egg-yolk diluted semen. The head to head agglutination effect of serum was believed to be responsible for the prolonged acrosomal maintenance. Yanagimachi (1970) reported that complement-fixed serum induced both head to head agglutination and the acrosome reaction in hamster spermatozoa.

The significant first order interactions ejaculate x hour, ejaculate x diluter and ejaculate x day indicate how variable single ejaculates respond to these independent variables. Such effects should have been expected. However it should be noted that there was no ejaculate x treatment interaction signifying that the ejaculates responded uniformly to the treatment. The diluter x day interaction is primarily due to the faster deterioration of the acrosome in skimmilk samples than in the egg-yolk diluted samples on day 3. The diluter x hour interaction was probably caused by the proportionately larger drop in percent intact acrosomes between 0 and 3 hr and 3 hr and 6 hr in the skimmilk samples whereas there was a more uniform rate of deterioration in the egg-yolk samples (Table 4, Appendix).

The variety of differences between treatments appearing at 6 hours of incubation on both days in the skimmilk samples cannot be explained. There were however no differences between serum treatments for the overall incubation mean on day 1 and day 3 or days 1 and 3 combined.

In comparing the two diluters on individual days, it is apparent that the non-specific effect of the serum was different for each diluter. Although the non-serum control was the lowest in percent intact acrosomes in egg yolk, it was not significantly different from the serum treatments until the 9-hr evaluation on day 3 (Table 4, Appendix). The skimmilk non-serum control was inferior to the serum treatments throughout day 1 incubation and least 10% less than any serum treatment at 9 hr of that incubation (Table 3, Appendix). However, on day 3, there was no difference between the non-serum and the serum treatment in skim-

milk diluter. Such contrast between semen diluters could have resulted from the more favorable environment provided by the egg yolk. Spermatozoa diluted in egg yolk were not stressed enough to show differences between samples until the evaluation on day 3. On the contrary, Senger (1974) found significant differences between non-serum controls and serum samples after 3 hr of 37°C incubation (comparable to day 1 in this study) in freeze-thawed egg-yolk diluted semen. However, such semen samples were more severely stressed due to the freeze-thaw process. Skimmilk samples did not respond to the serum agglutinating effect as well as egg-yolk diluted samples. Senger (1974) postulated that only healthy cells are capable of engaging in head to head agglutination. Once spermatozoa are agglutinated they maintain their acrosomal cap considerably longer than the non-agglutinated cells. Consequently, due to the inferior environment of skimmilk compared to egg yolk, less spermatozoa qualified for head to head agglutination and the acrosomal caps began to deteriorate much earlier in skimmilk. Thus the beneficial serum effects were apparent on day 1. The lack of any differences in skimmilk between the non-serum control and the serum treatments on day 3 cannot be explained.

It must be emphasized that the diluter differences presented above are only general differences between diluters in their response to the non-specific effects of serum. Such differences were not between treatments where any specific effects of the anti-egg-yolk antibodies would exist.

Since head to head agglutination is a function of serum addition,

only comparisons can be made between the serum treatments. There were significant differences between serum treatments at various hours of incubation on both days. However there was no treatment x diluter interaction indicating that treatments responded similarly in both diluters. Again there were no specific effects accountable to the titer of egg-yolk antibody. The agglutination effect was not detrimental to the spermatozoa since the serum treatments were significantly superior to the non-serum controls with regard to the other criteria measured (percent intact acrosomes and percent motility).

Agglutination was positively correlated with intact acrosomes ($r = 0.463$). Such results are acceptable on the basis of the findings of Menge and Protzman (1967) and Metz and Anika (1970). Since they definitively demonstrated that agglutination was not the causative factor of infertility. Incubation of bovine spermatozoa in anti-egg yolk sera did not alter the pattern of agglutination from non-specific serum induced head agglutination to mixed agglutination induced by immune serum possessing antibodies to egg yolk, as was observed by Griffin et al (1971).

