AN IN VITRO PROCEDURE FOR EVALUATING LEUCOCYTIC PHAGOCYTOSIS OF SPERMATOZOA IN THE BOVINE

by

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INTRODUCTION

The desire to make more rapid progress in the genetic improvement of animal populations had led to an increase in the use of artificial insemination. Artificial insemination enables us to distribute more efficiently the germ plasm of superior males. Since efficient reproduction is also important to domestic animal production, it has become important to develope techniques which enable us to accurately determine the fertility of these superior males, particularly the ability of their semen to undergo processes of collection, dilution, freezing and thawing, and still retain the ability to achieve high conception rates. From a practical standpoint, it would be most economical with respect to time and resources if the techniques used to assess semen fertility could be employed in the laboratory using visual or chemical criteria.

At the present time the more widely used criteria in the determination of semen quality are measures of cell viability (motility, acrosomal integrity, vital staining) and morphological normality (discriminating against the occurrence of cytoplasmic droplets, abnormal heads, abnormal tails, etc.). These parameters, especially motility and acrosomal integrity, have been useful in the evaluation and selection of males as well as the developement and improvement of methods of semen preservation and handling. However, the correlation of these criteria with fertility is not absolute. A source of potential error in predicting fertility from laboratory tests of semen quality may reside in the yet uninvestigated area involving the interaction between male gametes and the environment of the female reproductive tract.

Results of several investigators have shown that shortly after insemination, sperm disappear from the female tract (Dobrowolski and Hafez, 1970; Lineweaver, et al, 1970; Braden, 1953). A primary means of sperm cell loss is thought to be phagocytic removal by leucocytes which invade the female reproductive tract at estrus (Austin, 1957; Menge, et al, 1962; Yanagimachi and Chang, 1963; Bedford, 1965; Haynes, 1967). A phenomenon which had been reported by several authors, and which may be related to phagocytic removal of sperm cells, is the production of unequal ratios of offspring following heterospermic insemination of females with equal cell numbers from two or more males (Edwards, 1955; Beatty, 1957; Napier, 1961; Beatty, et al, 1969; Overstreet and Adams, 1971). Offspring ratios from heterospermic insemination not only coincide with fertility based on homospermic results and conventional laboratory measures of semen quality (Napier, 1961) but are also capable of detecting differences among males whose homospermic performance and semen quality are relatively equal (Beatty, 1960; Dziuk, 1965; Overstreet and Adams, 1971). The possibility has been suggested that the means by which the female is able to consistently select or favor the spermatozoa of one male over another is through a differential process of phagocytic removal of unqualified or less viable sperm cells (Overstreet and Adams, 1971).

The phagocytic process begins with a complex system of chemotaxis followed by recognition of the particle to be ingested. These events are then followed by attachment, ingestion and degradation. The recognition process probably involves a surface identification of the particle to be ingested by the phagocyte. Certain macromolecules within the membrane of the foreign particle may be responsible for this recognition (Boyden, 1963). Fawcett (1975) has shown from his studies of the sperm cell membrane, using freeze-fracture techniques and electron microscopy, that the spermatozoan surface is indeed quite complex, varying from one region to another. Because of this complexity there are undoubtedly characteristics of the spermatozoan membrane which are involved in determining its viability or acceptability to phagocytes and which cannot be assessed through gross microscopic observation and viability measurments such as motility and acrosomal integrity.

Another indication that the spermatozoan surface or membrane may be an important factor in semen evaluation is the process of agglutination. Senger and Saacke (1976) have shown that the addition of blood sera to diluted semen results in a very close head to head association between sperm cell membranes. Further, they have observed that only cells with intact cell and acrosomal membranes qualify for agglutination, and that the association between agglutinated cells is restricted to the cell membrane overlying the acrosome. Of more interest, however, is the observation that some sperm cells which have intact acrosomes are still unqualified for agglutination. Such cells undergo acrosomal deterioration more rapidly than agglutinated cells. Thus, loss of a cell's ability to agglutinate appears to be a prelude to structural deterioration. This would indicate that there is some characteristic of the spermatozoan membrane which is important to cell viability and fertility but cannot be assessed with conventional laboratory procedures and optics.

Since phagocytosis is a major cause of sperm cell loss from the female, and cell recognition is a surface phenomenon, it appears that an examination of this process may open new approaches to semen evaluation. A laboratory procedure which would enable us to identify or describe, even indirectly, those sperm characteristics which discourage phagocytosis could provide an additional means of developing new semen quality tests.

It was the objective of this study to develope in _in vitro_ procedure whereby evaluation of leucocytic phagocytosis of spermatozoa could be measured. Procedures have been described for _in vitro_ measurement of phagocytosis of bacteria and yeast by leucocytes from blood (Guidry, _et al_, 1974) and milk (Kent and Newbould, 1969; Paape, _et al_, 1977). Several factors are important in reaching optimum levels of phagocytosis in an _in vitro_ system. Newbould (1970; 1973), using milk leucocytes, reported that the addition of glucose to the incubation medium enhanced phagocytosis of staphylococci. Addition of serum containing specific antibodies to staphylococci had a more limited effect on increasing phagocytosis and non-immune serum had no effect. Hanks (1940) indicated that the ratio of leucocytes to particulate matter must be kept constant across incubations. Guidry, _et al_, (1974) reported an optimum leucocyte to yeast cell ratio of one to ten.

This work was undertaken to develope an *in vitro* system for measuring phagocytosis of spermatozoa by bovine mammary leucocytes. Three experiments were designed to accomplish the following objectives:

(1) to determine the effect of 1.25% glucose (w/v) on phagocytosis,

(2) to determine the rate and level of phagocytosis during incubation,

(3) to determine the effect of three sperm:leucocyte ratios (1:1, 2:1 and 1:2) on the rate and level of phagocytosis,

(4) to compare the variation in phagocytic level and rate among leucocyte populations from a single donor and from different donors,

(5) to compare phagocytic measurements made on unfixed wet smears with those made on fixed, stained preparations, and

(6) to evaluate the phagocytosis of live vs freeze-thaw killed populations of spermatozoa.

REVIEW OF LITERATURE

Disappearance of Spermatozoa from the Female Reproductive Tract

The number of spermatozoa which can be recovered from the female tract following insemination decreases rapidly with relatively few sperm actually reaching the site of fertilization in the fallopian tubes. Braden (1953) reported that, in rabbits mated with bucks ejaculating from 50 to 700 x 10^6 cells, only about one percent could be recovered one hour after insemination. Austin (1957) observed that the mean number of spermatozoa which could be recovered from mouse uteri decreased from 20 x 10^6 at two to six hours post insemination to 0.4 x 10^6 at 20 to 24 hours. Quinlivan and Robinson (1969) recovered approximately 0.1% of 25000 x 10^6 sperm from control ewes and 0.007% from progesterone-synchronized ewes 24 hours post insemination.

Percent recovery of cells is partially dependent upon both the condition of the sperm cell (Menge, et al, 1962; Lineweaver, et al, 1970; Baker and Degan, 1972; Pursel, et al, 1978) and upon the endocrine status of the female (Menge, et al, 1962; Mahajan and Menge, 1965; Haynes, 1967). Lineweaver, et al (1970) recovered only half as many cells from bovine reproductive tracts inseminated with frozen semen as compared to fresh semen. Baker and Degan (1972) recovered significantly fewer dead cells from gilt tracts which had been inseminated with live flourescent-stained and dead unstained spermatozoa.

Pursel, _et al_ (1978) recovered significantly fewer sperm cells from the oviducts, uterotubal junction and uterus of gilts inseminated with frozen semen, as opposed to those which had been inseminated with fresh semen. Using live and cold-shock killed rabbit and bull spermatozoa, Menge, _et al_ (1962) recovered significantly fewer dead than live bull spermatozoa after 20 to 38 hour _in vivo_ incubations in both estrus and luteal phase rabbit uteri. However, Haynes (1967) found no significant difference in the rate of disappearance of bull spermatozoa from aliquots which had been washed (containing a large percentage of dead or immotile cells) compared to those which had not been washed, following _in vitro_ incubations with leucocytes and flushings from rabbit uteri.

Several theories have been proposed for the mechanism of sperm removal from the female genital system. Blandau and Odor (1949) and Mattner and Braden (1969) have reported that in the rat and ewe, many sperm are lost through backflow into the vagina and eventual evacuation to the exterior following natural mating. Absorption of spermatozoa into the uterine mucosa and eventual dissolution was reported by very early workers (Kohlbrugge, 1910; 1913; Kuchner, 1954). Austin (1957) failed to confirm these observations in the mouse, and Vojtiskova (1956) reported the appearance of sperm cells within the uterine mucosa to be a result of tissue preparation for sectioning and histological evaluation. Later work by Austin (1960) showed that embedding of sperm cells into the uterine glands occurs in those species such as the bat, hedgehog and mole, where sperm are stored in the female tract for long periods of time. The only instance in which Austin (1960) observed actual penetration of spermatozoa into the mucosal epithelium was in conjunction with acute infection and tissue trauma. However, more recent observations by Phillips and Mahler (1977a) indicate a phagocytic action of the vaginal mucosal epithelium in the rabbit. Sperm cells were observed within vaginal epithelial cells up to seven days post coitus.

Another means of sperm removal which has gained increasing attention is phagocytosis by neutrophilic polymorphonuclear leucocytes which infiltrate the female reproductive tract in abundant numbers during estrus. This phenomenon has been observed by Austin (1957), Menge, _et_ _al_ (1962), Yanagimachi and Chang (1963), Bedford (1965), Marcus (1966) and Haynes (1967).

Leucocyte Infiltration of the Female Reproductive Tract

Austin (1957) reported that a decline in the concentration of sperm found in the uterus of the mouse was accompanied by a corresponding increase in the concentration of leucocytes. At two to six, eight to 12, 14 to 18 and 20 to 24 hours post insemination, the sperm to leucocyte ratio was 1000:3.6, 10:6.7, 1:2.3 and 1:8.0, respectively. The mean number of leucocytes increased from 0.07 x 10^6 at two to six hours post insemination to 25 x 10^6 at 14 to 18 hours. The majority of sperm recovered 14 hours after insemination were undergoing phagocytosis.

In heifers, Mahajan and Menge (1965) observed a maximum leucocyte infiltration of the reproductive tracts 12 hours following artificial insemination. Phillips and Mahler (1977a) have reported the appearance of increased leucocyte numbers in venules of the rabbit vagina 45 minutes after mating, with subsequent migration to the vaginal lumen within three hours.

Mattner (1968), in a study using mated and unmated goat does, observed a greater number of leucocytes present in the cervix, uterine lumen and oviducts of mated does. The number of leucocytes flushed from mated vs unmated females was 315,000 vs 56,000, 521,000 vs 158,000 and 470 vs 320 for the cervix, uterine lumen and oviducts, respectively. Yanagimachi and Chang (1963) observed that the maximum infiltration of leucocytes into the uteri of unmated female hamsters occurred in very early metestrus, while maximum infiltration in mated females occured approximately six hours earlier. They further demonstrated that accelerated leucocyte invasion in mated females was not a result of cervical or vaginal stimulation, nor of distention of the uterine lumen, and concluded that the semen itself was chemotactic to leucocytes.

Marcus (1966) reported that epididymal spermatozoa elicited a greater leucocyte response than did either Ringer's solution (sterile or unsterile) or seminal plasma in both estrogen- and progesterone-treated ovariectomized hamsters. No differences were observed between the chemotactic abilities of either motile or non-motile, or between rat and hamster spermatozoa. Using a modification of Boyden's technique for measuring chemotaxis, Maroni, et al (1972) found that both sperm and seminal plasma attract leucocytes in the presence of blood serum or plasma, but not in their absence. Seminal plasma was also found to be chemotactic in the presence of complement-fixed serum. Maroni, et al (1972) have suggested that this cytotaxigenic activity may act through either (1) the conventional complement system, (2) a non-complement-dependent pathway via proteolytic splitting of serum proteins and subsequent release of chemotactically-active products, or (3) the presence of bacterial endotoxins.

Effect of Reproductive Phase on Leucocytic Infiltration and Phagocytic Activity

The inflammatory response in the female reproductive tract has been shown by several investigators to be dependent on reproductive phase, with more efficient bacterial removal and resistance to infection occuring during estrus (Rowson, et al, 1953; Black, et al, 1954; Hawk, 1958; 1959; Broome, et al, 1960; Hawk, et al, 1960). Killingbeck (1963) observed *in vitro* that inert starch particles are phagocytosed more efficiently by leucocytes obtained from the estrus uterus than those obtained from either the pseudopregnant uterus or the peritoneal cavity.

Several studies have been conducted to determine the effect of the endocrine state of the female on leucocyte infiltration (Menge, *et al*, 1962; Marcus, 1966; Howe, 1967; Haynes, 1967) and phagocytosis of spermatozoa, both *in vivo* (Menge, *et al*, 1962; Mahajan and Menge, 1965; Bedford, 1965; Haynes, 1967) and *in vitro* (Bedford, 1965; Haynes, 1967).

Howe (1967), studying the leucocytic response in estrus and pseudopregnant rabbit does, found a greater number of leucocytes in estrus females following insemination. Haynes (1967) also observed that the rate of leucocyte infiltration was greater for estrus than for pseudopregnant uteri of rabbit does, and that the degree of infiltration was inversely correlated to the number of spermatozoa recovered following *in vivo* incubation. Marcus (1966), using ovariectomized hamsters treated with estrogen or progesterone, found the leucocytic response following insemination to be much less in pregesterone-treated females than in those treated with estrogen.

Contrary to the results reported by most authors, Menge, *et al* (1962) observed similar numbers of leucocytes in the uteri of both estrus and pseudopregnant rabbits following insemination. These observations, however, were made 20 to 38 hours post insemination, while those of other authors were made following much shorter incubation intervals, post insemination.

Mahajan and Menge (1965) studies the recovery of bull spermatozoa from ligated uteri and oviducts of luteal and estrus heifers at six, 12, 18 and 24 hours post insemination. At all time intervals, fewer sperm were recovered from estrus than from luteal phase females. These authors also observed that the maximum leucocytic response occured at 12 hours post insemination in estrus heifers as opposed to 18 hours for luteal heifers.

Menge, *et al* (1962) reported differences in the recovery of rabbit and bovine spermatozoa after *in vivo* incubation for 20 and 38 hours in estrus and pseudopregnant rabbit uteri. Their results showed faster removal of bull spermatozoa from the estrus uterus of the rabbit than from the pseudopregnant uterus, while the opposite was observed for rabbit sperm.

The results of Menge, *et al* (1962) indicate that leucocytes invading the female reproductive tract may have the capacity to differentiate between species of spermatozoa. However, results of later studies conducted by Haynes (1967) are in contradiction with those of Menge, *et al* (1962). Haynes (1967), using *in vitro* incubations of bovine and rabbit spermatozoa with uterine leucocytes and flushings from estrus and pseudopregnant rabbit does, observed a more rapid decline in the number of rabbit spermatozoa incubated with leucocytes from estrus uteri, while the disappearance of bull spermatozoa was the same for leucocytes from both estrus and pseudopregnant rabbit uteri. In another experiment utilizing *in vivo* incubations of bull and rabbit sperm in estrus and pseudopregnant rabbit uteri, Haynes (1967) found that both bull and rabbit spermatozoa disappeared more rapidly from the estrus uterus. However, these *in vivo* incubations still showed no significant difference between the rate of disappearance of bull and rabbit spermatozoa. It is important to note that, while the aforementioned authors did make some observations of acutal ingestion of spermatozoa by leucocytes, for the most part, quantitation of phagocytosis, if made, was done only indirectly by measurements of sperm disappearance.

