

Attempts to Isolate Highly Viable, Morphologically Normal,  
Y-Chromosome-Bearing Bovine Spermatozoa

by

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## INTRODUCTION

In the past, many techniques have been designed in an effort to separate spermatozoa based on either chromatin content or viability. The separation of X- and Y-chromosome-bearing spermatozoa would be of practical significance for those species in which artificial insemination is possible. The ability to separate X- and Y-chromosome-bearing human spermatozoa offers parents the ability to choose the sex of their child before conception. In the livestock industry, the predetermination of the sex of a calf would offer tremendous economic benefit. Despite many grandiose claims, the success of methods proposing to isolate a population of either X- or Y-chromosome-bearing spermatozoa has not yet been proven.

Separating spermatozoa in order to isolate a population of highly viable sperm cells for artificial insemination could be useful for increasing conception rates associated with artificial insemination. For example, cows bred with semen from bulls of questionable fertility could have a greater chance of conceiving if they were bred with spermatozoa selected for their viability.

A method of separation that could isolate a population of Y-chromosome-bearing bovine spermatozoa as well as a population of highly viable sperm cells would obviously be advantageous to the livestock industry. Both the producer

and the consumer could benefit from the determination of such a method. The experiments in this thesis were designed to determine if a discontinuous bovine serum albumin gradient could isolate a population of highly viable, morphologically normal, Y-chromosome-bearing bovine spermatozoa.

## REVIEW OF LITERATURE

Collection of bull semen with an artificial vagina, followed by dilution and freezing semen for artificial insemination is a common practice and is used routinely throughout the cattle industry. Collection and extension of an average ejaculate from a superior sire produces more than 200 units of semen for artificial insemination. This gives many producers the advantage of introducing outstanding genetic potential into their herds without purchasing expensive sires.

### Semen Quality Evaluation

Semen quality tests are measures of various semen quality traits but do not necessarily reflect the fertility of a semen sample. However, significant predictive values of fertility are evident with some combinations of pre-freeze and post-thaw semen quality characteristics (Wells and Awa, 1970). According to Elliott (1979), the motility of bull semen from a fresh ejaculate is not a reliable predictor of fertility or freezability, however, the post-thaw motility of frozen semen is often used to adjust the concentration of spermatozoa per insemination unit when the semen is used for artificial insemination. As post-thaw motility decreases, the number of sperm cells/unit is

increased in an effort to maintain high conception rates.

Although the estimation of number of live cells by live-dead staining is not any better predictor of fertility than motility estimates, it is a more objective method of quality assessment.

The acrosome, a membrane-bound sac covering the anterior end of the sperm nucleus, contains hydrolytic enzymes and is involved in the fertilization process (Garner and Hafez, 1980). Saacke and White (1972) reported that acrosomal maintenance in post-thaw spermatozoa was more indicative of potential fertility than sperm motility estimates. Analysis of acrosomal integrity after post-thaw incubation at 37 C for varying intervals indicated that measurement of acrosomal cap maintenance after 2 h of incubation was more closely related to fertility ( $r=.6$ ) than measurement of acrosomal cap maintenance measured immediately post-thaw ( $r=.42$ ) or motility estimates measured at any interval post-thaw ( $r=.42$ ).

Morphological abnormalities of sperm cells were first identified as an indication of subfertility in bulls by Williams and Savage (1925) and Lagerlof (1934). Several factors, such as disease (Williams and Savage, 1925) heat or cold stress (Rathore, 1970; Chang, 1943), and exposure to adverse environmental conditions (Wells et al., 1972; Erb et al., 1942) influenced the frequency of abnormal sperm cells within an ejaculate. Williams and Savage (1925) reported

that when the percentage of morphologically abnormal sperm cells reached 18%, fertility was impaired. According to Sullivan (1979), ejaculates that total more than 20% abnormal spermatozoa, or that possess more than 10% spermatozoa with abnormalities of the head and midpiece should not be used in commercial artificial insemination.

Morphologically abnormal spermatozoa are often categorized by the portion of the sperm cell affected with the abnormality and (or) the effect of the abnormality on the fertilizing ability of the sperm cell. Primary sperm cell abnormalities have been classified as those that occur as a result of faulty spermatogenesis, while secondary abnormalities are those which occur after the sperm cells have left the testes (Lagerloff, 1934). Blom (1972) reclassified spermatozoal defects into major abnormalities that induce sterility as a result of testes or epididymis dysfunction and minor abnormalities. Major defects of the head included pyriform, tapered, small, or free abnormal heads and knobbed, incomplete, or ruffled acrosomes. Other major abnormalities included corkscrew, filiform, or truncated midpieces, midpieces containing protoplasmic droplets, tightly coiled tails (dag defect) and decapitated sperm cells. Minor sperm defects included small, but normal shaped heads, free normal heads, giant or narrow heads, abaxial implanted midpieces, distal droplets of the midpiece, bent tails and coiled tails.

Sperm cell abnormalities affecting the acrosome have been studied in detail due to their adverse effects on fertility. The "knobbed" acrosomal cap of spermatozoa, characterized by a rounded thickening in the anterior part of the head, is formed by an abnormal apical body (Hancock, 1949). High frequencies of this abnormality are related to sterility in bulls, although cells with this defect may possess normal motility (Hancock, 1953; Blom and Birch-Anderson, 1962). Inseminations with semen possessing different frequencies of knobbed spermatozoa resulted in corresponding levels of subfertility (Saacke et al., 1968).

The "distintegrated", "decapitated", or "tailless" sperm cell defect has been reported in Hereford (Williams, 1965; Blom, 1977) and Guernsey (Hancock and Rollinson, 1949) bulls. Ejaculates containing this defect have free heads and free motile tails, however, ejaculates containing distintegrated sperm cells with nonmotile tails have been isolated from bulls with testicular hypoplasia (Williams, 1965). The separation of heads from tails usually occurs in the caput epididymis and often involves 100% of the ejaculated spermatozoa, resulting in total sterility (Hancock, 1955).

Abnormalities associated with the midpiece of spermatozoa have been described by Munro et al. (1961), Blom (1959), Savage and Isa (1963) and Lagerloff (1934). Sperm cells with filiform midpieces (midpieces without the

mitochondrial helix) were observed by Savage and Isa (1963). Sperm cells with corkscrew shaped midpieces were observed by Blom (1959) and Munro et al. (1961) and related to subfertility in the bull. Blom (1959) showed electromicrographs of irregular agglomerates of mitochondrial material in cells with the corkscrew defect as apposed to the regular mitochondrial helix of normal cells.

Ejaculated spermatozoa which retain their cytoplasmic droplets are considered morphologically abnormal (Hancock, 1959) and the proportion of the cells with droplets has been related to reduced fertility in the bull (Campbell et al., 1960; Saacke and White, 1972). Spermatozoa usually lose their cytoplasmic droplets during maturation in the epididymis (Cummins and Orgebin-Christ, 1971; Hancock, 1959). Spermatozoa obtained from the caput epididymis have proximally located cytoplasmic droplets and are less fertile than those from the cauda epididymus where the droplet has become distally located or shed. This result suggests that the translocation and loss of the cytoplasmic droplet during epididymal maturation coincides with the achievement of fertilizing capacity and that abnormal maturation of the spermatozoa in the epididymis may be a cause of increased numbers of ejaculated cells with droplets in certain bulls.

Bent and coiled tails are considered common anomalies of bull semen and are associated with reduced fertility (Sullivan, 1979). They may develop after spermatozoa have

migrated to the cauda epididymis (Swanson and Boyd, 1962) and decrease in frequency with successive ejaculations (Campbell et al., 1960).

Abnormalities affecting the head midpiece or tail of the sperm cell vary in intensity and in influence on the fertility of the spermatozoa. A bull possessing a high frequency of abnormal spermatozoa should probably not be used for breeding, regardless of the type of abnormality possessed by his spermatozoa. Separating morphologically abnormal bovine spermatozoa from morphologically normal sperm cells would be beneficial to the cattle industry. However, the separation of bovine spermatozoa by morphology has received less attention than separation procedures based on other sperm cell characteristics.

#### Separation of Spermatozoa

Experimental procedures have been designed to separate spermatozoa based on either their morphology, viability, all surface properties or chromatin content. The most profitable type of separation would be that permitting predetermination of the sex of offspring. As a result, methods designed to separate X- and Y-chromosome-bearing spermatozoa have been studied more frequently than those procedures designed to separate spermatozoa based on other characteristics.

Separation of X- and Y-Chromosome-Bearing Spermatozoa

Repeated attempts to separate X- and Y-chromosome-bearing spermatozoa using any methodology have been unsuccessful. However, the literature has been plagued with results of individual studies reporting methods capable of sex-ratio alteration. Since differences between the two types of sperm cells exist, separation attempts will continue until a successful method of separation is discovered.