The interaction of diluter x day is primarily due to the more rapid deterioration of agglutination on day 3 in skimmilk samples compared to the more gradual decline in the egg-yolk samples. The low percent agglutination at 0 hr on day 1 in egg yolk samples accounts for the fact that agglutination on day 1 was lower than on day 3 in egg yolk diluted samples. The remainder of the evaluations at 3, 6 and 9 hours were similar on both days. This also accounts for the significant diluter

x hour interaction. Skimmilk diluted samples were also quite low at 0 hr on day 1 but not to the same degree as the egg-yolk treatments (Table 5, Appendix).

Senger (1974) conducted a thorough investigation into the effects of serum upon spermatozoa. He demonstrated that serum samples varied in the degree of agglutination they induced. Variation was due to differences among sera from different animals and sera from the same animal. The significant differences observed in the present study between serum treatments in percent agglutination is probably due to this same variation. Serum for each treatment was from a different heifer. The use of homologous serum from many females would have been the ideal, however, obtaining identically titered serum from a group of animals is difficult.

The egg yolk diluted treatments had at least a 9% higher agglutination than did similar treatments diluted in skimmilk. This suggests that the egg-yolk diluent provided a more optimum environment for spermatozoa. As mentioned before, the higher agglutination in egg yolk samples may have been due to a higher population of healthy intact spermatozoa with the converse being the case for skimmilk treatments.

The third evaluation, percent motility, has been an established criterion for evaluating viability of spermatozoa. However, due to the interference of agglutination with accurate estimations of percent motility, motility was not as reliable a means of evaluation as anticipated. Nevertheless, the crude estimations of motility did substantiate the findings of other investigators.

There were no significant differences in percent motility between serum treatments, that is, no effect upon spermatozoa due to the antibody titer. However, all serum treatments regardless of antibody titer maintained motility longer over each incubation period than did the non-serum treatments. Such prolonged motility was of a less vigorous nature than that observed in the non-serum treatments. Swanson and Hunter (1969) observed that the percentage of motile rabbit sperm was not significantly affected by treatment with immune sera. However they did not compare control samples without serum with the immune serum samples. Griffin et al (1971) failed to detect any immobilization of spermatozoa using in-vitro tests to determine the effect of immune sera or genital fluids upon semen. Yamagimachi (1970) noted that hamster spermatozoa incubated with complement-fixed homologous or heterologous blood sera remained quite motile for more than 5 hr. Serum not complement fixed caused immobilization immediately.

It is concluded from evaluations made in this investigation that humoral antibodies specific against egg-yolk-citrate diluter do not have any detrimental effect upon the viability of bovine spermatozoa diluted in egg yolk as determined by the following criteria: percent motility, percent intact acrosomes and percent agglutination. Nevertheless, tissue-fixed antibodies against the diluent that are produced in the female genital tract may have a significant adverse effect upon spermatozoa and fertility.

REFERENCES

Ackerman, D. R. 1967. Antibodies of the ABO system and the metabolism of human spermatozoa. Nature. 213:253.

Ackerman, D. R. and R. Gongalez-Enders. 1969. Metabolism of rabbit spermatozoa in homologous immune serum and oviductal fluid. Biol. Reprod. 1:407.

Barros, C., J. Arrau and E. Herrera. 1971. Induction of the acrosome reaction of golden hamster spermatozoa with blood serum collected at different stages of the oestrous cycle. J. Reprod. Fertil. 28:67.

Baum, J., B. Boughton, J. L. Mongar and H. O. Schild. 1961. Autosensitization by sperm in guinea pigs. Immunology. 4:95.

Berstein, A. and G. Lazarev. 1933. Studies on the physiology of spermatozoa. Transactions of the Chair of Veterinary Pathology, Orenburg, Veterinary Inst., No. 1 Samara 156. Cited from: Anim. Breed. Abstr. 3:479.

Boyden, S. V. 1951. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination with antiprotein sera. J. exp. Med. 93:107.

Bratanov, K. 1972. Immunological problems in reproduction. Proc. VIIth Int. Congr. on Reprod. and A.I. (Munich) 3:2189.