From electron micrographic studies, Bedford (1965) observed differences in the phagocytic behavior of leucocytes from estrus and luteal phase uteri and leucocytes from the peritoneal cavity. Both intact and damaged spermatozoa were phagocytosed by leucocytes after incubation in estrus uteri of rabbits, while only damaged sperm cells were observed within leucocytes following incubation in pseudopregnant uteri or the peritoneal cavity. *In vitro* incubation of spermatozoa with leucocytes obtained from the estrus uterus resulted in phagocytosis of only damaged cells. Bedford (1965) postulated that some factor present in the estrus uterus, perhaps involved in the capacitation process, renders all spermatozoa acceptable to leucocytic phagocytosis, while a phagocytic inhibitor present in the progesterone dominated uterus leads to removal of only seriously damaged cells. This would also explain the less efficient phagocytosis observed by other investigators in the luteal or pseudopregnant uteri.

Selective Removal of Spermatozoa from the Female Reproductive Tract

Evidence for selective removal of sperm cells from the female reproductive tract is seen in the results of competitive breeding studies. Several investigators have observed the production of unequal ratios of offspring when females are inseminated with equal numbers of cells from two or more males (Edwards, 1955; Beatty, 1957; 1960; Napier, 1961; Roche, _et al_, 1968; Beatty, _et al_, 1969; Overstreet and Adams, 1971). Edwards (1955) and Beatty (1957; 1960) have observed that the superiority of males in competitive matings is constant and reproducible across experiments.

The mechanism by which one male is able to repeatedly sire a greater proportion of offspring is not clear. It has been suggested by Beatty (1957) that the ejaculate of the superior male contains a greater proportion of viable cells, however, he does not define viability. Beatty (1957) has shown that many males which are inferior when used competitively are still capable of normal homospermic fertilization. However, differences in homospermic fertility are generally magnified when males are ranked based on competitive fertilization (Beatty, 1957). He further demonstrated, in rabbits, that the unequal numbers of offspring from competitive matings are not a result of embryonic mortality, since the average litter size was not significantly reduced when the proportion of semen from inferior males was increased. In a later study, Beatty, _et al_ (1976) also demonstrated that fresh semen from bulls which were of equal fertility became unequal following freezing at -196° C, indicating that males differ in the ability of their semen to undergo freezing and thawing procedures.

Experiments which have involved sperm transport in conjunction with heterospermic insemination provide more evidence for differential removal of spermatozoa by phagocytosis. Overstreet and Adams (1971) flushed different segments of the female tracts of rabbit does six and 13 hours following heterospermic insemination of equal cell numbers from two bucks. A fluorescein-isothiocyanate marker was used to differentiate the cells of one male from the other. At six hours post insemination the ratio of cells recovered was still equal. However, by 13 hours post insemination there was a significantly greater proportion of sperm from the superior buck recovered from all segments of the female tract. Similar observations were made by Ferreira and Graves (1972). Segments of female reproductive tracts of rabbits inseminated competitively and flushed 17 to 18 hours later yielded a significantly greater proportion of spermatozoa from the bucks which were heterospermically superior. Since the inequality between the number of cells recovered for males used competitively was not observed until 13 hours or 17 to 18 hours post insemination in both of these studies, it is doubtful that differential sperm transport is responsible, but rather, the cells of the superior male were retained within the female tract for a longer period of time while those of the inferior male were removed.

Selectivity of Phagocytosis

Selective phagocytosis then, through more active or efficient removal of cells of an inferior male, especially if those cells are structurally damaged, represents a possible mechanism by which one male exhibits superiority over another in competitive breeding. It may be that spermatozoa differentially interact with the fluid constituents of the female reproductive tract, resulting in less viable cells being more susceptible to phagocytic removal. Mattner (1969) using an in vitro incubation system consisting of a glass microscope slide held at 37° C with a stage warmer, observed that leucocytes obtained from the bovine estrus cervix in the presence of cervical mucus phagocytosed motile spermatozoa, while dead (immotile) spermatozoa were not not observed to be phagocytosed under these same conditions. Subsequent incubations in the presence of cervical mucus which had been "liquified", however, resulted in phagocytosis of both live and dead spermatozoa. Symons (1967), using immunofluorescence to observe the attachment of serum globulins to rabbit spermatozoa recovered from the reproductive tract of rabbit does, observed that a certain proportion of sperm recovered remained strongly motile and non-fluorescent. Dead cells showed the strongest fluorescence. Attachment of serum globulins resulting in fluorescence was an indication of cell injury and deterioration based on weak motility and enlargement and loosening of the acrosome. Symons (1967) also observed that those spermatozoa recovered from the female tract which showed fluorescence were either adherent to leucocytes or in the process of being ingested.

It is difficult at this time to determine if leucocytes engage in selective removal of spermatozoa, since the mechanisms of chemotaxis, recognition and attachment, especially in the absence of a specific opsonin, are poorly understood. There may be other substances (proteins) present in the reproductive tract fluids which are degraded by enzymes present in the seminal plasma, releasing products which can act as non-specific cytotaxigens or opsonins (Maroni, et al, 1972). According to Stossel (1974), once leucocytes reach a site of inflammation they do exhibit a definite selectivity with regard to particulate matter. He postulates that the selectivity is a matter of recognizing certain surface characteristics on the particles (in this case bacteria) to be ingested. However, the specific "trigger" for attachment is very subtle and has yet to be clearly identified. In the case of bacteria, specific antibodies are a major factor in the recognition and attachment of phagocytes. Mechanisms of recognition where a specific antibody is present, as in the case of bacteria, involve the complement system and/or the attachment of specific serum globulins (IgG), however, even this process needs further clarification (Stossel, 1977). Stossel (1972) has also noted that the acceptability of particles to phagocytic cells is influenced by changes in their net surface charge and in the hydrophobic properties of their plasma membrane. It is interesting to note that certain bacteria, especially those with polysaccharide capsules, are capable or preventing or eluding phagocytosis. Berlin (1972) has demonstrated that treatment of rabbit peritoneal leucocytes with Concanavalin A (Con A) significantly reduced in vitro phagocytosis of inert (polyvinyltoluene) particles. This inhibition of phagocytosis was shown to be reversible by the addition of certain sugars, which have previously been demonstrated to bind specifically to Con A *in vitro* (Goldstein, *et al*, 1965). These observations suggest that leucocytes may possess membrane binding sites which are important in the initiation of phagocytosis (recognition and attachment) and which are inactivated or occupied by the Con A molecules (Berlin, 1972).

Although a better comprehension of the mechanisms involved in phagocyte "recognition" and the membrane characteristics of the spermatozoon is needed, evidence indicates that selective removal of spermatozoa from the female reproductive tract following insemination can be a major factor in determining fertility.

EXPERIMENTAL PROCEDURE

Induction of Diapedesis and Collection of Milk Leucocytes

An inflammatory response was induced in the mammary gland of the cow by infusion of a sterile 0.5% solution of oyster glycogen in accordance with the technique described by Paape, *et al* (1977).

Physiological saline containing 0.85 g of NaCl in 100 ml of distilled water was sterilized by autoclaving at 15 psi and 121° C for ten minutes in 125-ml serum vials. Five tenths gram of oyster glycogen (Sigma, Type II) in a 125-ml serum vial, fitted with a rubber stopper, was autoclaved at 15 psi and 121° C for 20 minutes. The sterile saline and oyster glycogen were stored at 5° C for one to four weeks until needed for infusion, at which time 100 ml of the sterile saline was transferred into the 125-ml vial containing the oyster glycogen.

All quarters of the donor cow were milked out as usual and the cow was prepared for infusion of the antigen by thoroughly cleaning the teat end with cotton balls soaked in 70% ethanol. A 100-ml dose of saline-glycogen solution was infused into one or more quarters through the external teat orifice using a 50-ml sterile syringe and 14 gauge blunt cannulation needle, which had been sterilized with absolute ethanol. Following infusion of the glycogen solution, the teat was again cleaned thoroughly with 70% ethanol, followed by dipping in *Nolvasan* or a dilute solution of *Clorox*.

Twelve hours after infusion, milk was removed from all quarters, regardless of whether all quarters had been infused. Three hours after milk removal (approximately 15 hours post infusion), ten IU of oxytocin (Sigma) was administered intramuscularly. The mammary gland was washed with warm water and dried. The teats of the infused quarters were cleaned with 70% ethanol, and milk from treated quarters only was collected into a clean, silicone-treated 500-ml Erlenmeyer flask. Following milk collection, the teat end was again cleaned with 70% ethanol.

Leucocytes were recovered from the milk as outlined by Paape, *et al* (1977), with modifications. The collected milk was initially filtered through two layers of cheese cloth to remove large cell clots and debris. The filtered milk was then transferred to 50-ml silicone-treated centrifuge tubes and centrifuged at 120 g and 5° C for 15 minutes. Following centrifugation the supernate was removed by aspiration and all visible fat removed from the sides of the centrifuge tube using a *Kimwipe*. The cell pellets were resuspended in 3-4° phosphate buffered saline (PBS; pH 7.5) with mild vortexing. The resuspended pellets were combined and centrifuged at 120 g and 5° C for 15 minutes. The supernate was removed by aspiration and the cell pellet was again resuspended in a minimum (amount depending on the size of the cell pellet) of 3-4° PBS with mild vortexing. The third wash with PBS (recommended by Paape, *et al*, 1977) was omitted to minimize cell loss and clumping. All resuspended cells were combined in a cold silicone-treated Erlenmeyer flask.

The concentration of the leucocyte suspension was determined by pipetting 0.1 ml of the cell suspension into a test tube containing 7.9 ml cold PBS. This 1:80 dilution was counted using a hemacytometer under phase contrast optics. The leucocytes were then diluted to appropriate concentrations using 3-4° C PBS. The diluted leucocyte suspension was brought to room temperature just prior to incubation.

Collection and Dilution of Semen

Semen was collected from bulls at the VPI Dairy Cattle Research Center one to three days prior to the collection of leucocytes. Semen was pooled from two or three bulls and the concentration determined colorimetrically. Pooled semen of not less than 60% initial motility was diluted to appropriate concentrations using skimmilk diluter (Thacker and Almquist, 1953). The diluted semen was cooled gradually to and stored at 5° C until the day of incubation, at which time it was allowed to reach room temperature just prior to initiation of the incubation period. A split ejaculate (of pooled ejaculates) technique was used throughout this work.

Skimmilk diluter was prepared from milk obtained from the bulk tank at the VPI Dairy Cattle Research Center. The milk was centrifuged at 4080 g for five minutes after which the cream layer was removed. The supernate was collected into a 1000-ml Erlenmeyer flask, which was placed in a large beaker containing water. The skimmilk was heated to a temperature of 96° C for ten minutes (Thacker and Almquist, 1953), cooled to room temperature and antibiotics added at a rate of 1000 units of potassium penicillin G and 1000 μg dihydrostreptomycin sulfate per ml of diluent. The skimmilk was transferred to 15-ml disposible culture tubes with caps and frozen until needed for dilution of semen.

Procedure for Assessing Phagocytosis

All incubations were carried out in a 37° C water bath with agitation. Spermatozoa and leucocyte cell suspensions were pipetted into silicone-treated Erlenmeyer flasks in the appropriate cell ratios. According to the experimental design, treatment flasks were momentarily removed throughout the incubation to obtain aliquots from which smears were prepared. The aliquots were placed in five-ml silicone-treated test tubes containing 0.1 to 0.2 ml anticoagulant (citric acid-citrate-dixtrose; Paape, et al, 1975) and vortexed briefly to break cell clumps.

Dry smears to be fixed were prepared the same as blood smears. A small drop of the aliquot (sperm-leucocyte mixture) was spread the length of a glass microscope slide, coated with Mayer's Albumen, using the back edge of a second slide. Smears were dried quickly on a 55° C hotplate with a fan blowing air across the surface. Smears were permitted to remain on the hotplate for 30 to 40 minutes and then were placed in 100% methanol for three to 24 hours. The fixed smears were stained using Hematoxylin and alcoholic Eosin according to the procedure described in Appendix B. Three smears were made for each incubation interval sampled from each treatment flask.

Wet smears were prepared from the same aliquot as the fixed-stained smears. A small drop of the sperm-leucocyte mixture was placed on a glass slide and gently covered with a 1.5 coverslip such that the cell suspension spread evenly to the extremities of the coverslip. For the wet smears, counts were carried out within five to ten minutes of smear preparation.

Counts of wet smears were made using phase contrast optics at 900 X (oil immersion), while those of fixed-stained smears were made using brightfield optics at 1000 X (oil immersion) magnification. Two measurements of phagocytosis were made on both wet and fixed-stained smears. Percent phagocytosis (PP) was obtained by making differential counts of 100 neutrophils, and recording those containing spermatozoa as opposed to those which did not. Measurments of spermatozoa per ingesting leucocyte (S/L) were obtained by counting the number of spermatozoa within each phagocytosing leucocyte and dividing by the number of leucocytes (per 100 cells counted) ingesting spermatozoa. Two counts of 100 cells were carried out on each of two smears for every incubation interval sampled, and the average value for each smear was recorded. All smears were coded prior to evaluation to minimize bias.

Experiment I : Effect of Glucose on Phagocytosis

This experiment was conducted to evaluate the effect of an additional energy source (glucose) on phagocytosis. Four incubations were conducted, each on a separate day using different populations of leucocytes collected from the same cow, and pooled ejaculates from two or three bulls. Each incubation will be referred to as a replicate.

A 1:1 ratio of spermatozoa to leucocytes was obtained by combining 15 ml of a 50 x 10^6 cells per ml suspension of each cell type. Each replicate consisted of two treatment flasks. To one of the flasks was added 1.25% (w/v) dextrose (d-glucose). In the first two replicates, the glucose was added to the sperm-leucocyte suspension. The addition of glucose in replicates three and four was made prior to the addition of the cells, with the sperm suspension being added prior to the addition of the leucocytes. Alternately, at each of the four replicates, one of the incubations (treatment flasks) was initiated 30 minutes after the other to allow time for evaluations of wet smears to be made at one-hour intervals. Wet counts began after 60 minutes of incubation. Dry smears were made at 10, 20, 30, 40, 60, 120, 180 and 240 minutes of incubation. Counts of both PP and S/L were made on fixed-stained smears. Only determinations of PP were made on wet smears.

Data from the counts made on wet and fixed-stained smears were transformed to natural logrithms and subjected to analysis of variance with sources of variance being: Replicate (leucocyte-sperm population), Treatment (with or without glucose) and Time of incubation. Replicate was considered a random effect, while all other sources of variation were considered fixed.

Experiment II ; Sperm Cell to Leucocyte Ratio

The effect of sperm cell to leucocyte ratio was evaluated in two series of incubations. Each incubation will be referred to as a replicate. The first series (Experiment IIA: within cow) consisted of four replicates carried out using leucocytes from the same cow collected on different days. The second set (Experiment IIB: among cows) consisted of seven replicates carried out using leucocytes collected from a different cow for each replicate. Because of the variability in leucocyte populations with respect to levels of phagocytosis encountered in Experiments I and IIA, viability estimates using Trypan Blue vital staining (Newbould, 1967) were made for the leucocyte populations (replicates) in Experiment IIB.