X- and Y-chromosome-bearing spermatozoa differ phenotypically due to the mere presence of either an X- or Y-chromosome. Differences in size between the sex chromosomes have been estimated from drawings of the chromosomes of bovine spermatozoa (Makino, 1944), estimates of the relative DNA content (Garner et al., 1982) and characterization of the chemical composition of spermatozoa (Benedict et al., 1967). It has been presumed that the X-chromosome-bearing spermatozoa are either larger and/or heavier than Y-chromosome-bearing spermatozoa. Differences in chromosomal mass of X- and Y-chromosome-bearing bovine spermatozoa have been estimated at 2% (Sumner and Robinson, 1976) and 4% (Garner et al., 1982), and differences in volume have been estimated at 1% (Lindahl, 1971). Since the differences between X- and Y-chromosome-bearing spermatozoa were expected to provide the necessary differences in volume

or specific density to allow for separation of the two types of cells, many attempts to separate X- and Y-chromosome-bearing spermatozoa by centrifugation and(or) sedimentation have been conducted.

Separation of spermatozoa by sedimentation.

Equilibrium sedimentation in a density gradient and differential rate sedimentation are two methods researchers have employed to separate spermatozoa by specific gravity, weight, or volume. Equilibrium sedimentation requires differing specific gravities between the two types of spermatozoa. Centrifugation of spermatozoa in a density gradient causes sperm cells to come to rest at the point where the specific gravity of the cell is equal to that of the medium. Differential rate sedimentation is based on the assumption that cells with differing weights or volumes will sediment at different rates. However, differences in the physical shape of X- and Y-chromosome-bearing spermatozoa may also cause differences in sedimentation rates (Lindahl, 1971).

Benedict et al. (1967) used centrifugation as a method for separating bovine spermatozoa. Their experiments were designed to separate sperm cells on the basis of differences in buoyant densities of the cells. Several materials were used for the gradients including sodium chloride, glycerol, sucrose, Ficoll and bovine serum albumin (BSA). They reported that spermatozoa were separated into two density

classes and that the number of cells in each density varied with maturity, chemical and physical treatment of the cells, and permeability of the cell to the medium. Cells in the high-density fractions of the gradient were less motile than cells isolated from the low-density fractions. When cells in the high-density fractions were subdivided further, they appeared to differ in dry weight compositions which was made more pronounced by the replacement of cellular water with external solvent.

Luderer et al. (1982) reported the separation of bovine spermatozoa using water insoluble Newtonian gels as a medium for sedimentation. The gels were capable of separating ejaculated spermatozoa into different fractions according to their densities. Because the specific gravities of spermatozoa differed between bulls but were similar in several ejaculates from the same bull, the density of the gels were varied to accomodate the specific gravity characteristic of each bull. Therefore, before the specific gravity of the medium to be used for separation could be chosen, each bull had to be characterized regarding the specific gravity of its spermatozoa. Separated spermatozoa maintained adequate motility and acrosomal integrity both before and after freezing and conception rates obtained with fractionated semen were similar to those obtained with unfractionated semen, however, no deviation from the normal sex ratio was apparent. These authors concluded that this

system may be useful in separating spermatozoa of various densities and for removing extraneous matter, but not for separating X- and Y-chromosome-bearing spermatozoa. Similarly, unsuccessful results using equilibrium sedimentation as a method of separating X- and Y-chromosome-bearing spermatozoa have been reported by O'Donnell (1968; bull), Beatty (1969; rabbit) and Braham (1970; rabbit).

Drevius and Eriksson (1966) observed a slight, reversible swelling of spermatozoa subjected to a hypotonic medium. Apparently, some of the media used in sedimentation experiments were hypotonic. According to Lindahl (1971), those cells that underwent swelling usually died from the stress of centrifugation and did not sediment. As a result, those spermatozoa less resistant to swelling had the highest rate of sedimentation and were probably viable, but were not necessarily X-chromosome-bearing spermatozoa.

Non-equilibrium sedimentation or differential rate sedimentation is a method of separating spermatozoa in which the rate of movement is dependent on size, shape and specific gravity of the cells. The first attempts using centrifugation to separate X- and Y-chromosome-bearing rabbit spermatozoa by differential rate sedimentation were reported by Lush (1925), but were unsuccessful. Lindahl (1956) inseminated cows with bovine spermatozoa separated into different fractions by counter-streaming centrifugation designed to offset the short distance of sedimentation

attained in a centrifuge. Although initial results indicated a successful separation (11 male calves from 11 cows), Lindahl (1958) could not confirm these results in subsequent trials.

Bhattacharya et al. (1966) described a method for separating bull spermatozoa involving the sedimentation of immotile sperm cells in a diluent under the influence of gravity. Spermatozoa were rendered immotile by cooling to 1 C before being applied to the sedimentation column and then permitted to penetrate the column for 12 h. This procedure was effective in altering the normal sex ratio of the rabbit (Bhattacharya, 1958; 1962), however, Bhattacharya (1966) observed no differences in the sex ratio of calves born to heifers inseminated with untreated or fractionated bull semen.

Schilling (1966) described a method for separating bull spermatozoa into "light" and "heavy" fractions. He used a different medium than that described by Bhattacharya (1966) which preserved the motility and viability of the spermatozoa during separation. Over a 3-year period, 86 calves were born to cows inseminated with semen isolated from the "heavy" fractions of the gradient. Sixty-nine percent of the calves were females, a significant deviation from the normal sex ratio.

Krzanowski (1970) attempted to separate sperm cells by weight using a sedimentation process. Bovine sperm cells

were layered on a liquid column containing the same media used by Bhattacharya (1966). Inseminations of cows with spermatozoa from the lower fractions of the column altered the sex ratio of the offspring. Cows inseminated with spermatozoa from the lower BSA fractions produced more female than male calves. These results supported those of Schilling (1966) and Knaack (1968), however, cows inseminated with sperm from the upper fractions of the gradient (which should have contained a higher percentage of Y-chromosome-bearing spermatozoa) also gave birth to a lower percentage of males than cows inseminated with unseparated spermatozoa.

Roberts (1972) developed a theory of motion for spermatozoa under gravity which may account for the failures to completely separate X- and Y-chromosome-bearing spermatozoa by sedimentation and centrifugation. According to Roberts (1972), there are two forces exerted on nonmotile cells: a downward sedimentation velocity and a torque, the latter of which depends on the shape of the cell. Schilling (1971) reported that measurements of spermatozoa from 10 bulls indicated differences among bulls in the length, width and area of the sperm heads, but no differences were detected in the size of the acrosomes or the length of the midpieces and tails. Variations in head size and tail length among spermatozoa can influence sedimentation velocity up to 20%, which is greater than the 1% difference

in sedimentation velocity due to differences in the mass of the X and Y-chromosome, differences in the sedimentation rate of X- and Y-bearing-spermatozoa may be masked by the effects of variation in size and shape of spermatozoa within or among ejaculates (Roberts, 1972).

Failure to obtain consistent separation of X- and Y-chromosome-bearing spermatozoa by sedimentation is likely for reasons other than those noted by Roberts (1972). Sedimentation does separate "heavy" spermatozoa from "light" spermatozoa, but the efficiency of this method of separation is affected because the density of spermatozoa is altered by aging and swelling. Aging and swelling are difficult to control during the sedimentation process. They can influence the density of the sperm cell as much or more than the different sex chromosomes. Results have indicated that these factors associated with the loss of the acrosome from a sperm cell render sedimentation inappropriate as a method of separating X- and Y-chromosome-bearing spermatozoa since a sperm cell without an acrosome would be non-viable.

(O'Donnell and Symons, 1970).

Separation of spermatozoa by electrophoresis. Sperm separation by electrophoresis is based on the theory that differences in the electrical charge of the cell membranes of X- and Y-chromosome-bearing spermatozoa will cause them to migrate differentially to either the anode or cathode, depending on the temperature, pH, and ionic strength of the

buffer used to suspend the semen (Hafs and Boyd, 1971).

The first electrophoretic separation of X- and Y-chromosome-bearing rabbit spermatozoa was reported by Koltzoff and Schroder (1933). One doe inseminated with spermatozoa isolated from the anode produced a litter of females; another doe inseminated with spermatozoa from the cathode produced four males and one female. A third doe inseminated with the fraction of spermatozoa between the two poles produced two males and two females. Results since that time using semen from other species have been less encouraging.

Electrophoresis of bovine spermatozoa using a cell requiring  $\text{Cu-CuSO}_4$  electrodes was conducted by Vesselinovitch (1959). Both whole semen and semen diluted in various buffers were exposed to different potential gradients. Observations were made only on the electrokinetic characteristics of the spermatozoa and not the fertilizing capacity or chromosomal content. At any pH of the Modified-Schroder buffer, and regardless of the voltage applied, all immotile spermatozoa migrated toward the cathode and the motile spermatozoa moved in all directions. The motility of the spermatozoa was directly proportional to the intensity of the current applied, up to 180 volts. Above 180 volts, motility ceased. Vesselinovitch (1959) concluded that only inactive and dead spermatozoa followed electrophoretic drag. Actively motile

spermatozoa responded to current in several ways depending on their original motility and the potential forces applied.