Bratanov, K. and V. Dikov. 1959. Investigations on spermoisoagglutination in connection with the fertilization of cows. Nauchni Trud. naucnoizled. Inst. Razvad. Bol. izkustv. Osemen. selskotop. Zivotn (Sofia), 1:11. Cited from: Anim. Breed. Abstr. 30, No. 965.

Bratanov, K., V. Dikov and Y. Popova. 1964. Vet. Med. Nauki (Sofia). 1:31. Cited from: Serological Studies on Fertility. Swanson, L. V. 1967. M. S. thesis. Univ. of Minn.

Bratanov, K., V. Dikov and A. Tornjov. 1968. Sur la specificite des antigenes de spermatozoides dans le sperme du taureav. Proc. VIth Int. Congr. on Reprod. and A.I. (Paris) 2:973.

Burrell, R. and C. C. Mascoli. 1970. Experimental Immunology. (3rd ED). Burgess Co., Minneapolis.

Chang, M. C. 1947. The effects of serum on spermatozoa. J. Gen. Physiol. 30:321.

Edwards, R. G. 1960. Antigenicity of rabbit semen, bull semen, and egg yolk after intravaginal or intramuscular injections into female rabbits. J. Reprod. Fertil. 1:385.

Edwards, R. G. 1970. Immunology of conception and pregnancy. Br. med. Bull. 26:72.

Griffin, J. F. T., W. R. Nunn and P. J. Hartigan. 1971. An immune response to egg-yolk semen diluent in dairy cows. J. Reprod. Fertil. 25:193.

Henle, W., G. Henle and L. A. Chambers. 1938. Studies on the antigenic structure of some mammalian spermatozoa. J. Exp. Med. 68:335.

Hunter, A. G. and H. D. Hafs. 1964. Antigenicity and cross-reactions of bovine spermatozoa. J. Reprod. Fertil. 7:357.

Hunter, A. G. and D. J. Alsum. 1969. Immunological suppression of fertility in rabbits inseminated with egg yolk citrate semen extender. J. Dairy Sci. 52:922.

Katsh, S. 1959. Immunology, fertility, and infertility: A historic survey. Am. J. Obstet. Gynecol. 77:946.

Katsh, S. and D. W. Bishop. 1958. The effects of hemologous testicular and brain and heterologous testicular homogenates combined with adjuvant upon the testis of guinea pigs. J. Embryol. Exp. Morphol. 6:94.

Katsh, S. K. and G. F. Katsh. 1965. Perspectives in immunological control of reproduction: Past, present and future. Pacific Med. Surg. 73:28.

Kiddy, C. A., W. H. Stone and L. E. Casida. 1959. Immunological studies on fertility and sterility. II. Effects of treatment of semen with antibodies on fertility in rabbits. J. Immun. 82:125.

Kiddy, C. A., W. H. Stone, W. T. Tyler and L. E. Casida. 1959. Immunological studies on fertility and sterility. III. Effect of isoimmunization with blood and semen on fertility in cattle. J. Dairy Sci. 42:110.

Landsteiner, K. 1899. Zentralbl. Bakt. 25:546. Cited from: The Biochemistry of Semen and of the Male Reproductive Tract. T. Mann. 1964. Methuen and Co. Ltd. London. p. 366.

Lindahl, P. E. 1960. Some factors influencing the biological activity of sperm antagglutins. J. Reprod. Fertil. 1:3.

Lindahl, P. E. and J. E. Kihlstrom. 1954a. An antiagglutinic factor in mammalian sperm plasm. Fertil. and Steril. 54:241.

Lindahl, P. E., J. E. Kihlstrom and S. Ross. 1956. On some properties of the aromatic constituent of male sperm antagglutin and its "Active Group". Acta Chem. Scand. 10:1597.

Mann, T. 1964. The Biochemistry of Semen and of the Male Reproductive Tract. Methuen and Co. Ltd. London.

Matousek, J. 1964. Antigenic characterictics of spermatozoa from bulls, rams, and boars. III Absorption analysis, precipitins and fructolysis in relation to the antigenicity of bull spermatozoa. J. Reprod. Fertil. 8:13.