This experiment consisted of three treatment flasks, each containing one of three sperm to leucocyte ratios, 1:1, 2:1 or 1:2. Two cell concentrations, 100 x 10^6/ml and 50 x 10^6/ml, were made of both sperm cells and leucocytes. Each incubation mixture consisted of equal volumes of sperm and leucocyte suspensions at appropriate concentrations for each ratio. This resulted in a total cell concentration of 75 x 10^6/ml for ratios of 2:1 and 1:2, and a concentration of 50 x 10^6 cells per ml for the 1:1 ratio.

All three incubations were initiated simultaneously and carried out for a total of five hours. Dry smears were made from each incubation flask at ten-minute intervals for the first hour and 30-minute intervals thereafter. Wet smears and counts were made alternately every 30 minutes for each treatment ratio, resulting in a total of three evaluations of each ratio at 1.5-hour intervals. Determinations of PP and S/L were made on both wet and fixed-stained smears.

Data from fixed-stained smears were transformed to natural logrithms and subjected to analysis of variance with sources of variance being: Replicate (sperm-leucocyte population), Treatment (sperm:leucocyte ratio) and Time of incubation. Replicate was considered a random effect, while all other sources of variation were considered fixed. Data collected from within cow replicates (Experiment IIA) and among cows replicates (Experiment IIB) were analyzed separately.

Experiment III : Phagocytosis of Live vs Killed Populations of Spermatozoa

This experiment consisted of four replicates (incubations) carried out using leucocytes collected from different cows and semen from two different bulls at each replicate.

All procedures for the recovery of leucocytes were the same as those described for Experiments I and II with the exception that the raw milk was pre-diluted 1:1 with PBS (37° C) prior to the first centrifugation. This was done in an attempt to increase the number of leucocytes recovered (Phipps and Newbould, 1966).

Treatments consisted of leucocytes combined with live or freeze-thaw killed spermatozoa from each of two different bulls in a ratio of 2:1, sperm to leucocytes. This resulted in a total of four incubation flasks for each replicate.

All incubations were initiated simultaneously and carried out for a total of 2.5 hours. Fixed smears were made at ten-minute intervals during the first hour and at 30-minute intervals thereafter. Wet smears were not evaluated in this experiment. Determinations of both PP and S/L were made on fixed-stained smears.

Data from counts were transformed to natural logrithms and subjected to analysis of variance with sources of variance being: Replicate (sperm-leucocyte population), Bull, Treatment (live vs killed spermatozoa) and Time of incubation. Replicates were analyzed both separately, to determine bull effect, and together to determine the effect of live vs killed spermatozoa. Replicates were considered random while all other sources of variation were considered fixed.

RESULTS

Experiment I : Effect of Glucose

The addition of 1.25% glucose to the incubation medium significantly depressed the percent phagocytosis (PP) as determined from both wet and fixed-stained smears ($P < .05$) as well as the mean number of spermatozoa per ingesting leucocyte (S/L) which was measured from fixed-stained smears only ($P < .05$).

The mean PP and standard deviations with and without glucose is presented in Table 1. With glucose, PP after four hours of incubation was 24.2 ± 9.1 (fixed smears) and 23.6 ± 4.7 (wet smears), as opposed to 42.8 ± 8.3 (fixed smears) and 46.3 ± 7.7 (wet smears) without glucose.

The mean S/L and standard deviations with and without glucose, as measured from fixed-stained smears only, is presented in Table 2. After four hours incubation, S/L in the presence of glucose was 1.09 ± 0.06 compared to 1.17 ± 0.10 without glucose.

A comparison of PP in the presence and absence of glucose throughout the four-hour incubation is presented graphically in Figure 1. During the initial 30 minutes of incubation the change in PP over time was 1.86 percent per minute and 2.53 percent per minute in the presence and absence of glucose, respectively. Between 30 and 240 minutes of

Table 1. Percent Phagocytosis (PP) in the Presence and Absence of Glucose as Determined from Wet and Fixed-Stained Smears (means ± SD of four replicates*).

	Treatment			
	With Glucose		Without Glucose	
Time (min)	Fixed	Wet	Fixed	Wet
10	5.2 ± 3.4		13.7 ± 3.9	
20	8.9 ± 2.9		28.9 ± 7.2	
30	13.6 ± 5.4		36.1 ± 6.4	
40	16.2 ± 6.2		35.9 ± 4.2	
60	17.6 ± 7.8	15.1 ± 7.2	40.1 ± 7.3	35.9 ± 2.3
120	15.2 ± 3.7	20.4 ± 5.5	42.0 ± 9.5	41.1 ± 6.1
180	20.6 ± 7.8	22.7 ± 5.4	47.4 ± 9.4	44.9 ± 7.5
240	24.2 ± 9.1	23.6 ± 4.9	43.8 ± 8.3	46.3 ± 7.7
$\overline{X}$	15.2 ± 8.2	20.4 ± 6.5	35.7 ± 12.3	42.0 ± 7.2

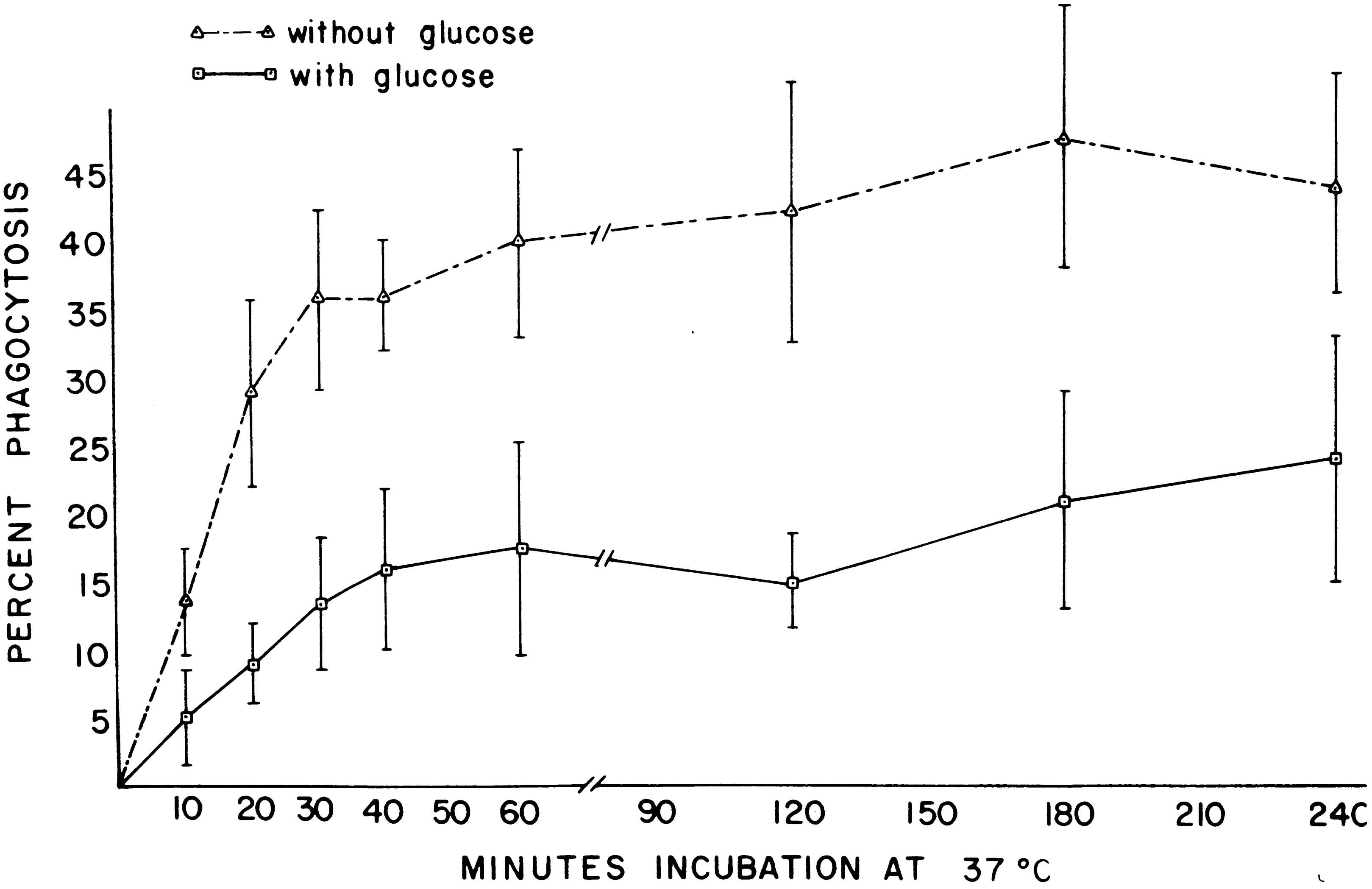
* each replicate represents a population of leucocytes from a single donor cow, taken on different days, and pooled ejaculates from two or three bulls.

Table 2. Number of Spermatozoa Per Ingesting Leucocyte (S/L) in the Presence and Absence of Glucose, Based on Evaluations of Fixed-Stained Smears (means ± SD of four replicates*).

Time (min)	Treatments	
	With Glucose	Without Glucose
10	1.00 ± 0.00	1.01 ± 0.01
20	1.00 ± 0.00	1.09 ± 0.04
30	1.03 ± 0.02	1.12 ± 0.07
40	1.04 ± 0.02	1.14 ± 0.09
60	1.03 ± 0.02	1.17 ± 0.05
120	1.06 ± 0.06	1.19 ± 0.04
180	1.06 ± 0.04	1.22 ± 0.11
240	1.09 ± 0.06	1.17 ± 0.10
$\bar{X}$	1.04 ± 0.04	1.14 ± 0.09

* each replicate represents a population of leucocytes from a single donor cow, taken on different days, and pooled ejaculates from two or three bulls.

Figure 1. Percent phagocytosis (PP) of spermatozoa in the presence and absence of glucose (means ± SD of four replicates, based on evaluations of fixed-stained smears).


without glucose
with glucose
PERCENT PHAGOCYTOSIS
45
40
35
30
25
20
15
10
5
10
20
30
40
50
60
90
120
150
180
210
240
MINUTES INCUBATION AT 37 °C

incubation the change in PP over time declined to 0.20 and 0.10 percent per minute in the presence and absence of glucose, respectively.

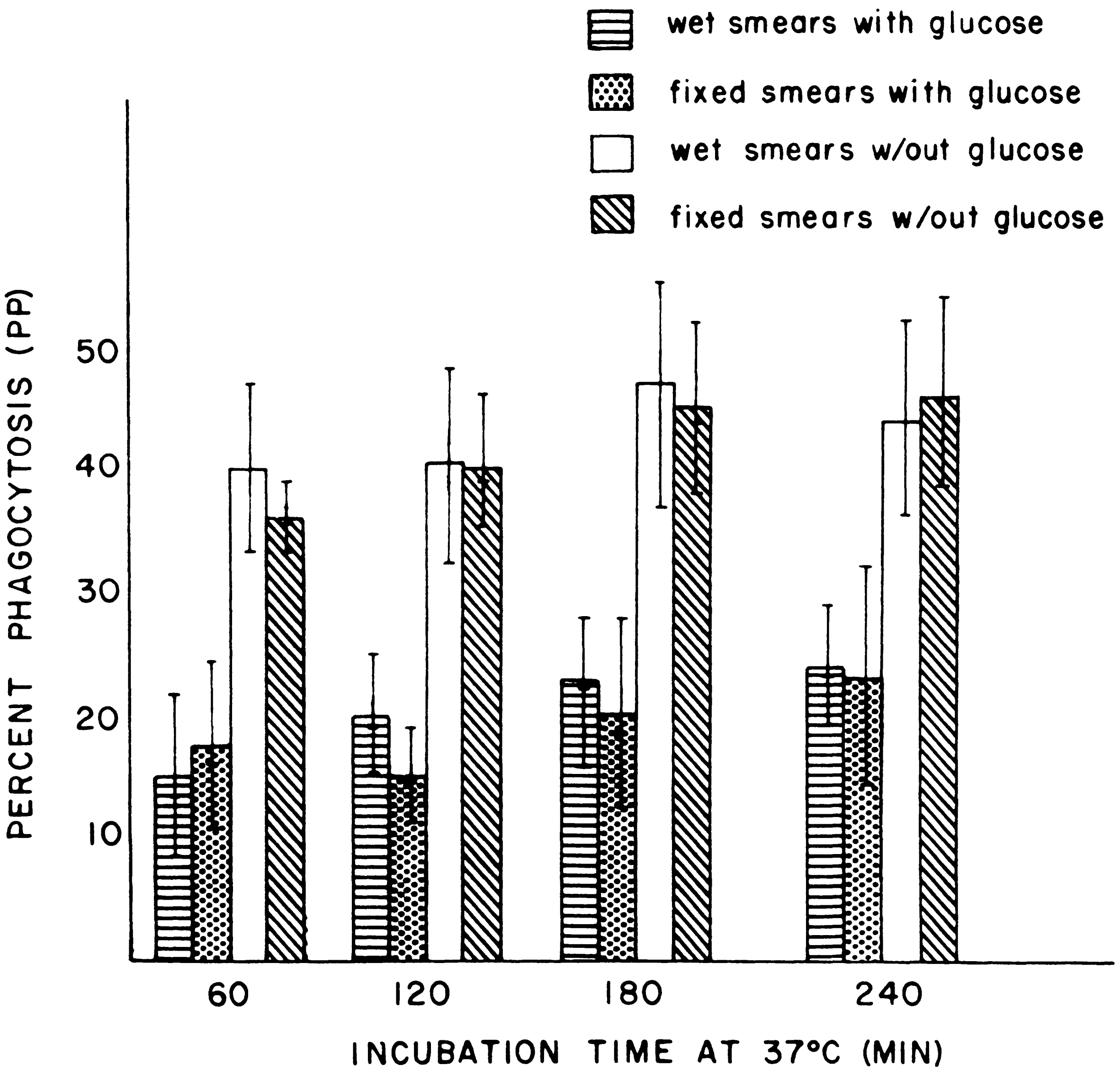
Analysis of variance for PP of spermatozoa based on evaluations of fixed-stained smears is presented in Table 1 of Appendix A. Significant effects were due to replicate, time ($P < .01$) and glucose addition ($P < .05$). There was also significant variation due to a replicate by glucose interaction ($P < .01$). However this interaction was due to the magnitude of phagocytic response to glucose among replicates and not different responses.

Analysis of PP based on counts from wet smears is presented in Table 2 of Appendix A. Again, significant effects were due to glucose, time ($P < .05$) and a replicate by glucose interaction ($P < .01$).

Analysis of variance of S/L, based on evaluations of fixed-stained smears is presented in Table 3 of Appendix A. Only time of incubation ($P < .01$) and glucose treatment ($P < .05$) were significant.

The correlation between counts made on wet vs fixed-stained smears for PP across all replicates and incubation times and treatments was significant, with $r = .87$ ($P < .01$). The correlation between wet and fixed-stained smears with the variation due to glucose removed was also significant, ($P < .01$) with $r = .53$ in the presence of glucose and $r = .64$ in the absence of glucose. A comparison of wet and fixed-stained smears in the presence and absence of glucose is illustrated in the histogram in Figure 2.

Figure 2. Comparison of wet vs fixed-stained smears in the evaluation of percent phagocytosis (± SD) in the presence and absence of glucose.


wet smears with glucose
fixed smears with glucose
wet smears w/out glucose
fixed smears w/out glucose
PERCENT PHAGOCYTOSIS (PP)
10
20
30
40
50
60
120
180
240
INCUBATION TIME AT 37°C (MIN)

The correlation between the two measurements of phagocytosis (PP and S/L), across all replicates, times and treatments, based on evaluations of fixed-stained smears only, was also significant, with $r = .79$ ($P < .01$).