Hafs and Boyd (1971) and Sevinc (1968) have reported successful separations of rabbit spermatozoa by electrophoresis. They suggested that the cell membranes of X- and Y-chromosome-bearing rabbit spermatozoa possessed different electrical charges. However, Hafs and Boyd (1971) were unsuccessful in separating X- and Y-bearing spermatozoa when these procedures were applied to bovine semen. Unlike that of the rabbit, the viability of bovine spermatozoa decreased dramatically when separated by electrophoresis. In contrast, Sevinc (1968) found motility and viability of bovine spermatozoa separated by electrophoresis to be satisfactory, but no sex ratio experiments were conducted with the separated semen. Unsuccessful attempts to separate the X- and Y-chromosome-bearing bovine spermatozoa by electrophoresis have been reported by Macpherson and Vesselinovitch (1959).

Visual identification of X- and Y-chromosome-bearing spermatozoa. The procedure of determining the sex ratio among progeny after artificial insemination with separated semen is tedious and, when applied to livestock, expensive. The ability to determine the X- or Y-chromosome-bearing spermatozoa by visual observation could simplify the evaluation of procedures designed to separate them. According to Sumner et al. (1971) and Barlon and Vosa (1970)

about half of the human spermatozoa contain a fluorescent spot called an "F-body." Sumner et al. (1971) and Sumner and Robinson (1976) have reported that those spermatozoa which stain with the quinacrine fluorescence stain have less DNA than those spermatozoa that do not stain. The F-body procedure has been accepted as a labelling method for Y-chromosome-bearing spermatozoa in humans. Initially, an F-body could not be identified in the spermatozoa of other species. However, Bhattacharya and Gunther (1976) claimed to have modified the F-body technique to identify the Y-chromosome-bearing spermatozoa in semen of the bull, stallion, and boar and have called it the "B-body" test.

Using the "B-body" test as an endpoint, Bhattacharya et al. (1977) attempted to solve the problem of sex control in cattle by developing a particle-free semen extender which controlled the metabolism of the spermatozoa, and a sedimentation technique, which caused the cells to move downward. Further purification of the sample was attempted through the use of galvanic cell current. Eighty-three percent of the separated semen migrated to the anode of the galvanic cell and 81% of the anode-separated spermatozoa were considered to be Y-chromosome-bearing based on the "B-body" test. Although this method of separation and the "B-body" test appear promising as tools for the successful separation of X- and Y-chromosome-bearing bovine spermatozoa, they have yet to be scrutinized by other

researchers in the field.

#### Separation of spermatozoa by chromatography.

Spermatozoa with differing charge characteristics react differently when passed through a bed of ion-exchange resin. Downing et al. (1976) reported differences in the effect of ion-exchange chromatography on spermatozoa collected from several species. Rabbit and bull spermatozoa recovered from ion-exchange columns were of higher viability than initial semen samples indicating that nonviable spermatozoa from these species were retained in the resin. In contrast, the treatment tended to kill a large portion of human spermatozoa and failed to separate X- and Y-chromosome-bearing spermatozoa when tested by the "F-body" technique.

Hahn et al. (1974) separated bull semen using ion-exchange columns. The separation of Y-chromosome-bearing spermatozoa was achieved within the ejaculate of a bull, but no separation was confirmed from all ejaculates among bulls. Correspondingly, Quinlivan et al. (1982) reported an increase in motility (62 to 84%) and in the proportion of X-chromosome-bearing spermatozoa (60 to 74%) in human semen fractions separated with Sephadex columns. However, the degree of separation varied among men or ejaculates.

#### Separation of Spermatozoa Based on Their Motility

Examination of the migration pattern of human X- and Y-

chromosome-bearing spermatozoa in cervical mucus has indicated differences in motility between the two types of spermatozoa (Rohde et al., 1973). As sperm cells penetrated the mucus, a significant increase in the Y-chromosome-bearing spermatozoa (as determined by quinacrine staining) was observed in the frontal zone of sperm cell migration. This suggested that Y-chromosome-bearing spermatozoa were more progressively motile than x-chromosome-bearing spermatozoa.

Roberts (1972) hypothesized that separation of X- and Y-chromosome-bearing spermatozoa occurred in suspensions containing motile spermatozoa. He claimed that when motile cells were placed on top of a vertical column containing a liquid medium, they oriented themselves downward due to gravity. In theory, the two types of spermatozoa then progressed down the column at different rates due to their differences in shape, the Y-chromosome-bearing spermatozoa moving faster. Experiments performed by Goodall and Roberts (1976) confirmed this hypothesis, however, the process of isolation was dependent on two major factors: 1) spermatozoa had to be extended in a media less dense than the one on which they were overlayed and 2) the differences in viscosity had to be great enough to provide an interface which separated motile from nonmotile and normal from abnormal spermatozoa. The normal and motile X- and Y-chromosome-bearing spermatozoa could then penetrate the

interface where the Y-chromosome-bearing spermatozoa could outdistance the X-chromosome-bearing spermatozoa. Based on this theory, Ericsson et al. (1973) proposed a method for the isolation of human semen fractions rich in Y-chromosome-bearing spermatozoa which also provides an approach to improving semen quality.

The separation method developed by Ericsson et al. (1973) involved layering .5 ml of human semen ( $50 \times 10^6$  sperm cells) over a discontinuous gradient of BSA and allowing the spermatozoa to penetrate the medium. This method simultaneously increased the proportion of Y-chromosome-bearing spermatozoa, as determined by quinacrine staining, and the proportion of motile sperm cells in the lower fractions of the gradient. Dmowski et al. (1979) reported similar observations after using the same techniques. Ross et al. (1975) and Evans (1975) attempted to confirm these findings by repeating the procedure designed by Ericsson et al. (1973) but they failed to detect an increase in the percentage of Y-chromosome-bearing spermatozoa in separated semen over that of control semen. However, Ross et al. (1975) reported that a higher percentage of spermatozoa recovered from the column were motile than in the unseparated semen.

Using a column modified from that designed by Ericsson et al. (1973), Quinlivan et al. (1982) reported a 22% increase in the proportion of human Y-chromosome-bearing

spermatozoa recovered from the bottom fractions as compared to unseparated semen. However, the mean motility of the spermatozoa recovered from the column was 8% lower than the motility of the parent semen. Glaub et al. (1976) reported that the viability of sperm cells recovered from a BSA column was similar to that reported by Ericsson et al. (1973). Human sperm cell freeze-preservation techniques were applied to semen samples and separated sperm cell fractions. Post-thaw percentage motility and percentage survival were higher for the separated fractions than for the parent semen.

Ericsson (1980) reported the results of a large scale field trial conducted to test the sex ratio of offspring sired by bovine spermatozoa isolated using a BSA gradient. He failed to detect an alteration in the sex ratio of calves produced by cows inseminated with separated semen, although the semen isolated from the column contained a greater proportion of motile and viable cells than the unseparated semen. Ericsson (1980) indicated that by using a conservative one-step procedure to preserve viability of the spermatozoa instead of a more rigorous separation with several gradients, contamination of the sample with X-chromosome-bearing spermatozoa was inevitable.

Ericsson et al. (1973) and Ross et al. (1975) reported an increase in the percentage of morphologically normal cells recovered from the discontinuous BSA gradient when

compared to the percentage of normal spermatozoa recovered from unseparated semen. Ross et al. (1973) noted that the variation in the percentage of normal cells recovered was due to the percentage and type of abnormality present in the original sample. Since the isolation of more viable spermatozoa was based on the motility of the cell, then those abnormalities affecting motility were more likely to be isolated in the upper fractions of the gradient. Glaub et al. (1976) repeated these same procedures and indicated that the amount of seminal debris also declined considerably in successive fractions of the gradient. Lower fractions were essentially devoid of extraneous matter.

#### Separation Methods to Enhance Semen Quality

Isolation of the population of sperm cells within an ejaculate possessing qualities indicative of high fertility could result in a greater percentage of potentially fertile cells available for artificial insemination. Singer et al. (1980) applied human semen samples to columns of discontinuous gradients of BSA in an attempt to enhance semen viability. No differences in motility were observed between unseparated and separated semen. However, motility estimates of semen recovered from the bottom fractions of the gradient were higher than those estimates of motility from semen isolated from the middle fractions. Furthermore,

the percentage of morphologically normal cells isolated in the bottom fractions of the gradient was greater than that of either the middle fractions or original sample.

Illyes et al. (1977) reported an increase in the percentage of motile bovine spermatozoa collected from the lower fractions of a BSA gradient. Motility estimates from the middle fractions, bottom fractions and original sample were 74, 81 and 50%, respectively. Goodeux and Kreider (1978) also reported a significant improvement in motility of stallion semen isolated from both top and bottom fractions of the two-layer, discontinuous BSA gradient.

Jeulin (1980) used a Laser Doppler Velocimeter to objectively determine the percentage of motile human spermatozoa and to characterize the velocity of the sperm cells isolated in each fraction of discontinuous BSA gradients. One milliliter of extended semen ( $100 \times 10^6$  spermatozoa) was layered on a two-layer gradient of either 5 over 7.5%, 10 over 15%, or 20 over 25% BSA. The semen count and percentage of sperm cells recovered from the three types of columns that were motile were  $8.5 \times 10^6$  and 26%,  $6.5 \times 10^6$  and 20%, and  $3 \times 10^6$  and 9%, respectively. In all columns, 90% of the spermatozoa from the bottom fractions were motile. The mean velocities for spermatozoa in the 5, 7.5 and 10% BSA fractions (83, 85, and  $81 \mu\text{m/s}$ , respectively) were higher than the mean velocities of the spermatozoa in Tyrodes solution ( $69 \mu\text{m/s}$ ) or seminal fluid

(58  $\mu\text{m/s}$ ). However, there was a decrease in the number of spermatozoa with high velocities in columns with BSA concentrations above 15%. Jeulin (1980) concluded that columns of BSA retained non-motile and slow-moving spermatozoa.