Menge, A. C. 1967. Induced infertility in cattle by iso-immunization with semen and testis. J. Reprod. Fertil. 13:445.

Menge, A. C. 1970a. Immune reactions and infertility. J. Reprod. Fertil. Suppl. 10:171.

Menge, A. C., W. H. Stone, W. J. Tyler and L. E. Casida. 1962b. Immunological studies on fertility and sterility. IV. Fertility of cattle and rabbits inseminated with semen treated with antibodies produced against semen, spermatozoa, and erythrocytes. J. Reprod. Fertil. 3:331.

Menge, A. C. and W. P. Protzman. 1967. Origin of the antigens in rabbit semen which induce antifertility antibodies. Reprod. Fertil. 13:31.

Metchnikoff, E. 1899. Ann. Inst. Pasteur 13:737. Cited from: The Biochemistry of Semen and of the Male Reproductive Tract. T. Mann. 1964. Methuen and Co. Ltd. London. p. 366.

Metz, C. B. and J. Anika. 1970. Failure of conception in rabbits inseminated with non-agglutinating antibody-treated semen. Biol. Reprod. 2:284.

Mittal, K., G. W. Salisbury, C. N. Graves and B. A. Rasmusen. 1965. Antigens of bovine semen and the influence of specific rabbit anti-bull semen serum on metabolic activity of bull spermatozoa. J. Reprod. Fertil. 10:29.

Moyer, R. F. and J. O. Almquist. 1962. Diluents for bovine semen X. Livability of spermatozoa stored at ambient room temperatures in diluents containing skimmilk. J. Dairy Sci. 45:383.

Nedyalkov, K. and M. Stayanova. 1966. Vet. Med. Nauki (Sofia) 3:1039. Cited from: Hunter, A. G. 1972. Proc. 4th Tech. Conf. Artificial Insem. and Reprod.

Olds, D. and N. L. VanDemark. 1957. The behavior of spermatozoa in luminal fluids of bovine female genitalia. Am. J. Vet. Res. 18: 603.

Peterson, H. P. 1965. Some influences of uterine secretions from normal and repeat breeding cows on bovine spermatozoa. Am. J. Vet. Res. 26:873.

Saacke, R. G. and J. M. White. 1970. Acrosomal alteration of freeze-thawed bovine sperm. J. Animal Sci. 31:229 (Abstr.).

Saacke, R. G. and J. M. White. 1972. Acrosomal cap maintenance and fertility of frozen bovine semen. J. Animal Sci. 35:253 (Abstr.).

Senger, P. L. 1974. Blood serum induced head to head agglutination in bovine spermatozoa. Ph.D. dissertation. VPI & SU

Senger, P. L. and R. G. Saacke. 1973. Effects of blood serum on bovine spermatozoa. J. Animal Sci. 37:328 (Abstr.).

Swanson, L. V. 1967. Serological studies on fertility. M. S. thesis. Univ. of Minnesota.

Swanson, L. V. and A. G. Hunter. 1969. Egg yolk antigens and their effect on fertility in rabbits. Biol. Reprod. 1:324.

Tyler, A. 1961. Approaches to the control of fertility based on immunological phenomena. J. Reprod. Fertil. 2:473.

Tyler, A., E. T. Tyler and P. C. Denny. 1967. Concepts and experiments in immunoreproduction. Fertil. Steril. 18:153.

VanTienhoven, A. and R. G. D. Steel. 1956. The effect of different diluents and dilution rates on fertilizing capacity of turkey semen. Poultry Sci., 36:473.

Weil, A. J. and J. M. Rodenburg. 1962. The seminal vesicle as a source of the spermatozoa coating antigen of seminal plasma. Proc. Soc. exp. Biol. Med. 109:567.

Yanagimachi, R. 1970. In-vitro capacitation of golden hamster spermatozoa by homologous and heterologous blood sera. Biol. Reprod. 3:147.

APPENDIX

Table 1. Analysis of Variance for Percent Intact Acrosomes and Percent Agglutination on Day 1. (mean of all hours of incubation and both diluters).