Experiment II : Sperm Cell to Leucocyte Ratio

This experiment was conducted to evaluate variations in phagocytosis due to different low sperm to leucocyte ratios. Two experiments were carried out (IIA and IIB), the first (IIA) comparing sperm to leucocyte ratios with leucocytes obtained from a single donor cow on different days (replicates within cow). In the second experiment (IIB), leucocytes from seven different cows were utilized (replicates among cows).

IIA Replicates Within Cow. Means and standard deviations of PP as evaluated from wet and fixed-stained smears for the three test ratios are shown in Table 3. Means and standard deviations for S/L based on evaluations of wet and fixed-stained smears for the three test ratios are shown in Table 4.

The means for PP across incubation times and replicates, based on counts from fixed-stained smears, are illustrated graphically in Figure 3. From this graphic illustration it may be noted that the change in PP with respect to time is greatest during the first 30 to 60 minutes and then declines markedly. The change in PP over time during the initial 30 minutes of incubation for sperm to leucocyte

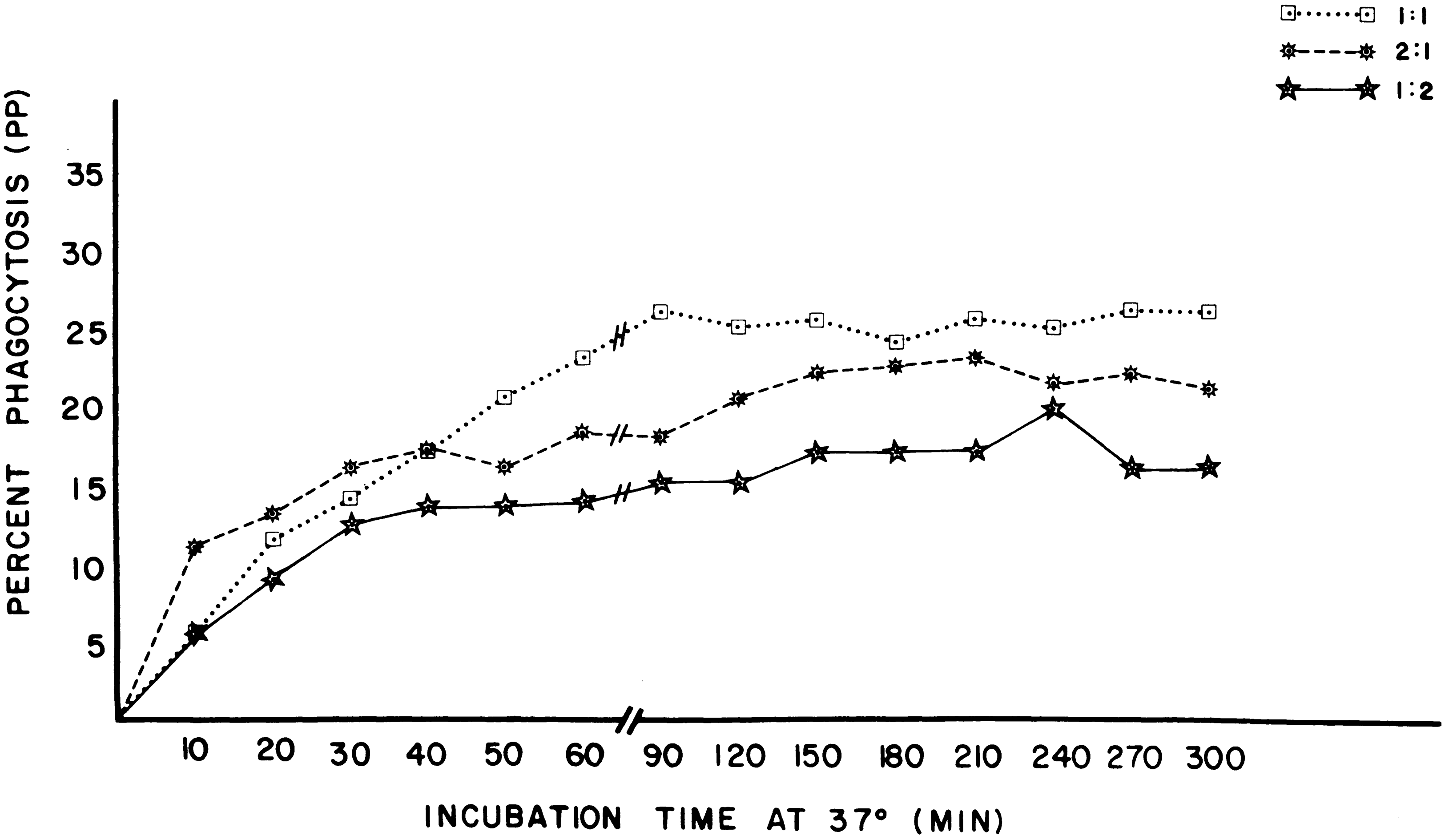
Table 3. Effect of Three Sperm to Leucocyte Ratios on Percent Phagocytosis (PP) as Determined from Wet and Fixed-Stained Smears (means ± SD of four replicates from Experiment IIA: Replicates Within Cow).

Time (min)	Sperm:Leucocyte Ratio					
	1 : 1		2 : 1		1 : 2	
	Fixed	Wet	Fixed	Wet	Fixed	Wet
10	5.6 ± 3.8		11.1 ± 5.0		5.4 ± 3.1	
20	11.6 ± 2.4		13.0 ± 4.7		8.8 ± 4.1	
30	13.7 ± 3.1		16.2 ± 4.2		12.4 ± 4.6	
40	16.9 ± 6.4		17.1 ± 6.9		13.6 ± 4.9	
50	20.4 ± 4.8		15.8 ± 3.0		13.6 ± 3.9	
60	23.0 ± 8.2	18.2 ± 1.8	18.3 ± 3.7	16.2 ± 8.1	13.7 ± 4.0	
90	26.1 ± 9.6		17.7 ± 5.3	15.7 ± 1.1	15.2 ± 5.5	15.5 ± 6.9
120	24.9 ± 10.8	24.4 ± 9.4	20.4 ± 6.3		15.1 ± 6.7	12.7 ± 1.8
150	25.3 ± 12.8	23.7 ± 1.8	22.0 ± 7.5	21.9 ± 9.6	16.9 ± 6.0	
180	23.8 ± 8.8		22.4 ± 8.2	20.5 ± .7	16.8 ± 3.9	20.5 ± 8.1
210	25.5 ± 9.0	28.7 ± 10.0	23.1 ± 6.8		16.9 ± 5.3	16.7 ± .3
240	24.6 ± 9.2	31.7 ± 1.1	21.4 ± 4.9	32.0 ± 8.7	19.7 ± 8.4	
270	26.3 ± 10.5		22.1 ± 5.8	20.2 ± .3	15.7 ± 8.3	28.1 ± 6.9
300	25.7 ± 8.8	39.9 ± 10.5	21.0 ± 7.9		16.0 ± 8.5	18.7 ± 1.8
$\overline{X}$	21.0 ± 10.0	29.4 ± 10.7	18.7 ± 6.6	21.3 ± 9.0	14.3 ± 6.5	20.0 ± 8.0

Table 4. Effect of Three Sperm to Leucocyte Ratios on the Number of Sperm Per Ingesting Leucocyte (S/L) as Determined from Wet and Fixed-Stained Smears (means ± SD of four replicates from Experiment IIA: Replicates Within Cow).

	Sperm:Leucocyte Ratio					
	1 : 1		2 : 1		1 : 2	
Time (min)	Fixed	Wet	Fixed	Wet	Fixed	Wet
10	1.02 ± .03		1.02 ± .03		1.00 ± .00	
20	1.03 ± .04		1.02 ± .02		1.01 ± .02	
30	1.02 ± .04		1.08 ± .04		1.03 ± .04	
40	1.08 ± .03		1.06 ± .05		1.07 ± .07	
50	1.09 ± .04		1.04 ± .04		1.09 ± .07	
60	1.10 ± .04	1.01 ± .02	1.11 ± .07	1.01 ± .03	1.09 ± .07	
90	1.09 ± .03		1.10 ± .05	1.06 ± .05	1.09 ± .06	1.04 ± .05
120	1.10 ± .07	1.08 ± .08	1.11 ± .07		1.09 ± .07	1.04 ± .01
150	1.08 ± .06	1.06 ± .03	1.07 ± .09	1.09 ± .05	1.09 ± .06	
180	1.07 ± .06		1.12 ± .04	1.08 ± .05	1.10 ± .07	1.05 ± .05
210	1.09 ± .07	1.06 ± .06	1.09 ± .05		1.07 ± .06	1.00 ± .00
240	1.09 ± .05	1.02 ± .03	1.10 ± .05	1.07 ± .05	1.08 ± .10	
270	1.09 ± .08		1.10 ± .05	1.01 ± .02	1.07 ± .07	1.04 ± .05
300	1.07 ± .04	1.08 ± .02	1.10 ± .08		1.10 ± .08	1.00 ± .08
$\overline{X}$	1.07 ± .06	1.06 ± .05	1.08 ± .06	1.06 ± .05	1.07 ± .07	1.04 ± .05

Figure 3. Effect of three sperm to leucocyte ratios on percent phagocytosis (PP) of spermatozoa based on evaluations of fixed-stained smears (means of four replicates from Experiment IIA: replicates within cow).


1:1
2:1
1:2
PERCENT PHAGOCYTOSIS (PP)
35
30
25
20
15
10
5
10
20
30
40
50
60
90
120
150
180
210
240
270
300
INCUBATION TIME AT 37° (MIN)

ratios of 1:1, 2:1 and 1:2 was 1.78, 2.10, and 1.16 percent per minute, respectively. The change in PP with time between 30 and 150 minutes of incubation for the same three ratios was 0.37, 0.19 and 0.14 percent per minute, respectively.

Analysis of variance of the data from fixed-stained smears (Table 4, Appendix A) showed no significant differences in PP and S/L for the three ratios tested. For PP, significant differences were due to replicates, time and the replicate by ratio interaction ($P < .01$). The interaction of replicate and ratio for PP for both fixed and wet smears may be seen in Table 5 and in Figure 4.

Analysis of variance of S/L (Table 5, Appendix A), based on evaluations of fixed-stained smears, indicated significant effects due to replicates and time ($P < .01$) and the replicate by ratio interaction ($P < .01$).

The correlation (r value) between counts made on wet and fixed-stained smears for measurements of PP across replicates and time was .57, .68 and .63 for sperm to leucocyte ratios of 1:1, 2:1 and 1:2, respectively ($P < .01$). The overall correlation between measurments of PP made from wet vs fixed-stained smears was significant, with $r = .67$ ($P < .01$). A comparison of wet and fixed-stained smears for each replicate may be seen in the histogram in Figure 4.

The overall correlation between the two measurements of phagocytosis made in this study, PP and S/L, was also significant, with $r = .60$ ($P < .01$). The overall correlation between PP and S/L, based on

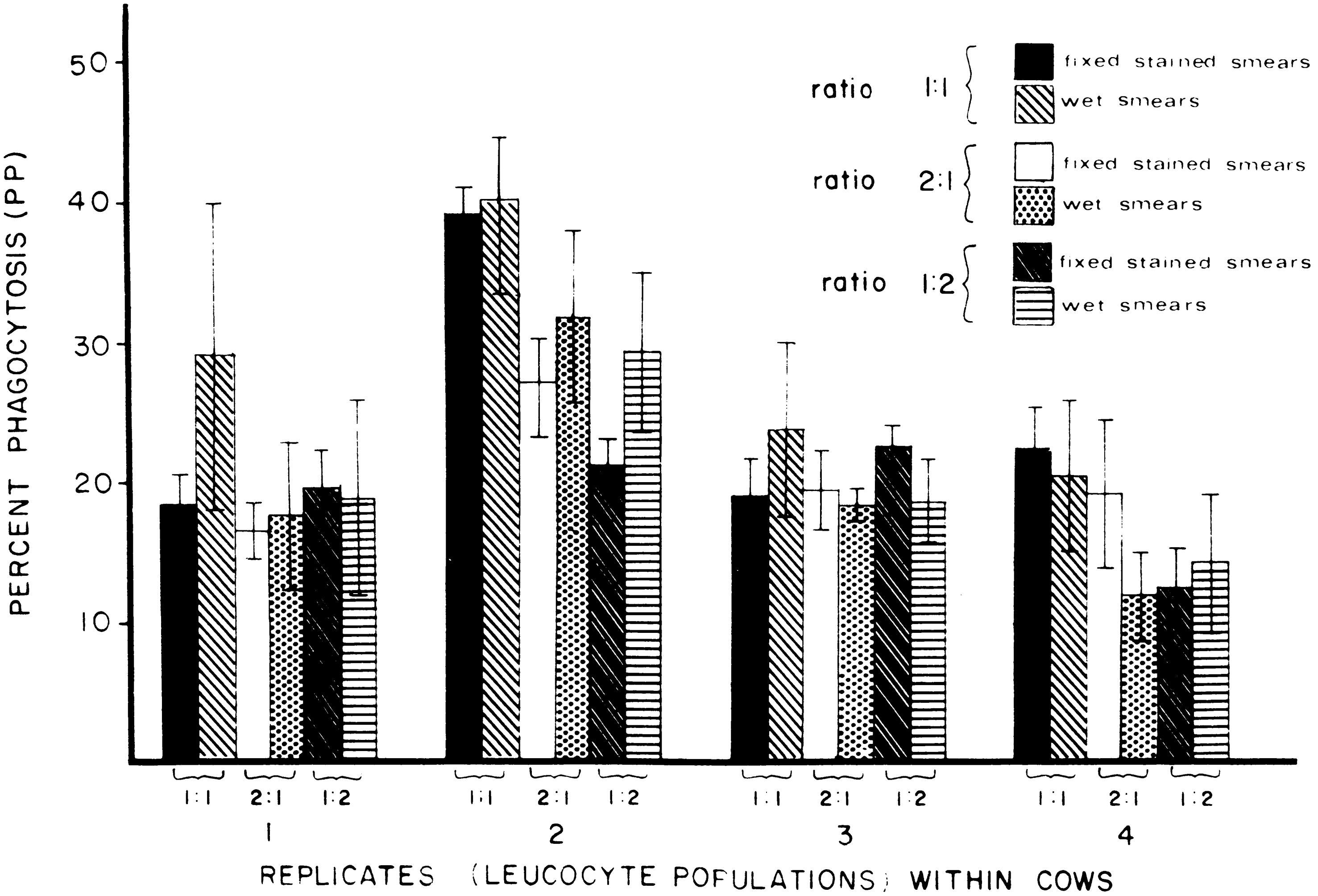
Table 5. Interaction Between Replicates and Three Sperm to Leucocyte Ratios (based on evaluations of percent phagocytosis from wet and fixed-stained smears, Experiment IIA: Replicates Within Cow).

Replicates		Ratios			Ranking of Ratios		
		1:1	2:1	1:2	1	2	3
1	W*	30.7	18.5	19.6	1:1,	1:2,	2:1
	F**	17.2	14.5	16.6	1:1,	1:2,	2:1
2	W	41.4	33.1	30.0	1:1,	2:1,	1:2
	F	32.1	25.2	20.7	1:1,	2:1,	1:2
3	W	24.6	18.8	16.1	1:1,	2:1,	1:2
	F	17.2	15.4	8.5	1:1,	2:1,	1:2
4	W	21.0	11.7	14.5	1:1,	1:2,	2:1
	F	17.3	19.7	11.3	2:1,	1:1,	1:2

* based on evaluations of wet smears

** based on evaluations of fixed-stained smears

Figure 4. Comparison of percent phagocytosis (PP) for four replicates (leucocyte populations) from the same cow (means ± SD for three sperm to leucocyte ratios).