Filtration of semen with glass wool or glass beads effectively isolates motile spermatozoa. The semen traverses the columns and leaves debris, agglutinated spermatozoa, and nonviable spermatozoa adhered to the glass beads (Bangham and Hancock, 1955) or glass wool (Paulson and Polakoski, 1977). The separation of live and dead bovine spermatozoa using glass bead columns was first reported by Bangham and Hancock (1955). They noted that the difference in optical density between filtered and unfiltered semen was correlated ( $r=.7$ ) to the percentage of live cells recovered after filtration. Maki-Laurila and Graham (1968) reported that the viability of bovine semen filtered through columns of glass wool was enhanced as dead spermatozoa were removed from the semen sample. Furthermore, an increase in fertility was noted when cows bred with filtered semen (72% vs 68%, filtered vs unfiltered 90 to 120 d non-return rates).

Another effective method for the isolation of motile spermatozoa is Sephadex filtration. Bull semen passed through a Sephadex column designed by Graham (1978) contained few dead or damaged cells. The percentage of

intact acrosomes increased from 66% in unfiltered semen to 93% in filtered semen. In addition, filtered semen was free of all abnormal sperm cells except those with bent ridges or ruffled acrosomes. Since viability of the abnormal cells was unknown, it could not be determined whether abnormal cells were being excluded because of their low viability or due to their abnormal morphology.

In summary, separation methods effective in the isolation of highly motile, morphologically normal spermatozoa have been well documented. In contrast, an effective method of separating X- and Y-chromosome-bearing sperm has not been discovered. Experiments designed to measure the effects of freezing and thawing and post-thaw aging on cells separated for high viability have not been conducted but could be of substantial benefit to the livestock industry. The underlying scheme of this thesis was to determine if a discontinuous BSA gradient (Ericsson et al., 1973), modified to maximize the percentage of sperm cells recovered, could isolate a population of highly viable, morphologically normal, Y-chromosome-bearing bovine spermatozoa.

## PURPOSE AND SPECIFIC OBJECTIVES

Isolation of a population of highly motile, morphologically normal bovine spermatozoa for artificial insemination (AI) could provide an opportunity to improve the fertility of cattle bred by AI. In addition, if the isolation method allowed the ability to predetermine the sex of calves produced, tremendous economic benefits could result for calf producers.

The following study was conducted to characterize the motility, morphology and sex-chromosome content of spermatozoa isolated using a discontinuous bovine serum albumin (BSA) gradient. The specific objectives of this study were:

- to determine the concentration of spermatozoa that could be applied to the gradient in order to optimize the efficiency of the separation,

- to determine the effects of differences in the concentration of spermatozoa applied on the motility and the percentage of spermatozoa recovered from the gradient,

- to determine if the gradient could exclude spermatozoa with abnormal morphology,

- to characterize the viability of spermatozoa immediately after it was recovered from various fractions of the column and compare the viability of freshly separated spermatozoa with that of unseparated spermatozoa,

-to compare the viability of separated and unseparated spermatozoa after freezing and thawing and after periods of post-thaw aging, and

-to determine if the gradient had the ability to separate X- from Y-chromosome-bearing spermatozoa.

## METHODS OF PROCEDURE

### Experiment 1: The motility and percentage of applied spermatozoa recovered from fractions T, 1 and 6

An initial experiment was conducted to test the feasibility of a discontinuous BSA gradient for the separation of bovine semen. In experiments of Ericsson (1973; 1980) and Wall et al. (1980) only  $50 \times 10^6$  sperm cells could be applied to a discontinuous BSA gradient for separation. Since this represented only <1% of an average bovine ejaculate, our intention was to design a larger gradient, capable of separating a greater portion of an ejaculate.

Ejaculates from 3 bulls were split, pooled equally, evaluated for percent progressive motility, and either 1, 3, 5, 7, 14 or  $21 \times 10^9$  sperm cells in 26 ml of Tris buffer (pH 6.8; 310 mosm) were layered on top of a discontinuous bovine serum albumin (BSA) gradient (figure 1). The gradient consisted of 4% BSA (2.4 g/60 ml of Tris buffer; Sigma Chemical Co, St. Louis, Missouri; pH adjusted to 6.8) layered over 10% BSA (6.0 g/60 ml; pH adjusted to 6.8) using a biruet attached to 2 ft of silicone tubing. Gravity forced the BSA through the biruet and tubing. Care was taken to establish a clear interface between the 4 and 10% albumin layers. Flow rates were controlled by adjusting the

# BSA COLUMN

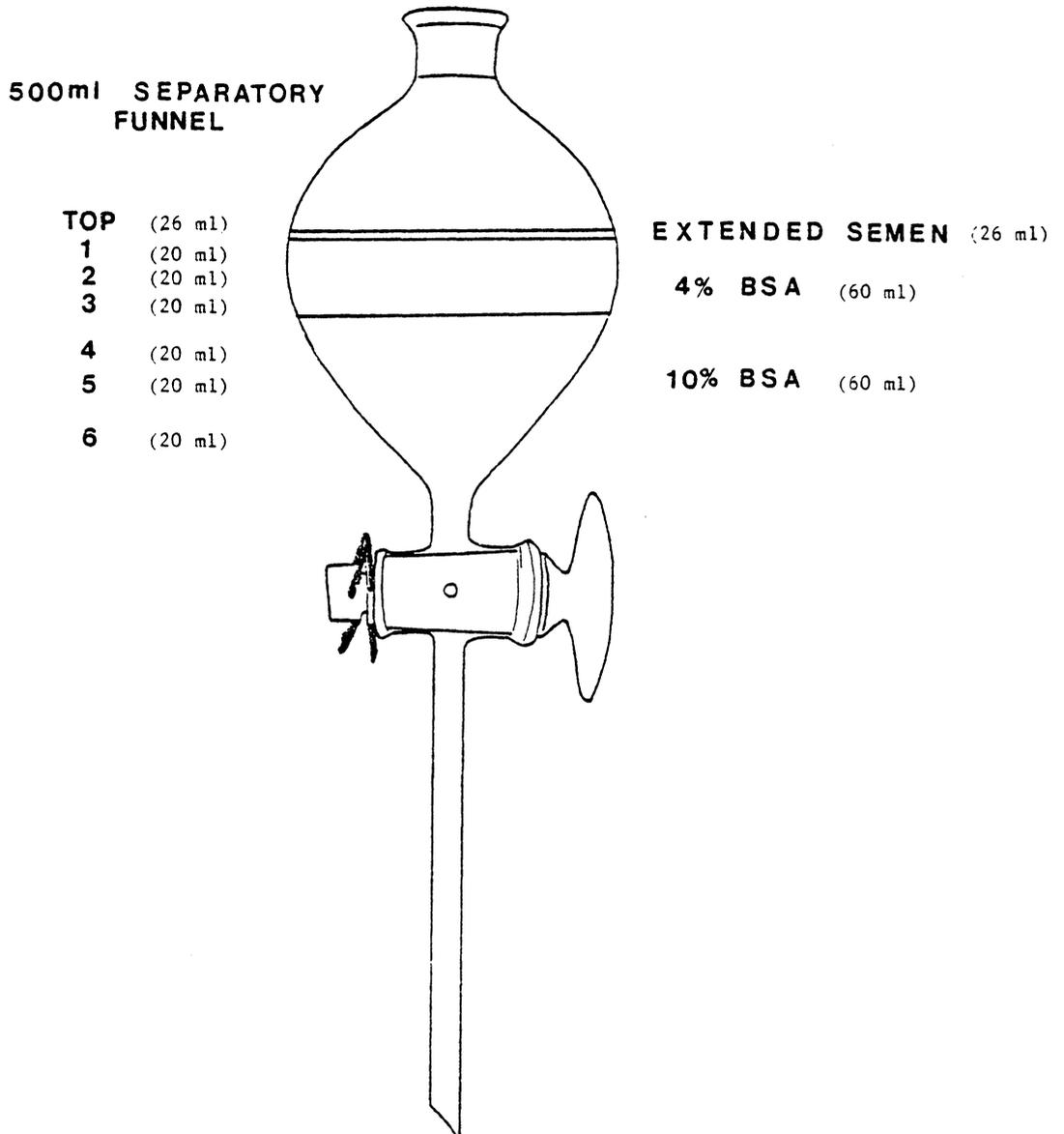


Figure 1. Bovine serum albumin column used for separating semen.

stopcock on the biuret. Three gradient separations (replicates) were conducted for each concentration of sperm cells.

After 1 h of sperm cell migration, six 20-ml aliquots and the remaining 26 ml were collected from the bottom of the column. Those seven aliquots were identified as fractions T, 1, 2, 3, 4, 5 and 6. Fraction 6 was the first aliquot collected from the bottom (10% BSA) of the column, fraction T, the last. Fractions T, 1 and 6 were evaluated for concentration and percentage of progressively motile sperm cells.