Source of Variation	Degrees of Freedom	Mean Square	
		Percent Intact Acrosomes	Percent Agglutination
Ejaculate	6	458.369**	697.315**
Treatment	3	241.040**	2865.218**
Hour	3	1213.954**	9066.147**
Diluter	1	2709.112**	704.381**
Ejaculate x Treatment	18	17.18	88.85
Ejaculate x Hour	18	20.63	237.719**
Ejaculate x Diluter	6	110.341**	176.836
Hour x Diluter	3	103.013**	1205.710**
Residual	96	19.126	101.412

** significant at P < .01

Table 2. Analysis of Variance for Percent Intact Acrosomes and Percent Agglutination on Day 3 (mean of all hours of incubation and both diluters).

Source of Variation	Degrees of Freedom	Mean Square	
		Percent Intact Acrosomes	Percent Agglutination
Ejaculate	6	618.022**	1230.315**
Treatment	3	119.647*	3910.996**
Hour	3	4277.872**	6297.486**
Diluter	1	9118.806**	11492.323**
Ejaculate x Treatment	18	30.412	224.037**
Ejaculate x Hour	18	150.823**	187.427**
Ejaculate x Diluter	6	183.871**	263.694**
Hour x Diluter	3	774.209**	1206.315**
Residual	96	37.782	80.893

** significant at $P < .01$
* significant at $P < .05$

Table 3. Comparison of Treatment Means between Diluters for Percent Intact Acrosomes at 0, 3, 6 and 9 hr of Incubation on Day 1. (Mean of 7 ejaculates)

Treatment	Diluter	Hours of Incubation at 37°C				
		0	3	6	9	X
Control	Egg Yolk	89.85	85.85	83.42	79.00	84.53
	Skimmilk	84.71	78.57	73.92^{bc}	64.07^{b}	75.32^{b}
1-Negative Serum	Egg Yolk	91.00	88.21	86.57	83.21	87.25
	Skimmilk	86.50	84.14	78.57^{ab}	74.35^{a}	80.89^{a}
2-Serum titered 1:640	Egg Yolk	88.42	89.35	85.50	83.64	86.73
	Skimmilk	87.78	80.00	74.42^{bc}	75.57^{a}	79.44^{a}
3-Serum titered 1:2560	EggYolk	90.92	87.78	85.71	83.64	87.01
	Skimmilk	85.92	85.21	81.78^{a}	75.28^{a}	82.05^{a}

different superscripts by hr indicate significant differences between treatments within diluter as tested by Duncan's Multiple Range Test ($P < .05$).

Table 4. Comparison of Treatment Means between Diluters for Percent Intact Acrosomes at 0, 3, 6 and 9 hr of Incubation on Day 3. (Mean of 7 ejaculates)

Treatment	Diluter	Hour of Incubation at 37°C				
		0	3	6	9	$\bar{X}$
Control	Egg Yolk	88.00	85.35	83.50	69.21[b]	81.52[b]
	Skimmilk	86.35	75.14	60.92[b]	59.71	70.50
1-Negative Serum	Egg Yolk	89.78	88.42	84.28	80.71[a]	85.80[a]
	Skimmilk	88.28	74.07	62.14[b]	57.07	70.39
2-Serum titered 1:640	Egg Yolk	89.64	88.85	84.07	79.85[a]	85.60[a]
	Skimmilk	85.78	77.42	69.85[a]	58.57	72.91
3-Serum titered 1:	Egg Yolk	91.42	86.45	81.00	80.92[a]	84.95[a]
	Skimmilk	88.00	74.85	70.21[a]	58.92	73.00

different superscripts by hr indicate significant differences between treatments within diluter as tested by Duncan's Multiple Range Test ($P < .05$).