PERCENT PHAGOCYTOSIS (PP)
50
40
30
20
10
ratio 1:1
fixed stained smears
wet smears
ratio 2:1
fixed stained smears
wet smears
ratio 1:2
fixed stained smears
wet smears
1:1
2:1
1:2
1
2
3
4
REPLICATES (LEUCOCYTE POPULATIONS) WITHIN COWS

data from wet smears was also significant, with r = .53 ($P < .01$).

IIB Replicates Among Cows. The means and standard deviations of percent phagocytosis (PP), based on evaluations of wet and fixed-stained smears are presented for each of the three experimental ratios in Table 6. Means and standard deviations of sperm per ingesting leucocyte (S/L), based on counts made from wet and fixed-stained smears, for the three experimental ratios, are presented in Table 7. Means of PP, based on counts from fixed-stained smears, are illustrated graphically in Figure 5.

In this experiment, analysis of variance of data from fixed-stained smears showed that the ratio of sperm to leucocytes significantly affected percent phagocytosis ($P < .01$; Table 6, Appendix A). A Duncan's Multiple Range Test of the means for fixed-smear data indicated a sperm to leucocyte ratio of 2:1 resulted in significantly greater PP than a ratio of 1:2 ($P < .01$). PP for a ratio of 1:1 did not significantly differ from either a 2:1 or a 1:2 ratio ($P > .05$).

Analysis of variance for PP evaluated from fixed-stained smears is shown in Table 6 of Appendix A. Significant effects were due to replicate, ratio and time of incubation ($P < .01$). Replicate by ratio and ratio by time interactions were also significant ($P < .01$). The interaction of replicate by ratio may be seen in Table 8 and in the histogram in Figure 6. The interaction of ratio by time is most evident in Figure 5 where little difference among ratios is apparent at 60 minutes of incubation as compared to the earlier and later incuba-

Table 6. Effect of Three Sperm to Leucocyte Ratios on Percent Phagocytosis (PP) as Determined from Wet and Fixed-Stained Smears (means ± SD of seven replicates from Experiment IIB: Replicates Among Cows).

Time (min)	Sperm:Leucocyte Ratio					
	1 : 1		2 : 1		1 : 2	
	Fixed	Wet	Fixed	Wet	Fixed	Wet
10	7.4 ± 5.8		13.9 ± 7.0		7.5 ± 8.7	
20	14.3 ± 6.5		19.0 ± 9.4		11.4 ± 10.6	
30	14.6 ± 10.3		21.0 ± 9.6		14.2 ± 8.1	
40	16.9 ± 11.6		22.3 ± 11.8		14.4 ± 11.1	
50	19.0 ± 11.0		22.6 ± 11.6		16.1 ± 10.5	
60	18.8 ± 14.9	14.0 ± 9.7	20.0 ± 12.4	22.1 ± 15.7	15.9 ± 10.5	10.4 ± 2.0
90	18.2 ± 14.9	9.5 ± 7.1	23.2 ± 15.6	22.5 ± 9.6	19.2 ± 15.0	18.2 ± 9.1
120	21.1 ± 11.6	23.7 ± 8.4	24.9 ± 12.5	16.6 ± 1.9	16.0 ± 12.3	13.5 ± 2.0
150	23.2 ± 14.5	17.4 ± 7.1	28.5 ± 14.8	28.2 ± 15.9	15.2 ± 11.8	13.0 ± 4.7
180	25.0 ± 13.3	10.7 ± .3	28.0 ± 9.1	26.6 ± 6.1	18.2 ± 11.1	21.1 ± 9.5
210	23.6 ± 15.4	32.3 ± 16.1	29.0 ± 14.2	17.5 ± 3.1	15.9 ± 12.7	16.6 ± 3.7
240	27.0 ± 11.8	21.5 ± 3.3	28.0 ± 11.8	51.0 ± 2.1	15.6 ± 10.6	12.6 ± 4.5
270	29.0 ± 15.4	11.5 ± .7	29.8 ± 11.5	28.0 ± 4.2	17.2 ± 11.8	27.7 ± 11.4
300	30.6 ± 12.6	33.3 ± 10.7	28.4 ± 15.6	21.0 ± 2.6	17.7 ± 13.4	21.6 ± 4.2
$\bar{X}$	20.6 ± 13.5^{ab}	21.8 ± 11.9	24.2 ± 12.6^{a}	24.7 ± 10.6	15.3 ± 11.4^{b}	17.9 ± 8.5

different superscripts indicate significant differences between ratios as tested by Duncan's Multiple Range test ($P < .01$).

Table 7. Effect of Three Sperm to Leucocyte Ratios on the Number of Sperm Per Ingesting Leucocyte (S/L) as Determined from Wet and Fixed-Stained Smears (means ± SD of seven replicates from Experiment IIB: Replicates Among Cows).

	Sperm:Leucocyte Ratio					
	1 : 1		2 : 1		1 : 2	
Time (min)	Fixed	Wet	Fixed	Wet	Fixed	Wet
10	1.01 ± .03		1.02 ± .03		1.01 ± .02	
20	1.00 ± .06		1.05 ± .07		1.02 ± .04	
30	1.02 ± .05		1.09 ± .06		1.04 ± .06	
40	1.08 ± .08		1.09 ± .09		1.05 ± .07	
50	1.11 ± .10		1.09 ± .09		1.06 ± .07	
60	1.09 ± .09	1.03 ± .05	1.10 ± .11	1.10 ± .12	1.09 ± .09	1.01 ± .02
90	1.09 ± .12	1.00 ± .00	1.08 ± .09	1.07 ± .06	1.07 ± .10	1.04 ± .06
120	1.07 ± .11	1.07 ± .07	1.09 ± .08	1.02 ± .03	1.08 ± .10	1.04 ± .06
150	1.09 ± .11	1.07 ± .07	1.12 ± .10	1.14 ± .07	1.08 ± .09	1.01 ± .01
180	1.07 ± .08	1.00 ± .00	1.12 ± .08	1.06 ± .05	1.09 ± .08	1.06 ± .09
210	1.09 ± .07	1.04 ± .04	1.11 ± .09	1.02 ± .03	1.08 ± .12	1.04 ± .04
240	1.07 ± .06	1.06 ± .04	1.11 ± .10	1.16 ± .01	1.05 ± .09	1.02 ± .02
270	1.10 ± .10	1.02 ± .03	1.09 ± .07	1.09 ± .05	1.06 ± .06	1.06 ± .08
300	1.10 ± .09	1.06 ± .04	1.13 ± .10	1.00 ± .00	1.06 ± .09	1.04 ± .06
$\bar{X}$	1.07 ± .09	1.05 $.05^{ab}$	1.09 .09	1.07 $.07^{a}$	1.06 .08	1.04 $.05^{b}$

different superscripts indicate significant differences between ratios as tested by Duncan's Multiple Range test ($P < .05$).

Figure 5. Effect of three sperm to leucocyte ratios on percent phagocytosis (PP) of spermatozoa based on evaluations of fixed-stained smears (means of seven replicates from Experiment IIB: replicates among cows).

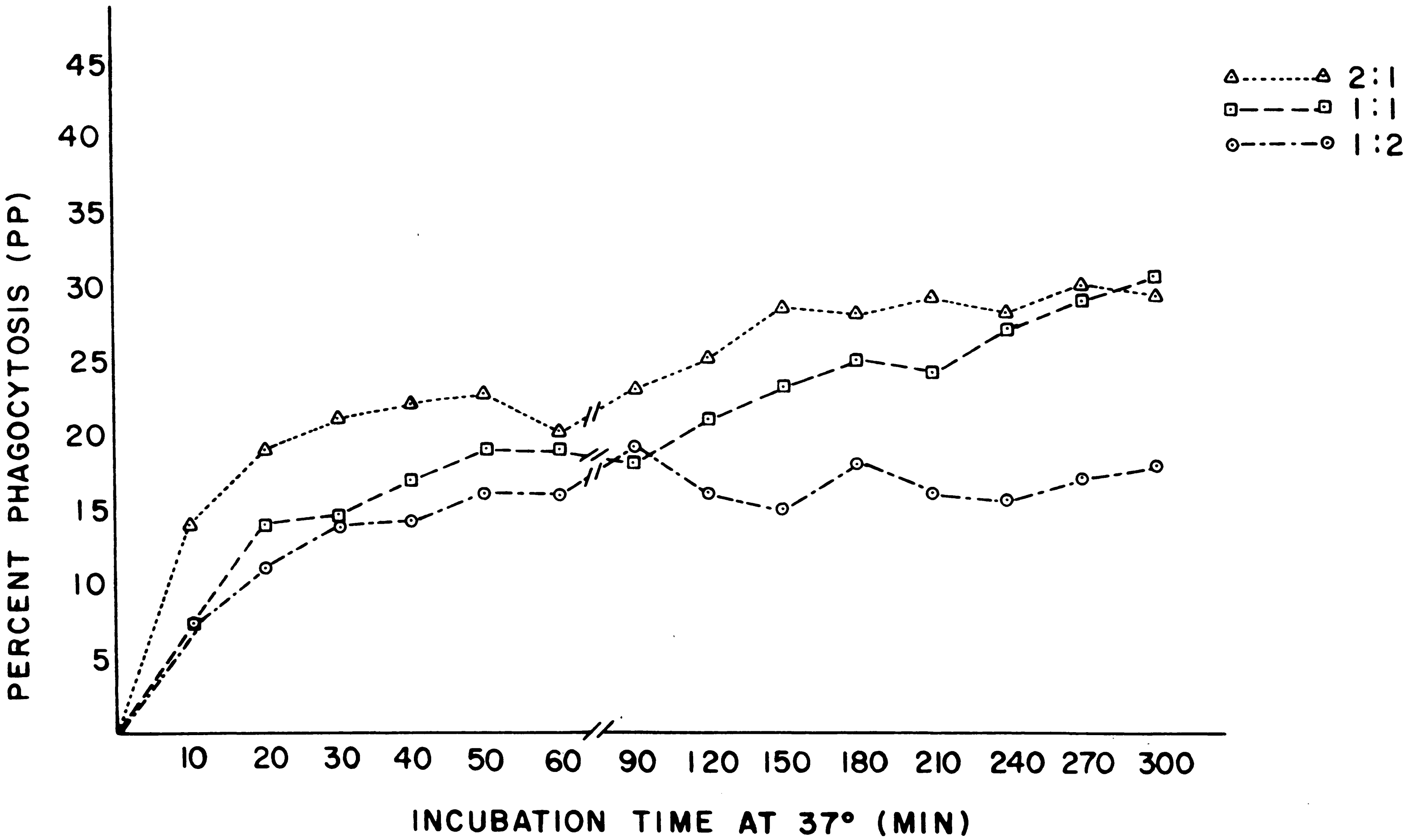

PERCENT PHAGOCYTOSIS (PP)
45
40
35
30
25
20
15
10
5
10
20
30
40
50
60
90
120
150
180
210
240
270
300
INCUBATION TIME AT 37° (MIN)
2:1
1:1
1:2

Figure 6. Comparison of percent phagocytosis (PP) for seven replicates (leucocyte populations) from different cows (means ± SD for three sperm to leucocyte ratios).

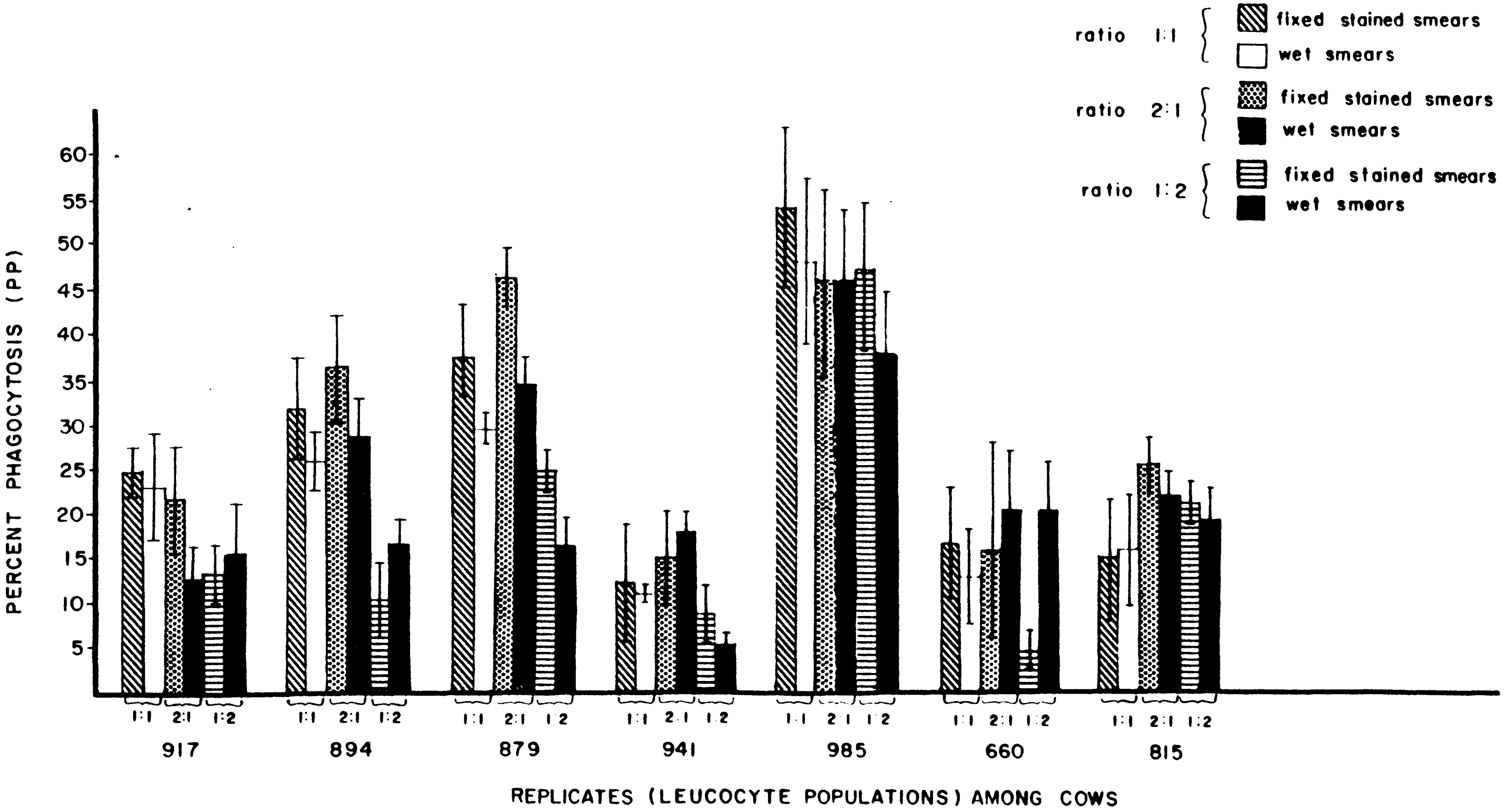

ratio 1:1
▨ fixed stained smears
☐ wet smears
ratio 2:1
▩ fixed stained smears
■ wet smears
ratio 1:2
▤ fixed stained smears
■ wet smears
PERCENT PHAGOCYTOSIS (PP)
5
10
15
20
25
30
35
40
45
50
55
60
1:1
2:1
1:2
917
894
879
941
985
660
815
REPLICATES (LEUCOCYTE POPULATIONS) AMONG COWS

Table 8. Interaction Between Replicates and Three Sperm to Leucocyte Ratios (based on evaluations of percent phagocytosis from wet and fixed-stained smears, Experiment IIB: Replicates Among Cows).