Concentrations of the fractions were determined through the use of a cell counter (Deibel et al., 1978). Fractions were diluted (1:200) in an electrolyte solution and then passed through an aperture to displace an equal volume of electrolyte, causing the resistance in the path to change. The number of changes within a specific length of time was proportional to the number of cells within the fraction.

Estimations of motility were made by diluting raw semen in a drop of Tris buffer on a microscope slide warmed to 37 C. Separated semen was not diluted when evaluated for motility. A phase contrast microscope (100x) equipped with a heated stage was used to estimate the percent progressive motility to the nearest 5%.

Statistical analysis. The effects of the different concentrations of spermatozoa applied to the column on the

percentage of spermatozoa recovered in fractions T, 1 and 6 were evaluated using analysis of variance (Snedecor and Cochran, 1979). The model included concentration and fraction as main effects and initial motility of the applied sample was applied as a covariate. Comparisons among fractions within concentrations were made using Tukey's studentized range test (Snedecor and Cochran, 1979).

Experiment 2: The morphology and viability of spermatozoa recovered from the BSA column

Six ejaculates with 30 to 60% morphologically abnormal sperm cells were collected, one from each of six bulls. Aliquots from each ejaculate were stained for assessment of viability using eosin-aniline blue stain (Shaffer and Almquist, 1948). Using a split ejaculate technique, semen was either extended and conventionally frozen in an egg-yolk-glycerol-citrate buffer ( $20 \times 10^6$  cells/ml) or extended in Tris buffer ( $1 \times 10^9$  sperm cells/26 ml) and applied to the BSA gradient described in Experiment 1. Spermatozoa from each ejaculate were used in two gradient separations (replicates). Following 1 h of sperm cell migration, fractions 3 and 6 (see figure 1) were recovered from the gradient. Fractions recovered were then diluted in Tris-egg-yolk buffer (30 ml added to each fraction) that had been clarified by passing the buffer through a millipore filter

(.45  $\mu$ m). Diluted fractions were cooled to 5 C over 1.5 h in a water bath. Following cooling, the diluted fractions were split equally, added to two 50 ml centrifuge tubes and centrifuged (630 x g) at 5 C for 10 min to form a soft pellet. Supernatants were then extracted with a 4 in needle (12 gauge) attached to a 60 ml syringe and recentrifuged (630 x g for 10 min). After centrifugation, all but 1 ml of the supernatant was removed and discarded. The remaining pellet was resuspended. Duplicate hemocytometer counts were conducted and each sample was extended to the volume necessary to achieve a concentration of  $30 \times 10^6$  sperm cells/ml by addition of Tris-egg-yolk buffer. Fractionated semen was glycerolated, stored overnight at 5 C, frozen in .5 ml French straws the following day, and stored at -196 C.

Four weeks following freezing, 6 straws from each of the two fractions collected from the BSA gradient and 6 straws of unseparated semen were thawed (45 s at 35 C) and evaluated by two independent observers. The percent progressive motility was estimated for each sample by observing two wet smears at 100x magnification using a phase contrast microscope equipped with a heat stage. Smears were coded to prohibit biased evaluation.

Acrosomal integrity was determined by the presence of an intact apical ridge using differential contrast microscopy (Saacke and White, 1972). One hundred sperm cells were assessed from each of two smears. If the

percentage of cells with intact acrosomes differed by more than 8%, a third smear was evaluated.

Sperm cell morphology was evaluated from thawed semen and semen stained with eosin-aniline blue using differential interference contrast optics (oil immersion). Two hundred cells were identified as either normal or abnormal. Each sample was evaluated twice. Abnormalities were categorized as the following:

Head - decapitated, tapered, pyriform, short, asymmetrical,  
head and droplet, crater, other

Midpiece - proximal, translocating and distal droplets,  
bent, truncated, other

Acrosomal

Broken neck

Tail - coiled, abaxial, bent, other

Statistical analysis. The main effects of ejaculate, separated or unseparated spermatozoa (treatment), the replication of separatory funnels (separatory funnel), and fractions 3 and 6 of the separated semen (fraction) on the proportion of morphologically abnormal sperm cells, the percentage of motile spermatozoa, and the proportion of sperm cells with intact acrosomes were evaluated using least squares analysis of variance (Snedecor and Cochran, 1979). The ejaculate by treatment interaction was included in the separatory funnel effect as a source of variation. Student's t-test (Snedecor and Cochran, 1979) was used to

test differences in least squares means between spermatozoa from either fraction 3 or 6 with unseparated spermatozoa.

Experiment 3: The post-thaw viability of separated and unseparated semen

Fifteen ejaculates with at least 50% motile sperm cells were collected from 6 bulls. No one bull contributed more than four ejaculates. Semen was either extended in Tris-yolk buffer ( $20 \times 10^6$  sperm cells/ml) and frozen or extended in Tris buffer ( $1 \times 10^9$  cells/26 ml) and applied to the BSA gradient described in Experiment 1. Portions of each ejaculate were used in two different gradient separations (replicates). After 1 h of sperm cell migration, fractions T, 1, 2, 3, 4, 5 and 6 were recovered. Fractions 2 and 5 were discarded. The remaining fractions were each evaluated to assess their relative motility and the porportion of cells with intact acrosomes. The sperm cells in each fraction were washed and frozen as described in Experiment 2.

Four weeks after freezing, 6 straws prepared from each fraction and 6 straws prepared from unseparated semen were thawed (45 s at 37 C). In order to assess latent injury, all samples were incubated (37 C) for 2 h post-thaw. Samples were evaluated for post-thaw motility, the percentage of sperm cells with intact acrosomes, average

sperm velocity, total motility (VMOTT), and progressive motility (VMOTP) at 0 and 2 h of incubation (37 C).

Assessment of velocity and VMOTT were made by using a videomicrographic system consisting of a video camera mounted on a phase microscope equipped with a heat stage and a video cassette recorder connected to a time-date generator and a television monitor (Katz and Overstreet, 1981). Samples were recorded at each incubation time for approximately 2 min in order to tape at least 5 fields of each sample. Videotapes were then replayed and the velocity and VMOTT determined. The swimming velocity ( $\mu\text{m/s}$ ) of 50 sperm cells from each replicate of each fraction were measured by overlaying a calibrated acetate grid on the screen of the monitor during replay. VMOTT was determined by observing the proportion of sperm cells moving within a 1-s time interval.

VMOTP was computed by multiplying the proportion of sperm cells in a sample which moved at a rate of  $\geq 25 \mu\text{m/s}$  by the percentage of moving sperm cells in a sample (VMOTT). Sperm cells moving backwards were noted but not included in either VMOTT or VMOTP.

Statistical analysis. The effects of ejaculate, unseparated semen and fractions T, 1, 3, 4, and 6 (sample) and post-thaw incubation time (time), on semen viability parameters were analyzed using least squares analysis of variance (Snedecor and Cochran, 1979). The effects of

ejaculate by time, sample by time, separatory funnel replicates, and the ejaculate by sample interaction on the semen parameters measure were not significant in a preliminary analysis. Therefore these were not included as sources of variation. Differences between samples were determined using Tukey's studentized range test for pairwise comparisons (Snedecor and Cochran, 1979).

To further characterize the effects of freezing and aging on motility and acrosomal integrity, the change in motility as a percentage of the motility either prior to freezing or immediately post-thaw, and the change in intact acrosomes as a percentage of the rate of intact acrosomes prior to freezing or immediately post-thaw in samples prepared from the 5 fractions of separated semen and in unseparated were compared by least squares analysis of variance. The change in the percentage of sperm cells with intact acrosomes or the proportion of motile sperm cells was calculated as a percentage of the measurement at the earlier evaluation:

$$\text{Change (\%)} = \frac{\text{Initial reading (\%)} - \text{final reading (\%)}}{\text{Initial reading (\%)}} \times 100$$

Variation due to different ejaculates (ejaculate) and

variation due to different fractions or unseparated semen (sample) were included as main effects. Differences in the percentage of change between samples were determined using Tukey's studentized range test.

Experiment 4: An attempt to isolate Y-chromosome bearing spermatozoa using a BSA gradient

In order to determine if the separation method cited in Experiment 1 had the ability to separate X- and Y-chromosome-bearing bovine spermatozoa, separated spermatozoa were recovered and used for artificial insemination.

Three Angus bulls from Select Sires, Inc. were collected on four occasions by artificial vagina. One billion sperm cells were isolated from each ejaculate and extended in 26 ml of Tris buffer. The extended semen ( $38 \times 10^6$  sperm cells/ml) was layered on top of the discontinuous BSA gradient described in Experiment 1. The remaining portion of the ejaculate was extended in egg-yolk-citrate buffer and frozen in .5 ml French straws (as routinely performed by Select Sires, Inc.). After 1 h of sperm cell migration the fifth and sixth fractions were collected, pooled, and frozen as described in Experiment 2.