Table 5. Comparison of Treatment Means between Diluters for Percent Agglutination at 0, 3, 6 and 9 hr of Incubation on Day 1. (Mean of 7 ejaculates)

Treatment	Diluter	Hour of Incubation at 37°C				
		0	3	6	9	$\bar{X}$
1-Negative Serum	Egg Yolk	20.14	66.07	53.92^{b}	61.36	50.37^{b}
	Skimmilk	27.64^{b}	57.37	51.29	38.86^{b}	43.79^{b}
2-Serum titered 1:640	Egg Yolk	35.86	75.50	73.71^{a}	68.71	63.45^{a}
	Skimmilk	48.14^{a}	63.86	60.71	63.21^{a}	58.98^{a}
3-Serum titered 1:2560	Egg Yolk	27.36	74.29	66.79^{ab}	58.29	56.68^{ab}
	Skimmilk	42.93^{ab}	66.86	60.14	51.86^{ab}	55.45^{a}

different superscripts by hr indicate significant differences between treatments within diluter as tested by Duncan's Multiple Range Test ($P < .05$).

Table 6. Comparison of Treatment Means between Diluters for Percent Agglutination at 0, 3, 6 and 9 hr of Incubation on Day 3. (Mean of 7 ejaculates).

Treatment	Diluter	Hour of Incubation at 37°C				
		0	3	6	9	$\bar{X}$
1-Negative Serum	Egg Yolk	64.79	66.64	53.00^{b}	48.00	58.11^{b}
	Skimmilk	64.36	47.00	31.29^{b}	21.21^{b}	40.97^{b}
2-Serum titered 1:640	Egg Yolk	80.07	79.78	70.79^{a}	64.85	73.87^{a}
	Skimmilk	79.93	55.50	49.21^{a}	49.00^{a}	58.41^{a}
3-Serum titered 1:2560	Egg Yolk	76.85	71.57	61.00^{ab}	62.50	67.98^{a}
	Skimmilk	74.50	55.64	45.14^{ab}	28.57^{b}	50.96^{a}

different superscripts by hr indicate significant differences between treatments within diluter as tested by Duncan's Multiple Range Test ($P < .05$).

The vita has been removed from the scanned document

EFFECT OF ANTI-EGG-YOLK-DILUENT SERA UPON THE VIABILITY OF BOVINE SPERMATOZOA IN EGG YOLK DILUENT

by

Michael L. O'Connor

(ABSTRACT)

The first experiment was designed to determine if the antigenicity of egg yolk diluter could be eliminated by the addition of specific antibodies. Two virgin heifers were immunized with 20% yolk - 2.1% citrate - 7% glycerol with penicillin and streptomycin. Non-immune serum and immune sera having titers of 1:640 and 1:2560 were collected and frozen. Serum antibody titers were determined by the passive hemagglutination test. Utilizing equivalent proportions test it was determined that 80 volumes of anti-egg-yolk-diluent sera titered 1:2560 was necessary to neutralize 1 volume of 20% egg yolk diluent.

The second experiment examined the effect of immune sera against egg yolk diluter upon the viability of bovine spermatozoa in that diluter. Using a split-ejaculate technique, 7 ejaculates from 3 bulls were diluted in egg yolk-citrate diluter or skimmilk diluter, cooled, glycerolated and stored at 5°C. On the first day after semen dilution, complement-fixed immune serum titered 1:2560, 1:640 and non-immune serum were added to aliquots of diluted semen (1:9 v/v). Aliquots of each diluter without serum served as controls. Each treatment was evaluated immediately after addition of serum and again after 48 hr storage at 5°C. Percent intact acrosomes, percent motility and percent agglutination were measured from unfixed smears at 0, 3, 6 and 9 hr of incubation at 37°C. Both immune and non-immune serum treatments were characterized by head to

head agglutination. Within both diluters, there were no significant differences in motility or intact acrosomes due to immune and non-immune sera. However, all serum treatments were significantly higher ($P < .01$) in motility and percent intact acrosomes than the non-serum controls. The overall percent intact acrosomes across all hours, days and diluters were 77.9, 81.1, 81.2 and 81.7 for control, non-immune serum, 1:640 serum and 1:2560 serum, respectively. Differences in the percent head to head agglutination were not due to anitbody titer of the serum.