Replicates		Ratios			Ranking of Ratios		
		1:1	2:1	1:2	1	2	3
1	W*	21.0	11.7	14.5	1:1	1:2	2:1
	F**	17.3	19.7	11.3	2:1	1:1	1:2
2	W	23.7	26.3	15.1	2:1	1:1	1:2
	F	23.1	31.2	11.2	2:1	1:1	1:2
3	W	25.9	31.9	15.1	2:1	1:1	1:2
	F	28.3	37.4	20.7	2:1	1:1	1:2
4	W	10.6	16.4	8.9	2:1	1:1	1:2
	F	8.5	13.1	7.1	2:1	1:1	1:2
5	W	44.7	42.7	34.7	1:1	2:1	1:2
	F	42.1	38.6	38.2	1:1	2:1	1:2
6	W	12.3	18.8	19.4	1:2	2:1	1:1
	F	11.8	10.0	4.9	1:1	2:1	1:2
7	W	14.7	20.3	17.8	2:1	1:2	1:1
	F	13.0	19.2	6.5	2:1	1:1	1:2

* based on evaluations of wet smears

** based on evaluations of fixed-stained smears tion periods.

As was observed in Experiment IIA, the change in PP with respect to time was greatest during the initial 30 to 60 minutes, then declined. During the initial 30 minutes of incubation, the change in PP over time for sperm to leucocyte ratios of 1:1, 2:1 and 1:2 was 1.59, 2.28 and 1.57 percent per minute, respectively. The change in PP over time between 30 and 150 minutes and between 150 and 300 minutes of incubation for the same three ratios was 0.23, 0.20 and 0.20 percent per minute and 0.16, 0.20 and 0.05 percent per minute, respectively.

Analysis of variance of S/L, based on counts from fixed-stained smears (Table 7, Appendix A), showed significant effects due to replicate, time ($P < .01$) and ratio ($P < .05$), as well as replicate by ratio and replicate by time interactions ($P < .01$).

The overall correlation of PP values measured from wet vs fixed-stained smears, was significant, with $r = .82$ ($P < .01$). The correlation between wet and fixed-stained smears for sperm to leucocyte ratios of 1:1, 2:1 and 1:2 was also significant, with $r = .93$, $r = .88$ and $r = .66$, respectively ($P < .01$). A comparison of wet and fixed-stained smears for each replicate is illustrated in Figure 6.

The correlation between PP and S/L, based on fixed-stained smears and wet smears across ratios, time and replicates, was significant, with $r = .76$ for fixed-stained smears and $r = .68$ for wet smears ($P < .01$).

Results of the Trypan Blue vital staining tests for replicates two, three, four, five and seven showed there were 96.0, 97.5, 98.5, 95.5 and 95.0 percent live leucocytes, respectively, at the initiation of each incubation period. Viability data was not collected on replicates one and six.

Experiment III : Phagocytosis of Live vs Killed Spermatozoan Populations

This experiment was conducted to evaluate the ability of leucocytes to distinguish a killed cell population of sperm from a live cell population. The live populations were represented by 50 to 70% motile cells (based on estimates) while the dead populations were of essentially zero to trace motility.

The means and standard deviations for percent phagocytosis (PP) of live and killed spermatozoa are presented in Table 9. Analyses of variance of PP (Table 8, Appendix A) and S/L (Table 9, Appendix A) indicated that differences in PP and S/L among live and dead sperm populations were not significant ($P > .05$). There were, however, significant differences between the phagocytosis of live and dead spermatozoa when the data from each replicate was analyzed separately (Tables 10 through 17, Appendix A). PP was significantly greater for live cells in replicates two and four ($P < .01$). S/L for live cells was significantly greater in replicate four ($P < .01$).

Table 9. Effect of Live vs Freeze-Thaw Killed Populations of Spermatozoa on Percent Phagocytosis (PP) (means ± SD of four replicates*).

	Treatments	
Time (min)	Live Spermatozoa	Killed Spermatozoa
10	12.1 ± 6.5	16.0 ± 8.1
20	18.0 ± 6.7	20.1 ± 14.3
30	21.6 ± 9.6	22.2 ± 16.9
40	22.1 ± 10.1	19.7 ± 8.5
50	23.0 ± 9.8	17.5 ± 7.3
60	26.2 ± 11.4	20.9 ± 14.9
90	26.5 ± 8.5	21.1 ± 11.2
120	29.1 ± 11.1	22.3 ± 12.2
150	29.5 ± 11.5	26.2 ± 13.4
$\overline{X}$	23.1 ± 10.7	20.6 ± 12.2

* each replicate represents a population of leucocytes, each obtained from a different donor cow, and pooled ejaculates from each of two different bulls.

Analysis of variance of PP (Table 8, Appendix A) showed significant variation due to replicates ($P < .01$) and time of incubation ($P < .05$). Significant interactions included replicate by treatment (live vs killed spermatozoa), replicate by time and treatment by time ($P < .01$). The treatment by time interaction may be seen in Figure 7. This interaction suggests that dead sperm are more readily phagocytosed in the early stages of incubation, while later stages favor phagocytosis of live spermatozoan populations.

Analysis of variance of S/L (Table 9, Appendix A) indicated significant variation due to replicate and time of incubation ($P < .01$). Also significant were replicate by treatment ($P <.01$) and replicate by time ($P <.05$) interactions. The replicate by treatment interaction for PP and S/L is shown in Table 10.

Means for PP of live and killed spermatozoan populations across replicates are presented graphically in Figure 7. The change in PP with respect to time was again greatest during the first 30 to 60 minutes of incubation. The change in PP with time during the initial 30 minutes of incubation was 2.43 and 2.50 percent per minute for live and killed cells, respectively. The change in PP decreased to 0.23 a and 0.11 percent per minute between 30 and 150 minutes of incubation for live and killed spermatozoa, respectively.

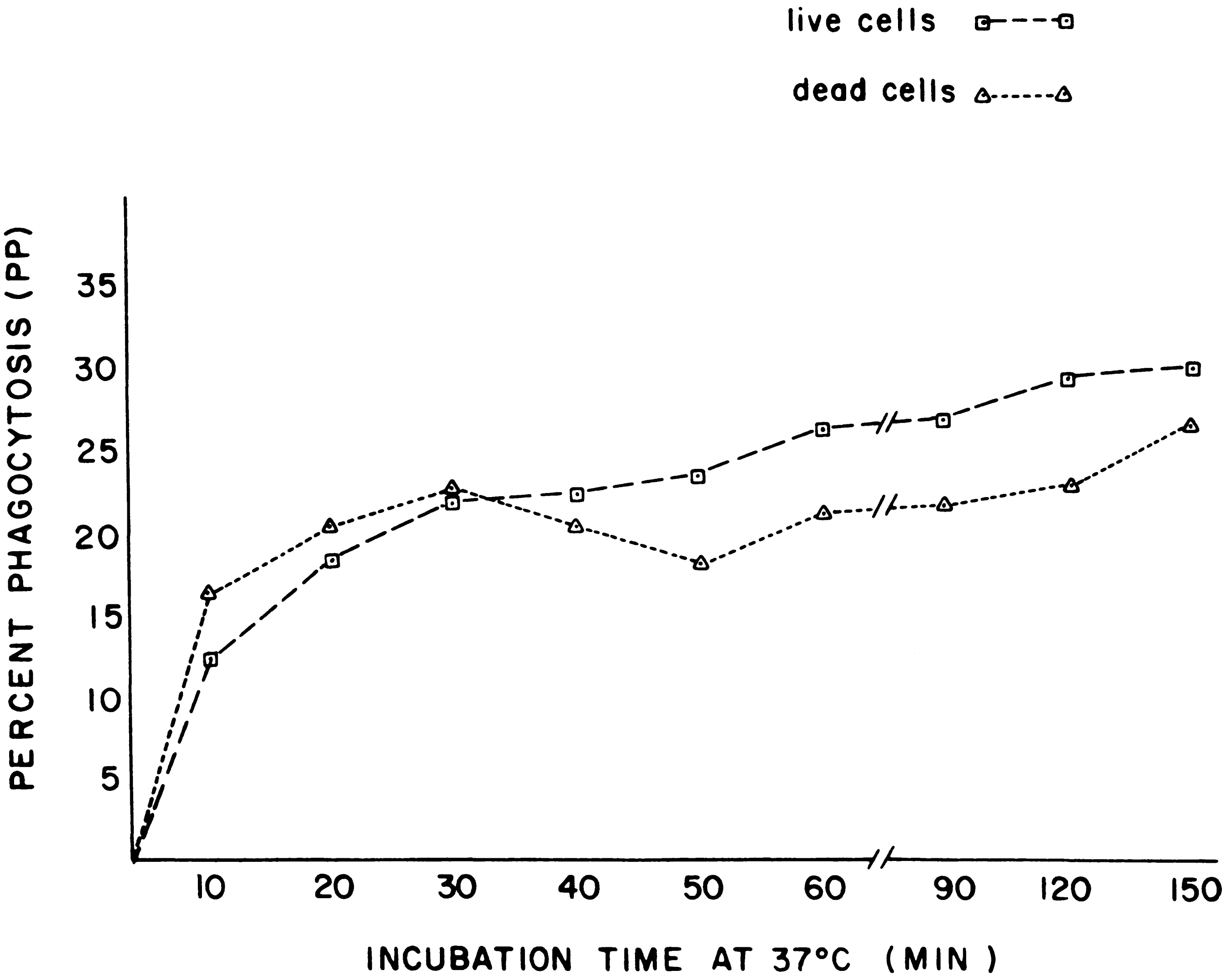
Analysis of variance for PP and S/L was also carried out separately on the data from each replicate (Tables 10 through 17, Appendix A) to test for differences between bulls (within leucocyte populations)

Table 10. Effect of Live and Freeze-Thaw Killed Populations of Spermatozoa on Percent Phagocytosis (PP) and Sperm Per Ingesting Leucocyte (S/L) (means ± SD for each of four replicates*).

Repetitions	Treatments			
	Live Spermatozoa		Killed Spermatozoa	
	PP	S/L	PP	S/L
1	29.94 ± 7.54	1.10 ± .06	33.00 ± 11.19	1.11 ± .09
2	11.47 ± 3.52	1.03 ± .05	7.78 ± 3.36	1.02 ± .03
3	25.82 ± 8.09	1.09 ± .06	24.92 ± 9.33	1.06 ± .06
4	25.29 ± 11.69	1.09 ± .10	16.93 ± 5.44	1.03 ± .04
$\bar{X}$	23.13 ± 10.74	1.08 ± .08	20.66 ± 12.25	1.06 ± .07

* each replicate represents a population of leucocytes, each obtained from a different donor cow, and pooled ejaculates from each of two different bulls.

Figure 7. Effect of live vs freeze-thaw killed populations of spermatozoa on percent phagocytosis (PP) based on evaluations of fixed-stained smears (means of four replicates).


live cells
dead cells
PERCENT PHAGOCYTOSIS (PP)
35
30
25
20
15
10
5
10
20
30
40
50
60
90
120
150
INCUBATION TIME AT 37°C (MIN)

with respect to phagocytosis. Significant bull variation was observed for both PP and S/L for replicates one and three ($P < .01$ for PP; $P < .05$ for S/L).

DISCUSSION

General Observations on the In Vitro Measurement of Phagocytosis of Spermatozoa

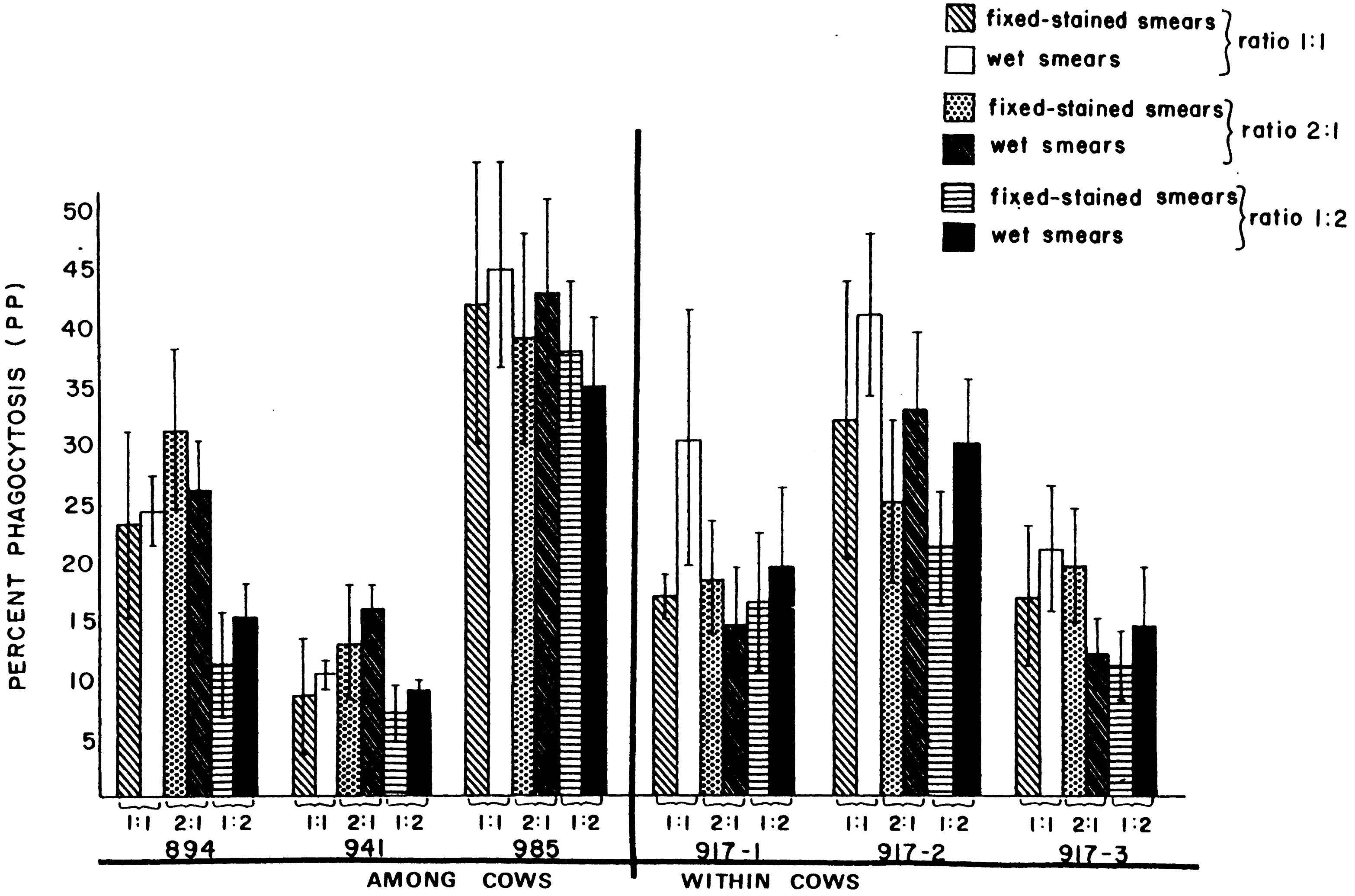
Data generated on several aspects of this study, such as the rate and level of phagocytosis, variation in leucocyte populations (within and among cows), comparisons of wet and fixed-stained smears and methods of evaluating phagocytosis (percent phagocytosis vs sperm per ingesting leucocyte) were concerned in general with the developement of procedures for measuring phagocytosis of spermatozoa in an *in vitro* system.