At least 4 wk following freezing, 180 crossbred beef cows at Neuhoff Farms Inc. (Foster's Falls, VA) and 65 cows at the Catawba Animal Research Station (Catawba, VA) were

treated with prostaglandin  $F_{2\alpha}$  for estrous synchronization and each animal was bred by artificial insemination 12 h after detection of estrus. Semen was thawed in a 35 C waterbath for 45 s. Cows that conceived to artificial breeding (135 Neuhoff; 59 Catawba) were maintained throughout gestation using common management practices. The sex of the calves was recorded at birth.

Differences between the sex ratio of calves born to cows inseminated with separated semen and the sex ratio of calves born to cows inseminated with unseparated semen were analyzed using Chi-square methods (Snedecor and Cochran, 1979).

Experiment 5: An attempt to isolate Y-chromosome bearing spermatozoa using a BSA gradient

Flow cytometry was used to determine the relative DNA content of separated and unseparated bovine spermatozoa (Garner et al., 1982). Three ejaculates from 3 Holstein bulls were pooled. A portion of the pooled ejaculates was applied to the BSA column as described in Experiment 1. The remaining semen was divided equally and either frozen or exposed to 10% BSA and frozen. However, semen was extended and washed in citrate and egg-yolk-citrate buffers instead of buffers containing Tris. Fractions 1 and 6 were recovered from the column and frozen as described in Experiments 1 and 2. The frozen samples were then shipped

to the Lawrence Livermore National Laboratory (Livermore, CA) where they were thawed and washed by centrifugation using a series of dimethyl sulfoxide solutions, fixed in ethanol, treated with papain and stained with 4-6-diamidino-2-phenylindole for fluorescence (Pinkel et al., 1982).

The DNA of the sperm cells was measured with an epillumination flow cytometer. Fluorescence from the stained spermatozoa was stimulated by a laser beam traveling perpendicular to the plane of the sample stream. One fluorescence detector was aimed directly into the laser beam and measured the DNA content from the flat side of the sperm cell. If the spermatozoa are properly oriented, another fluorescence detector made a simultaneous measurement on the thin edge of the sperm cell. The sperm cells must be oriented properly in order to obtain accurate measurements. If they were misoriented, they interfered with stain content measurements and were detected from the fluorescent detector measuring fluorescence on the thin edge. Bimodal histograms were generated by displaying the number of spermatozoa vs fluorescence intensity per sperm cell. Each bimodal histogram was analyzed by computer to determine the difference in proportion of X- and Y-chromosome-bearing spermatozoa in the sample.

## RESULTS AND DISCUSSION

### Experiment 1: The motility and percentage of applied spermatozoa recovered from fractions T, 1 and 6

The concentration of spermatozoa in the sample applied to the BSA gradient was varied in order to determine the effect of different concentrations on the final motility and the percentage of spermatozoa recovered from three fractions of the gradient. Since motility of the spermatozoa in the samples applied differed, the data for final motility and percent recovery were adjusted based on differences in initial motility. The effects of concentration applied, the fraction collected, and the concentration by fraction interaction were significant for both the motility and percentage of sperm cells recovered (tables 1 and 2).

To determine how the sperm cell concentration of the applied sample affected the percentage of sperm cells in the applied sample that were recovered, comparisons were made between the percentage of sperm cells recovered from each fraction at each concentration of the applied sample (figure 2). When  $3 \times 10^9$  sperm cells were applied to the column, the percentage of spermatozoa recovered from fractions T and 1 was greater than when  $1 \times 10^9$  sperm cells were applied to the column. The greater proportion of motile spermatozoa recovered coincided with an increase in the recovery of

sperm cells from these fractions. When  $5 \times 10^9$  sperm cells were applied, the percentage of spermatozoa recovered in fraction 1 was less ( $P < .05$ ) than the percentage of sperm cells recovered from fraction T. This difference was unexpected but indicated that the percentages of sperm cells recovered from two fractions were inversely related. Above  $7 \times 10^9$  sperm cells/26 ml, the percentage of sperm cells recovered from fractions T and 1 were similar. The slight decreases in percent recovery observed in fractions T and 1 when more than  $5 \times 10^9$  cells were applied may have been explained if the motility and percent recovery of fraction 2 were examined. Furthermore, the density of the semen when  $14 \times 10^9$  and  $21 \times 10^9$  spermatozoa were applied to the column was probably of sufficient magnitude to cause a portion of the applied semen to forcibly penetrate into the 4% BSA.

The proportion of the applied sample recovered from fraction 6 was greatest when  $1 \times 10^9$  sperm cells were applied to the column. Above that concentration, the percent recovery dropped dramatically. When one billion cells were applied, the percentage of spermatozoa recovered from fractions T, 1 and 6 was similar (19.9%, 15.4%, and 16.0%, respectively). At higher concentrations, fractions T and 1 each contained a greater ( $P < .05$ ) percentage of spermatozoa than fraction 6. Similar results were obtained by Jeulin (1980). She reported that approximately 20% of the applied sample was recovered in the 10% BSA fraction of the

Table 1

ANALYSIS OF VARIANCE OF THE EFFECTS OF INITIAL MOTILITY  
AND THE APPLIED CONCENTRATION OF THE SAMPLE ON THE  
FINAL MOTILITY OF THE FRACTIONS RECOVERED FROM  
THE COLUMN

Source	df	Mean Square	Level of Significance
		Final Mot	
Concentration	5	570.4	.0601
Fraction	2	6645.0	.0001
Conc X Frac	10	191.0	.0141
Initial motility	1	351.8	.0319
Error	35	70.42	

Table 2

ANALYSIS OF VARIANCE OF THE EFFECTS OF INITIAL MOTILITY  
AND THE APPLIED CONCENTRATION OF THE SAMPLE ON THE  
PERCENTAGE OF THE APPLIED SAMPLE RECOVERED FROM THE COLUMN

Source	df	Mean Square	Level of Significance
		Percent Recovery	
Concentration	5	86.72	.0011
Fraction	2	3367.	.0001
Conc X Frac	10	145.1	.0001
Initial motility	1	303.6	.0001
Error	35	16.66	