Guidry, *et al* (1974) reported no significant increase in the level of phagocytosis of yeast cells by blood leucocytes *in vitro* between samples incubated for 30 vs 60 minutes. These authors concluded that a 30-minute incubation period was sufficient to obtain maximum phagocytosis. The results of experiments conducted in this study were in reasonable agreement with respect to the faster rate of phagocytosis observed during early incubation periods, in that approximately 50 to 60% of the phagocytosis was completed by 30 minutes. However, by waiting until 60 minutes, 70 to 80% was complete with 20 to 30% occuring over the next three to four hours. Thus, measurements of phagocytosis of spermatozoa *in vitro* would be aided by measurements over at least a one-hour incubation period.

The absolute levels of phagocytosis obtained in this study were also highly variable from one replicate to the next and were accounted for by the highly significant replicate effect. This was true both for populations of leucocytes taken from the same cow (Experiment IIA) on different days, and for populations taken from several different cows (Experiment IIB). These variations in leucocyte populations are illustrated in the histogram in Figure 8. Although replicates utilized both leucocytes and spermatozoa from a different source, the significant replicate effect is probably due to leucocytes since Experiment III revealed that viability of spermatozoa did not influence *in vitro* phagocytosis and, with the exception of Experiment III, semen was pooled. While viability is the greatest source of variation in semen, other factors associated with the cells of certain males may be important to phagocytosis. Therefore, differences in replicates due to semen cannot be ruled out.

Newbould (1967) reported that leucocytes in milk samples taken from different quarters of the same cow varied significantly in their ability to phagocytose *Staph. aureus*. This is in agreement with the results of this study since the leucocytes for Experiment IIA were obtained not only on different days but also from different quarters of the same cow. Paape and Wergin (1977) however, questioned whether the variation observed by Newbould (1967) was a result of the inability of milk from different quarters to sustain phagocytosis, or a defect in the leucocytes per se. Paape and Wergin (1977) found no

Figure 8. Comparison of the variation in percent phagocytosis (PP) exhibited by leucocytes from the same cow (within cow) vs different cows (among cows) (means ± SD).


fixed-stained smears
wet smears
ratio 1:1
fixed-stained smears
wet smears
ratio 2:1
fixed-stained smears
wet smears
ratio 1:2
PERCENT PHAGOCYTOSIS (PP)
50
45
40
35
30
25
20
15
10
5
1:1
2:1
1:2
894
941
985
917-1
917-2
917-3
AMONG COWS
WITHIN COWS

significant differences in the phagocytosing ability of leucocytes from different quarters of the same cow when the leucocytes were suspended in homologous milk. In the present study the leucocytes were resuspended in PBS, therefore the environment was the same among replicates. This would suggest that a difference among the leucocyte populations, rather than the incubation medium is important. In agreement with the present findings, Paape and Wergin (1977) reported significant differences in phagocytosis between leucocyte populations obtained from different cows.

It is not clear at this time what causes the variability in leucocyte populations. The results of the Trypan Blue vital staining tests conducted in Experiment IIB indicate that cell viability is not the cause. It is possible that the *in vivo* exposure of leucocytes to milk constituents, especially lipid, may be an important factor in influencing phagocytic ability *in vitro*. Paape and Wergin (1977) have observed that the phagocytic ability of leucocytes isolated from milk was significantly less than leucocytes isolated from blood. It was further demonstrated that resuspension of blood leucocytes in whole milk and in serum plus cream reduced phagocytosis from 80% (as measured in 100% serum) to 44 and 56%, respectively. Ultrastructural studies (Paape and Wergin, 1977) of leucocytes isolated from milk revealed the presence of numerous lipid-filled phagocytic vacuoles. Thus, ingestion of lipid by milk leucocytes may be an important factor in determining the variability of phagocytic activity of leucocyte populations. This variation due to lipid ingestion could stem from two sources. Variations may be due to differences in milk fat percent or the period of time the leucocytes are in contact with the milk *in vivo*. While time intervals between infusion, milk removal and collection of cells was kept relatively constant in the present study, variations of up to 30 minutes could have occured as a result of the order in which the cows entered the milking parlor. It is interesting to note that leucocytes used in the present study were routinely observed to possess numerous lipid inclusions. However, these inclusions were apparent in leucocytes which also had phagocytosed spermatozoa as well as those which had not. While the presence of ingested lipid did not appear to inhibit phagocytosis, quantitation of leucocytes containing containing both lipid and spermatozoa vs those containing only lipid vacuoles was not made in this study.

Since the inherent phagocytic abilities of a leucocyte population are quite unpredictable, with respect to the use of an *in vitro* procedure to approach the problems described in this study, it is important to conduct simple experiments and to replicate such experiments using leucocytes from several animals.

The correlation between the two parameters of phagocytosis made in this study (percent phagocytosis and spermatozoa per ingesting leucocyte) was significant ($P < .01$). Measurements of PP tended to be a more sensitive indicator of phagocytic activity than measurements of S/L, as indicated by the greater range in response obtained for PP.

Guidry, et al (1974) reported that "mean phagocytosis" measurements, defined as the average or mean number of particles ingested per leucocyte, were a more accurate index of phagocytic activity than were measurements of percent phagocytosis. The mean phagocytosis measurement described by Guidry, et al (1974) is comparable to the S/L measurement made in the present study. The discrepancy between the results of this study and those of Guidry, et al (1974) in finding mean phagocytosis (or S/L) to be a more sensitive measure is probably a result of particle size. In the case of yeast cells, bacteria and particles of similar size, the range in the number of particles which may be ingested by a single phagocyte is much greater (0 to 10) than is the range in percent phagocytosis (96 to 100%) (Guidry, et al, 1974). The opposite was observed in the present study where, using a particle of much greater size (the spermatozoon) PP ranged from approximately five to 60%, whereas S/L only ranged from one to three. However, S/L would still be important in evaluating the overall influence of an environment on phagocytic behavior of leucocytes.

In Experiments I, IIA and IIB measurements of phagocytosis were made on both wet and fixed-stained smears. The correlation between evaluations made on the two preparations was significant ($P < .01$) both within and across treatments, with $r = .87$ (Experiment I, across treatments), $r = .53$ (Experiment I, with glucose), $r = .64$ (Experiment I, without glucose), $r = .67$ (Experiment IIA, across treatments) and $r = .82$ (Experiment IIB, across treatments). These significant correlations indicate that the differences in phagocytosis based on fixed-stained smears were real and not a result of fixation artifacts. However, correlations within treatments were lower than the overall correlations. This is expected since removal of differences due to treatment markedly reduces variation.

As can be noted from the experimental designs in Experiment I and II, wet smears must be evaluated during the incubation period, thus markedly decreasing the number of evaluations which can be made on each treatment. In addition, it was noted that the ability to assess the number of spermatozoa within leucocytes (for S/L measurements) on wet preparations was more difficult than on fixed-stained smears. These factors discourage the use of wet smears and favor fixed-stained preparations for evaluating phagocytosis.

Effect of Glucose Addition

Energy requirements of the neutrophil may be derived from both aerobic and anaerobic glycolysis of glucose, galactose, glycogen and fructose, with the majority being derived from the breakdown of glycogen granules stored in the cytoplasm (Cline, 1975; Murphy, 1976). At this time it is not known whether neutrophils are capable of utilizing any other extracellular sources of energy (Cline, 1975). The process of phagocytosis markedly increases the rate of energy utilization, and inhibitors of glycolysis will result in decreased phagocytic capacity (Cline, 1975).

Newbould (1970; 1973) reported an increase in the phagocytic activity of mammary leucocytes incubated _in vitro_ with the addition of .02% glucose to the incubation media. Further enhancement was not observed with additions of .04 and .06% glucose. The results of Experiment I in this study showed a significant inhibition of phagocytosis ($P < .05$) with the addition of 1.25% glucose.

The depression in phagocytosis observed in this study was probably a result of both the method of glucose addition and the relatively high level used. Drachman, _et al_ (1965) reported that high glucose levels (750 mg%) resulted in a decrease in the ability of neutrophils obtained from both normal and diabetic rats to phagocytose bacteria. Mowat and Baum (1971) however, reported that elevated blood glucose levels (300 to 900 mg%) did not impair chemotactic activity of neutrophils obtained from diabetic individuals.

While neutrophils which are engaged in phagocytosis do have higher energy requirements, they are also sensitive to adverse changes in osmolarity and pH of their environment. It has been reported (Cline, 1975) that certain diseases which result in high salt or glucose concentrations in the renal medulla result in an increased susceptability to bacterial infections of the kidney due to impaired phagocytic function. Although a 1.25% glucose concentration is not osmotically detrimental to spermatozoa (Moyer and Almquist, 1962), in the present study it may have had sufficient impact on the leucocytes to depress phagocytosis due to this factor alone. A dose response approach to the effect of glucose on phagocytosis would be an appropriate experiment to resolve the nutritional vs osmotic question.

Another means by which glucose addition may have reduced phagocytic activity is through an interaction with the membrane system of the spermatozoan and thereby interfering with the recognition mechanism between phagocyte and particle. Moyer and Almquist (1962) observed a decrease in spontaneous sperm agglutination with increasing levels of glucose added to a skimmilk diluent medium (0 to 1.5% glucose). This would seem to indicate that the glucose was in some way altering the membrane of the spermatozoan. The effect of glucose on sperm agglutination may also be a function of changes in osmolarity. This is doubtful, however, since the viability (motility) of spermatozoa was not affected by increasing levels of glucose.

Effect of Sperm to Leucocyte Ratio

Hanks (1940) reported that variations in the particle to leucocyte ratio and in total cell density within an *in vitro* system resulted in variations in the degree of phagocytosis observed, with greater variation resulting from differences in leucocyte to particle ratio than from differences in total cell concentration. Guidry, *et al* (1974) also reported a slight decrease in phagocytosis with dilutions up to 40%. Changing the concentration of yeast cells by ± two, five and ten percent, and thereby changing the ratio of yeast cells to leucocytes, did not significantly change phagocytosis. However, reducing the yeast cell concentration by 50% or increasing it by 50 or 100% significantly affected phagocytosis. A similar manipulation of the sperm to leucocyte ratio was carried out in this study with the 1:1 ratio. While the level of phagocytosis measured for this ratio cannot be compared directly with those of the 2:1 and 1;2 ratio because of the reduction in total cell concentration (75 x 10^6 vs 50 x 10^6 cells/ml), an indirect comparison can be made. With respect to the 2:1 ratio, the 1:1 ratio represents a 50% reduction in particle concentration with the leucocyte concentration held constant. Theoretically, reducing the availability of particles should reduce phagocytosis. The 50% decrease in spermatozoa in the 1:1 ratio of Experiment II, however, did not result in a significant reduction in phagocytosis. With respect to the 1:2 ratio, the 1:1 ratio represents a 50% reduction in leucocytes while particle numbers are held constant. Reducing the number of leucocytes competing for a fixed number of particles within a system should result in greater phagocytosis or a greater number of leucocytes containing particles. However, in this study the 50% reduction in leucocytes represented by the 1:1 ratio did not result in a significant increase in measurable phagocytosis. While significant changes in phagocytosis were not observed with the 50% decrease in either spermatozoa or leucocytes, the levels of phagocytosis for the 1:1 ratio did tend to fall between the other two ratios as would be expected.

The effect of sperm to leucocyte ratio was tested in four replicates using leucocytes from the same cow (Experiment IIA) and in seven replicates using leucocytes from different cows (Experiment IIB). Experiment IIA showed no significant difference between the three ratios, with a tendency to place the 1:1 ratio over the 2:1. However, the more extensive experiment, Experiment IIB, indicated that the 2:1 ratio was significantly superior, with respect to level of phagocytosis, to the 1:2 ratio. The 1:1 ratio in Experiment IIB, while not significantly different from the 2:1 or 1:2, was intermediate as would be expected. Because of the greater number of replicates and the highly significant replicate by ratio interaction, more emphasis should be placed on the results of Experiment IIB. Therefore, it was concluded that a sperm to leucocyte ratio of 2:1 would result in greater phagocytosis and was the ratio selected for Experiment III, since the greater the level of phagocytosis obtained, the greater would be the possibility of this *in vitro* system to measure variations in phagocytosis when treatment differences were imposed.

Effect of Live and Killed Spermatozoan Populations on Phagocytosis

Menge, *et al* (1962) reported significantly greater phagocytosis of dead than live spermatozoa based on a decrease in the number of dead spermatozoa which could be recovered following 20 to 38 hour *in vivo* incubations in both estrus and luteal phase rabbit uteri. Haynes (1967), however, reported no significant differences in the phagocytosis of sperm samples, which had been washed and contained a greater proportion of dead cells, as compared to control samples, after *in vitro* incubations with leucocytes and flushings from rabbit uteri. Mattner (1969) also observed equal phagocytosis of live and dead spermatozoa in the presence of "liquified" cervical mucus, while phagocytosis of live spermatozoa was observed to be greater in the presence of untreated cervical mucus.

The results of this study (Experiment III) showed no significant difference in the degree of phagocytosis (PP or S/L) between populations of live and freeze-thaw killed spermatozoa during a 2.5-hour *in vitro* incubation. The discrepancy between the results of the present study and between those of Haynes (1967) and Menge, *et al* (1962) with respect to differences in the phagocytosis of live vs killed populations of spermatozoa may be a result of using an *in vitro* environment (Haynes, 1967 and Experiment III of this study) as opposed to carrying out the incubations within the female reproductive tract (Menge, *et al*, 1962). This possibility is supported by the results of Lineweaver, *et al* (1970) and Pursel, *et al* (1978). Lineweaver, *et al* (1970) recovered significantly fewer spermatozoa from heifers which had been inseminated with frozen semen than from those which had been inseminated with fresh semen. Similar observation, with respect to the recovery of fresh and frozen semen, were made by Pursel, *et al* (1978) in gilts. Since the processes of freezing and thawing decrease the number of viable or live spermatozoa, the results of Lineweaver, *et al* (1970) and

Pursel, et al (1978) indicate that preferential loss or removal of dead or damaged cells in an in vivo environment is a possibility.

Results of in vivo studies indicate that there may be certain constituents present within the female reproductive tract which are important to the phagocytic removal of spermatozoa. The effect of female reproductive tract secretions or constituents thereof on in vitro phagocytosis of live vs dead populations of spermatozoa warrants further attention.

With respect to the results of the present study, it is interesting to note the significant time by treatment interaction ($P < .01$) observed for PP of live vs killed cells in Experiment III. It appears that the phagocytosis of killed spermatozoa was greater during the initial 30 minutes of incubation (Figure 7 and Table 9). The change in PP with respect to time during this incubation interval was 2.50 percent per minute for killed spermatozoa, as opposed to 2.43 percent per minute for live spermatozoa. It is not known, however, if these differences in phagocytic rate are significant.

Analyses of each of the four replicates in Experiment III, which were also carried out separately (Tables 10 through 17, Appendix A), indicate that each of the bulls used was a significant source of variation in the percent phagocytosis observed. While the spermatozoa of different bulls may in fact vary in their susceptability to phagocytosis, this conclusion cannot be drawn from this study due to the lack of replication among ejaculates within bulls.

In summary, this study found that the addition of glucose at a level of 1.25% (w/v) resulted in a significant inhibition of not only the number of leucocytes engaging in phagocytosis, but also on the extent of phagocytic activity exhibited by those cells engaged in phagocytosis as indicated by the mean number of sperm ingested by phagocytosing leucocytes (S/L). The procedure developed in this study, while it did not show a great sensitivity to the different sperm to leucocyte ratios tested, did result in greater phagocytosis for a 2:1 ratio than for a 1:2 ratio. There was no significant difference observed in the *in vitro* phagocytosis of live vs freeze-thaw killed spermatozoa. However, these results are not conclusive and the question of selective phagocytosis of dead or damaged spermatozoa by leucocytes warrants further investigation.

With respect to general procedures for measurement of phagocytosis of spermatozoa *in vitro*, it was observed that (1) the majority of phagocytosis appears to be complete within 60 minutes, (2) levels of phagocytosis are highly variable regardless of the source of milk leucocytes (within the same cow or among different cows) (3) fixed-stained smear preparations provide a reliable measurement of phagocytosis and (4) there is a positive correlation between measurements of PP and S/L.

Perhaps the most important result of this study is the success in achieving *in vitro* phagocytosis of spermatozoa with mammary leucocytes, and the ability to measure differences in the phagocytic activity of these cells due to changes in their environment (presence of glucose .

or ratio of cells). Further refinements, along with a better understanding of the physiology of the neutrophil and the phagocytic process, could help to make this procedure a useful tool in comprehending the interactions which take place between the male gametes and the environment of the female reproductive tract following insemination.

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APPENDIX A

ANOVA Tables

Table 1. Analysis of Variance for Percent Phagocytosis (PP) in the Presence and Absence of Glucose (based on fixed-stained smears).

Source of Variation	Degrees of Freedom	Mean^a Square	F Value	
Replicate	3	0.2093	19.17	**
Glucose	1	2.7923	20.00	*
Time	7	0.3440	49.14	**
Replicate x Glucose	3	0.1396	12.79	**
Replicate x Time	21	0.0073	0.67	
Glucose x Time	7	0.0124	1.14	
Residual	21	0.0109		

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

[a] based on log transformed data

Table 2. Analysis of Variance for Percent Phagocytosis (PP) in the Presence and Absence of Glucose (based on wet smears).

Source of Variation	Degrees of Freedom	Mean[a] Square	F Value
Replicate	3	0.0114	1.64
Glucose	1	0.9021	17.05 *
Time	3	0.0512	3.82 *
Replicate x Glucose	3	0.0529	7.58 **
Replicate x Time	9	0.0134	1.92
Glucose x Time	3	0.0091	1.31
Residual	9	0.0070	

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

[a] based on log transformed data

Table 3. Analysis of Variance for Spermatozoa Per Ingesting Leucocyte (S/L) in the Presence and Absence of Glucose (based on fixed-stained smears).

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value
Replicate	3	0.0037	2.37
Glucose	1	0.0440	17.39 *
Time	7	0.0065	4.33 **
Replicate x Glucose	3	0.0023	1.49
Replicate x Time	21	0.0015	0.95
Glucose x Time	7	0.0018	1.18
Residual	21	0.0017	

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

[a] based on log transformed data

Table 4. Analysis of Variance for Percent Phagocytosis (PP) for Three Sperm to Leucocyte Ratios (based on evaluations of fixed-stained smears from Experiment IIA: Replicates Within Cow).

Source of Variation	Degrees of Freedom	Mean[a] Square	F Value	
Replicate	3	0.3046	65.92	**
Ratio	2	0.4258	4.44	
Time	13	0.0247	42.57	**
Replicate x Ratio	6	0.0962	9.95	**
Replicate x Time	39	0.0065	0.67	
Ratio x Time	26	0.0164	1.69	
Residual	78	0.0097		

** significant at $P < .01$

[a] based on log transformed data

Table 5. Analysis of Variance for Sperm Per Ingesting Leucocyte (S/L) for Three Sperm to Leucocyte Ratios (based on evaluations of fixed-stained smears from Experiment IIA: Replicates Within Cow).

Source of Variation	Degrees of Freedom	Mean[a] Square	F Value	
Replicate	3	0.0087	45.98	**
Ratio	2	0.0002	0.16	
Time	13	0.0016	6.96	**
Replicate x Ratio	6	0.0013	6.72	**
Replicate x Time	39	0.0002	1.26	
Ratio x Time	26	0.0002	0.91	
Residual	78	0.0002		

** significant at $P < .01$)

[a] based on log transformed data

Table 6. Analysis of Variance for Percent Phagocytosis (PP) for Three Sperm to Leucocyte Ratios (based on evaluations of fixed-stained smears from Experiment IIB: Replicates Among Cows).

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value	
Replicate	6	2.7515	146.04	**
Ratio	2	1.4323	8.61	**
Time	13	0.3500	14.06	**
Replicate x Ratio	12	0.1663	8.83	**
Replicate x Time	78	0.0249	1.32	
Ratio x Time	26	0.0417	2.22	**
Residual	156	0.0188		

** significant at $P < .01$

[a] based on log transformed data

Table 7. Analysis of Variace for Sperm Per Ingesting Leucocyte for Three Sperm to Leucocyte Ratios (based on evaluations of fixed-stained smears from Experiment IIB: Replicates Among Cows).

Source of Variation	Degrees of Freedom	Mean[a] Square	F Value	
Replicate	6	0.0279	125.33	**
Ratio	2	0.0047	4.70	*
Time	13	0.0023	4.60	**
Replicate x Ratio	12	0.0010	4.71	**
Replicate x Time	78	0.0005	2.17	**
Ratio x Time	26	0.0002	1.11	
Residual	156	0.0002		

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

[a] based on log transformed data

Table 8. Analysis of Variance for Percent Phagocytosis (PP) for Live and Freeze-Thaw Killed Populations of Spermatozoa.

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value
Replicate	3	1.9732	127.26 **
Bull	1	0.0506	0.3174
Treatment	1	0.2539	2.60
Time	8	0.1203	2.85 *
Replicate x Bull	3	0.1594	10.28 **
Replicate x Treatment	3	0.0977	6.30 **
Replicate x Time	24	0.0422	2.72 **
Bull x Treatment	1	0.0149	2.97
Bull x Time	8	0.0149	0.96
Treatment x Time	8	0.0418	2.70 **
Residual	83	0.0155	

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

[a] based on log transformed data

Table 9. Analysis of Variance for Sperm Per Ingesting Leucocyte (S/L) for Live and Freeze-Thaw Killed Populations of Spermatozoa.

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value
Replicate	3	0.0063	20.24 **
Bull	1	0.0008	0.258
Treatment	1	0.0037	3.36
Time	8	0.0034	68.00 **
Replicate x Bull	3	0.0031	10.02 **
Replicate x Treatment	3	0.0011	3.67 **
Replicate x Time	24	0.00005	1.59
Bull x Treatment	1	0.0002	0.74
Bull x Time	8	0.0003	0.98
Treatment x Time	8	0.0003	0.90
Residual	83	0.0003	

** significant at $P < .01$

[a] based on log transformed data

Table 10. Analysis of Variance for Percent Phagocytosis (PP) for Replicate One of Experiment III: Effect of Live and Killed Populations of Spermatozoa on Phagocytosis.

Source of Variation	Degrees of Freedom	Mean[a] Square	F Value
Bull	1	0.2126	39.48 **
Treatment	1	0.0092	1.71
Time	8	0.0246	4.56 *
Bull x Treatment	1	0.00	0.00
Bull x Time	8	0.0139	2.58
Treatment x Time	8	0.0104	1.93
Residual	8	0.0054	

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

[a] based on log transformed data

Table 11. Analysis of Variance for Sperm Per Ingesting Leucocyte (S/L) for Replicate One of Experiment III: Effect of Live and Killed Populations of Spermatozoa on Phagocytosis.

Source of Variation	Degrees of Freedom	Mean[a] Square	F Value
Bull	1	0.0071	24.13 **
Treatment	1	0.0001	0.22
Time	8	0.0016	5.34 **
Bull x Treatment	1	0.0010	3.40
Bull x Time	8	0.0002	0.56
Treatment x Time	8	0.0004	1.40
Residual	8	0.0003	

** significant at $P < .01$

[a] based on log transformed data

Table 12. Analysis of Variance for Percent Phagocytosis (PP) for Replicate Two of Experiment III: Effect of Live and Killed Populations of Spermatozoa on Phagocytosis.

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value	
Bull	1	0.0651	2.75	
Treatment	1	0.3487	14.72	**
Time	8	0.0424	1.79	
Bull x Treatment	1	0.1355	5.72	*
Bull x Time	8	0.0291	1.23	
Treatment x Time	8	0.0563	2.38	
Residual	8	0.0237		

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

[a] based on log transformed data

Table 13. Analysis of Variance for Sperm Per Ingesting Leucocyte (S/L) for Replicate Two of Experiment III: Effect of Live and Killed Populations of Spermatozoa on Phagocytosis.

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value
Bull	1	0.0003	1.70
Treatment	1	0.0004	2.43
Time	8	0.0002	1.08
Bull x Treatment	1	0.0004	2.40
Bull x Time	8	0.0001	0.84
Treatment x Time	8	0.0003	1.60
Residual	8	0.0002	

[a] based on log transformed data

Table 14. Analysis of Variance for Percent Phagocytosis (PP) for Replicate Three of Experiment III: Effect of Live and Killed Populations of Spermatozoa on Phagocytosis.

Source of Variation	Degrees of Freedom	Mean[a] Square	F Value
Bull	1	0.2130	18.43 **
Treatment	1	0.0076	0.66
Time	8	0.0607	5.25 *
Bull x Treatment	1	0.0021	0.18
Bull x Time	8	0.0041	0.36
Treatment x Time	8	0.0070	0.60
Residual	8	0.0115	

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

[a] based on log transformed data

Table 15. Analysis of Variance for Sperm Per Ingesting Leucocyte (S/L) for Replicate Three of Experiment III: Effect of Live and Killed Populations of Spermatozoa on Phagocytosis.

Source of Variation	Degrees of Freedom	Mean[a] Square	F Value
Bull	1	0.0027	9.20 *
Treatment	1	0.0014	4.84
Time	8	0.0009	3.23
Bull x Treatment	1	0.0003	1.21
Bull x Time	8	0.0002	0.83
Treatment x Time	8	0.0003	1.03
Residual	8	0.0003	

* significant at $P < .05$

[a] based on log transformed data

Table 16. Analysis of Variance for Percent Phagocytosis (PP) for Replicate Four of Experiment III: Effect of Live and Killed Populations of Spermatozoa on Phagocytosis.

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value
Bull	1	0.0382	4.24
Treatment	1	0.1816	20.17 **
Time	8	0.1193	13.25 **
Bull x Treatment	1	0.0112	1.25
Bull x Time	8	0.0063	0.59
Treatment x Time	8	0.0289	3.22
Residual	8	0.0090	

** significant at P < .01

[a] based on log transformed data

Table 17. Analysis of Variance for Sperm Per Ingesting Leucocyte (S/L) for Replicate Four of Experiment III: Effect of Live and Killed Populations of Spermatozoa on Phagocytosis.

Source of Variation	Degrees of Freedom	Mean^{a} Square	F Value
Bull	1 1	0.00004	0.20
Treatment	1	0.0053	27.17 **
Time	8	0.0022	11.17 **
Bull x Treatment	1	0.00	0.00
Bull x Time	8	0.0002	1.09
Treatment x Time	8	0.0009	4.86 *
Residual	8	0.0002	

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

[a] based on log transformed data

APPENDIX B

Laboratory Procedures

I. Composition of Phosphate Buffered Saline

$NaH_2PO_4 \cdot H_2O$	1.2 g
$NaHPO_4 \cdot 7H_2O$	5.7 g
NaCl	24.0 g
Distilled H_2O	to bring volume to 3000 l

pH adjusted to 7.5 with 10N NaOH

filtered with 45μ millipore filter and sterilized at 121° C and 15 psi for 10 min.

II. Composition of ACD anticoagulant (Paape, et al 1975)

Dextrose	33.44 g
Citric Acid	19.98 g
Na Citrate	33.00 g
Distilled H_2O	1,000.00 ml

III. Composition of Alcoholic Eosin

Eosin Y	4.0 g
Phyloxine B	1.0 g
80% ETOH	to bring volume to 2000 ml
Glacial Acetic Acid (just prior to use)	7 drops/700 ml

IV. Composition of Harris Hematoxylin

Hematoxylin	5.0 g
100% ETOH	50.0 ml
Aluminum Amonium Sulfate	100.0 g
Distilled H_2O	1000.0 ml
Mercuric Oxide	2.5 g
Glacial Acetic Acid	10.0 ml

V. Staining Procedure

100% MEOH	60 min
Tap H_2O	5 min
Hematoxylin	5 min
Tap H_2O	rinse briefly
Acid Alcohol (1% conc. HCl in 80% ETOH)	5 dips
Tap H_2O with 4 drops conc. NH_4OH	until blue
Tap H_2O	3-4 min
Eosin	2 min
90% ETOH	6 dips
90% ETOH	6 dips
100% ETOH	2 min
100% ETOH	2 min
Xylene	3 min
Xylene	3 min
Xylene	3 min

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AN *IN VITRO* PROCEDURE FOR EVALUATING LEUCOCYTIC PHAGOCYTOSIS OF SPERMATOZOA IN THE BOVINE

by

Janice L. Waugh

(ABSTRACT)

Three experiments were conducted to develope an *in vitro* procedure for measuring leucocytic phagocytosis of bovine spermatozoa by mammary leucocytes. Experiment I was designed to determine the effect of adding 1.25% glucose (w/v) to the incubation medium on phagocytosis. Experiment II consisted of two series of incubations (replicates), IIA and IIB, and was designed to determine the effect of three sperm to leucocyte ratios (1:1, 2:1 and 1:2) on phagocytosis. Experiment IIA utilized leucocytes obtained from the same cow on four different days. Experiment IIB utilized leucocytes obtained from seven different cows. The effect of live vs freeze-thaw killed spermatozoa from two different bulls was evaluated in Experiment III.

Measurements of percent phagocytosis (PP) were made on wet and fixed-stained smear preparations in Experiments I and II and on fixed-stained smears only in Experiment III. Measurements of the mean number of spermatozoa per ingesting leucocyte (S/L) were made on fixed-stained smears only in Experiments I and III and on both wet and fixed-stained smears in Experiment II.

Both PP and S/L were significantly depressed ($P < .05$) as a result of glucose addition in Experiment I. Experiment IIA showed no significant effect due to different sperm to leucocyte ratios. Both

PP ($P < .01$) and S/L ($P < .05$) were significantly greater for a 2:1 ratio than for a ratio of 1:2 in Experiment IIB. The PP and S/L for the 1:1 ratio was intermediate to the other ratios and not significantly different from either. No significant differences were observed for phagocytosis of live vs freeze-thaw killed sperm populations, however, a significant ($P < .01$) time by treatment interaction was observed, with greater phagocytosis of dead cells occurring during the initial incubation period.

There was a significant correlation ($P < .01$) between measurements of phagocytosis made on wet vs fixed-stained smears with $r = .87$, .67 and .82 for Experiments I, IIA and IIB, respectively. There was also a significant correlation between measurements of PP vs S/L with $r = .79$, .60 and .76 for fixed-stained smears of Experiments I, IIA and IIB, respectively. Phagocytosis was most efficient during the initial 30 to 60 minutes of incubation. All experiments showed a highly significant effect due to different sperm-leucocyte populations (replicates).