# PERCENTAGE OF APPLIED SAMPLE RECOVERED

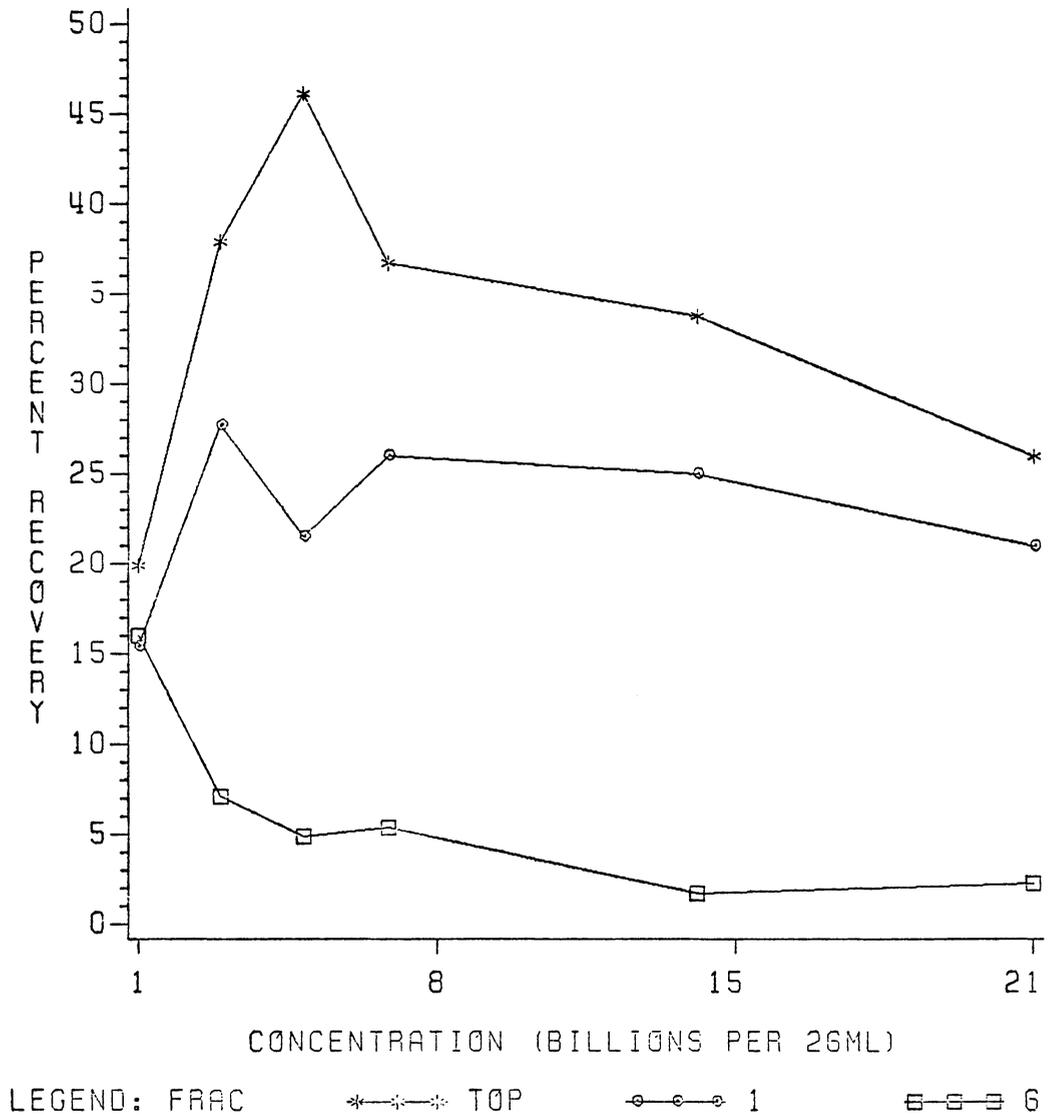


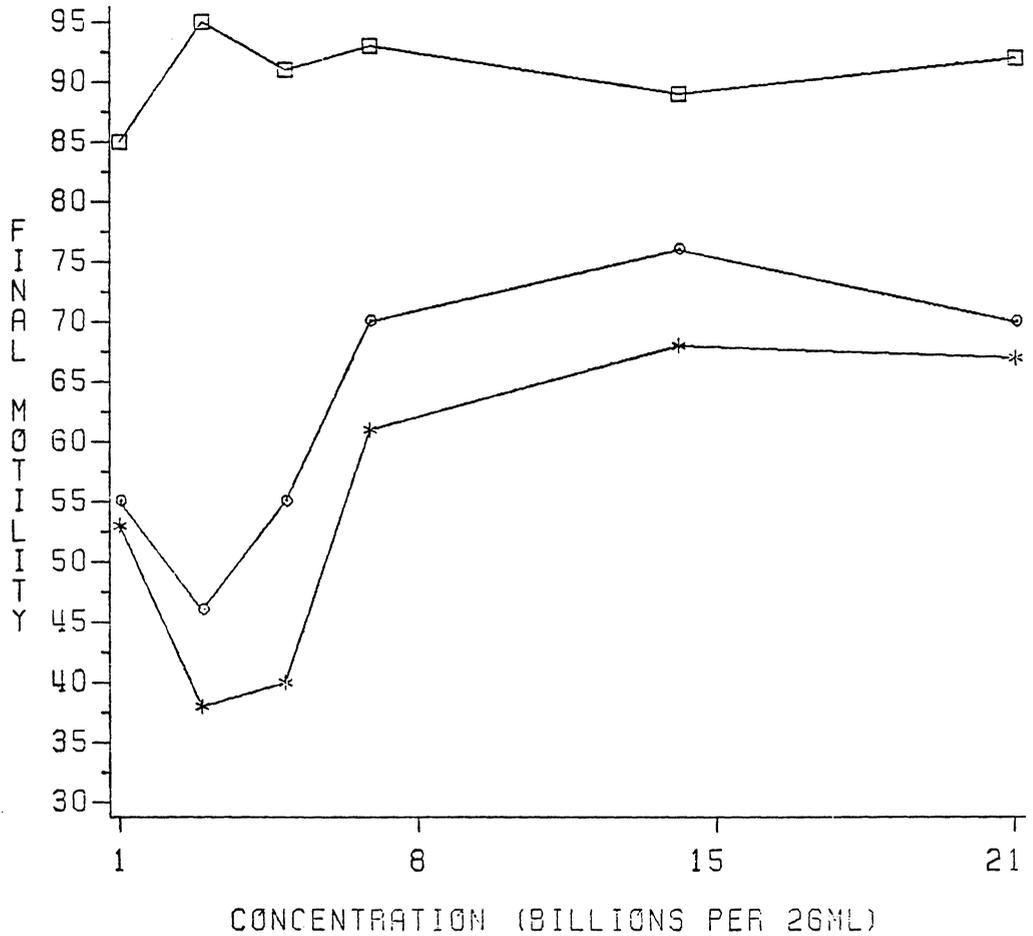
Figure 2. The percentage of the applied semen sample recovered at each concentration.

gradient.

For all concentrations evaluated, fraction 6 contained a higher percentage of motile spermatozoa than fractions T or 1 ( $P < .05$ ; figure 3). As the concentration of the applied sample increased beyond  $3 \times 10^9$ , the proportion of motile spermatozoa in fractions T and 1 increased. This trend was probably due to an insufficient amount of surface area at the interface of fractions T and 1 and fractions 3 and 4 of the gradient. The lack of surface area at these interfaces probably trapped non-motile spermatozoa causing them to form a barrier. This barrier then prohibited the passage of motile spermatozoa through the interface. In addition, an increase in the frequency of collisions between sperm cells of the applied semen was inevitable when large concentrations of spermatozoa were applied and may have caused the spermatozoa to become oriented in different directions other than those influenced by gravity.

Based on these data, motile spermatozoa apparently penetrated the BSA column and that the more dense the fraction, the greater the proportion of motile sperm cells within it (figure 3). When  $1 \times 10^9$  sperm cells were applied, the column was most efficient in isolating motile sperm cells in the bottom fraction. These results are similar to those of Ericsson et al. (1973), Dmowski et al. (1979), and Ross et al. (1975), but differ from those of Quinlivan et al. (1982) who observed lower motility in the most dense

## FINAL MOTILITY OF SPERMATOZOA RECOVERED



LEGEND: FRAC      \*-\*-\* TOP      ○-○-○ 1      □-□-□ 6

Figure 3. The final motility of the spermatozoa recovered at each concentration.

fractions.

The proportion of the applied sample recovered from fraction 6 was greatest when  $1 \times 10^9$  sperm cells were applied to the column. Fraction 6 also retained the highest percentage of motile spermatozoa. However,  $1 \times 10^6$  was the lowest number of cells applied to the column, therefore, future experiments should be conducted to determine if application of lower concentrations ( $< 1 \times 10^9$  sperm cells/26 ml) would increase the percentage of spermatozoa recovered from fraction 6. Other manipulations which may prove beneficial include: increasing the diameter of the column in order to increase surface area between the applied semen and the 4% BSA and the interface between the 4% BSA and the 10% BSA. The possibility of increasing the time permitted for spermatozoa to penetrate the gradient should also be investigated.

Ross et al. (1975), reported that the percentage of human sperm cells recovered from the bottom of a BSA column was affected by the morphology of the applied sample. He believed that spermatozoa with abnormal morphology would not penetrate the gradient although they may be motile. Morphology of the applied sample was not evaluated in this study. However, the ability of the column to separate abnormal sperm cells has been noted (see Experiment 2) and its affect on the percentage of sperm cells recovered may be of significance and should be considered in future studies.

Experiment 2: The morphology and viability of spermatozoa recovered from the BSA column

The discontinuous BSA gradient was capable of excluding the spermatozoa possessing tail or midpiece abnormalities from the lower fractions of the column, but had no effect on the spermatozoa possessing head abnormalities. Analysis of the effects of column separation on the frequency of sperm cells with abnormal morphology is depicted in table 3. Spermatozoa identified with acrosomal abnormalities were excluded from analysis due to scarceness (<1%). As expected, different types of abnormal sperm cells predominated in each ejaculate. As a result, the frequencies of sperm cells with head and midpiece abnormalities in separated semen differed among the ejaculates separated.

No difference in the frequency of head abnormalities was detected between unseparated and separated semen or between fractions 3 and 6 (table 4). Although it was not analyzed, a reduction in the frequency of decapitated heads (a subclass of head abnormalities) was observed in the separated semen. Sperm cells with decapitated heads were non-motile and were not expected to penetrate the gradient.

Katz et al. (1982) indicated that human sperm cells possessing normal head morphology moved faster than spermatozoa with abnormal head morphology. They reported

Table 3

RESULTS OF ANALYSIS OF VARIANCE OF THE EFFECT  
OF BSA COLUMN SEPARATION ON THE PERCENTAGE OF SPERMATOZOA  
POSSESSING HEAD, MIDPIECE OR TAIL ABNORMALITIES

Source	df	Mean Squares		
		Head	Midpiece	Tail
Ejaculate	5	2738.06*	760.78*	5.07
Treatment	1	18.41	320.13*	23.41*
Separatory funnel	11	65.84	31.01	2.01**
Fraction/funnel	1	63.38	384.0**	3.38*
Error	11	40.83	25.65	.284

\*P<.01

\*\*P<.005

Table 4

LEAST SQUARES MEANS ( $\pm$  SE) FOR PERCENTAGES OF SPERMATOZOA  
POSSESSING HEAD, MIDPIECE OR TAIL ABNORMALITIES IN  
UNSEPARATED AND SEPARATED SEMEN

	Head	Midpiece	Tail
Unseparated	26.5 $\pm 3.31$	20.3 <sup>a</sup> $\pm 2.27$	3.5 <sup>a</sup> $\pm .42$
Separated			
fraction 3	26.0 $\pm 1.84$	16.2 <sup>a</sup> $\pm 1.46$	1.7 <sup>b,c</sup> $\pm .15$
fraction 6	22.9 $\pm 1.84$	8.2 <sup>b</sup> $\pm 1.46$	.9 <sup>b,d</sup> $\pm .15$

a,b Means of unseparated and separated semen with different superscripts are significantly different ( $P < .05$ ).

c,d Means of unseparated and separated semen with different superscripts are significantly different ( $P < .05$ ).

that differences in velocity were attributed to greater flagellar beat frequencies of the normal cells rather than to any differences detected in flagellar beat shapes. These investigators suggested that sperm cells with abnormal head morphology also had abnormalities in the production and utilization of energy within the cell and, therefore, were less capable of maintaining viability for fertilization of the ova. From these observations and the results pertaining to head abnormalities in this study, one can conclude that the column may separate motile from non-motile spermatozoa, but that separation based on velocity differences due to head abnormalities (if they exist) may not be possible.

The proportion of spermatozoa with midpiece abnormalities (of which the majority were cells with translocating droplets) was greater in the unseparated semen than in the separated semen ( $P < .01$ ; table 3). The proportion of cells with abnormal midpieces was greater in fraction 3 than in fraction 6 ( $P < .05$ ; table 4). However, the frequency of spermatozoa with midpiece abnormalities was similar in fractions 3 and unseparated semen, but lower in fraction 6 than in unseparated semen. The interface between the 4% and 10% BSA was appeared essential for the column to separate semen with midpiece abnormalities. Since midpiece abnormalities alter the movement of the tail of the sperm cells and, consequently, their motility, those spermatozoa possessing midpiece abnormalities probably were excluded

from the bottom of the column because of their inability to penetrate the 10% BSA.

There were relatively few spermatozoa identified with tail abnormalities and, unlike the separation of other abnormal spermatozoa, the variation in ability to separate cells with abnormal tails among separatory funnel replicates was significant (table 3). This lack of repeatability appeared to be due to the fact that small numbers of abnormal spermatozoa (one or two) markedly altered the total percentage of spermatozoa identified with tail abnormalities. Hence, the variation between replicates may have been due to sampling errors rather than the replicate variation.

Separated spermatozoa recovered from the BSA gradient possessed fewer tail abnormalities than spermatozoa from unseparated semen. When evaluated independently, both fractions 3 and 6 had a lower frequency of sperm cells with tail abnormalities than unseparated semen (table 4). Fractions 3 and 6 differed significantly from each other in their ability to exclude spermatozoa possessing tail abnormalities. Therefore, although isolation of a population of spermatozoa with few tail abnormalities was possible with one column interface, further exclusion of sperm cells with abnormalities was possible due to the second interface located between the 4% and 10% BSA. Despite the statistical significance of the columns ability

to exclude cells with tail abnormalities, results of this study regarding tail abnormalities should be considered with some reservation due to the low frequency of spermatozoa with tail abnormalities and the statistically significant difference between separatory funnel replicates.

Morphologically abnormal human spermatozoa, isolated from either post-coital cervical secretions or fresh semen were slower moving and more frequently non-viable than morphologically normal sperm cells (Fredricsson et al., 1977; Overstreet et al., 1981). The percentage of viable spermatozoa in unseparated semen possessing abnormal morphology is depicted in table 5. A large proportion of sperm cells with either head, midpiece, or tail abnormalities were viable before separation. This indicates that the proportion of spermatozoa with tail or midpiece abnormalities were excluded from the column due to their abnormal morphology and not because they were non-viable. Conversely, majority of the spermatozoa with broken necks were non-viable. Therefore, it could not be ascertained as to whether or not spermatozoa with broken necks were isolated in the top fractions of the column because of their abnormal morphology or lack of viability.

The frequency of motile spermatozoa was greater in separated than unseparated thawed semen, although the proportion of motile spermatozoa in fractions 3 and 6 were similar (tables 6 and 7). Illyes et al. (1977) and Glaub

Table 5

THE PERCENTAGE OF VIABLE SPERMATOZOA IN UNSEPARATED  
SEMEN POSSESSING EITHER ABNORMAL OR NORMAL MORPHOLOGY

<u>Morphology of Spermatozoa</u>	<u>Percentage of viable spermatozoa (total number with abnormality)</u>
Normal	87.4 (714)
Head abnormality	62.5 (272)
Midpiece abnormality	95.6 (181)
Tail abnormality	60.0 ( 10)
Acrosomal abnormality	66.6 ( 3)
Broken neck	11.1 ( 9)

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Table 6

RESULTS OF ANALYSIS OF VARIANCE OF THE EFFECT  
OF BSA COLUMN SEPARATION ON THE PERCENTAGE OF VIABLE AND  
MORPHOLOGICALLY NORMAL SPERMATOZOA

Source	df	Mean Squares		
		Normal (%)	Motility (%)	Intact acrosomes (%)
Ejaculate	5	2016.64*	335.67	33.21
Treatment	1	755.01**	4955.06*	3857.35*
Separatory funnel	11	58.92	114.54	125.00*
Fraction/funnel	1	805.04**	208.33	22.01
Error	11	53.95	194.69	11.06

\*P<.001

\*\*P<.005

Table 7

LEAST SQUARES MEANS ( $\pm$  SE) FOR THE PERCENTAGE OF  
VIABLE AND MORPHOLOGICALLY NORMAL SPERMATOZOA IN  
UNSEPARATED AND SEPARATED SEMEN

	Motility	Intact Acrosomes	Normal
Unseparated	37.50 <sup>a</sup> $\pm 3.09$	65.60 <sup>a</sup> $\pm 3.22$	48.17 <sup>a</sup> $\pm 3.13$
Separated			
Fraction 3	60.00 <sup>b</sup> $\pm 2.85$	84.96 <sup>b</sup> $\pm .68$	54.9 <sup>a</sup> $\pm 2.06$
Fraction 6	64.17 <sup>b</sup> $\pm 2.85$	86.32 <sup>b</sup> $\pm .68$	66.5 <sup>b</sup> $\pm 2.06$

a,b Means of unseparated and separated semen with different superscripts are significantly different ( $P < .05$ ).

et. al. (1976) have also reported that separated semen possessed a higher percentage of motile spermatozoa after freezing and thawing. Enhancement of the number of of motile spermatozoa in the BSA fractions was similar to the enhancement observed in unfrozen semen in Experiment 1, further confirming the results of Ericsson et al. (1973), Dmowski et al. (1971) and Ross et al. (1973).

Concurrent with the enhancement of motile spermatozoa in separated semen was an additional increase in the percentage of spermatozoa with intact acrosomes in separated semen. Like the pattern exhibited for motility, the proportion of sperm cells with intact acrosomes in fractions 3 and 6 did not differ, but the difference between separated and unseparated semen was significant.

The frequency of morphologically normal spermatozoa was greatly enhanced by the discontinuous gradient separation procedure. Separated semen contained more morphologically normal spermatozoa than unseparated semen. The interface between the 4% and 10% BSA was more effective in isolating morphologically normal spermatozoa than the interface between the applied sample and 4% BSA since a greater percentage of morphologically normal spermatozoa were recovered from fraction 6 than from unseparated semen, but the proportions recovered from fraction 3 and unseparated semen were similar (table 7). These observations confirm results obtained by Singer et al. (1980) using a

discontinuous BSA gradient to filter human semen.

The isolation of a population of viable, morphologically normal spermatozoa using this separation procedure was obvious. If a large proportion of these separated spermatozoa maintain their enhanced viability through separation, freezing and post-thaw aging, they could represent a population of highly fertile sperm cells. This population of spermatozoa could be used to improve conception rates following artificial insemination, especially when compared to inseminations with semen from bulls with low fertility due to specific abnormalities.

Experiment 3: The post-thaw viability of separated and unseparated semen

The viability of spermatozoa prior to freezing is almost always greater than the viability to the same sample post-thaw. Other researchers have indicated that post-thaw viability is a better indication of the potential fertility of a semen sample than the pre-freeze viability.

Post-thaw viability parameters were evaluated for semen from 15 ejaculates (ejaculate) for five separated semen fractions and unseparated semen (sample) at 0 and 2 h post-thaw (time). Analysis of the effects of ejaculate, sample and time of incubation on motility, intact acrosomes, velocity, VMOTT, and VMOTP are presented in tables 8 and 9.

Table 8

RESULTS OF ANALYSIS OF VARIANCE OF THE VELOCITY,  
VMOTT, AND VMOTP OF SEMEN SAMPLES<sup>1</sup>

Sources	df	Mean Squares		
		Vel	VMOTT	VMOTP
Ejaculate	11	11624.8*	14551.9*	32401.5*
Sample	10	1542.8*	44104.8*	34440.8*
Time	1	10480.2*	420.8	4664.0**
Error	241	342.8	1438.1	1602.1

\*P<.0001

\*\*P<.1

<sup>1</sup>The effects of ejaculate x time, sample x time, separatory funnel replicates and the ejaculate x sample interaction on these parameters were not significant.

Table 9

RESULTS OF ANALYSIS OF VARIANCE OF THE MOTILITY  
AND ACROSOMAL INTEGRITY OF SEMEN SAMPLES<sup>1</sup>

Source	df	Mean Squares	
		Mot	PIA
Ejaculate	14	1295.4*	738.2*
Sample	10	4348.2*	4626.4*
Time	1	4378.2*	3283.9*
Error	304	109.3	78.7

\*P<.0001

<sup>1</sup>The effects of ejaculate x time, sample x time, separatory funnel replicates and the ejaculate x sample interaction on these parameters were not significant.

As expected, the effect of ejaculate on all parameters was significant. Post-thaw aging affected all viability parameters except VMOTT, and all samples responded similarly when aged, as indicated by the non-significant sample by time interaction.

Least squares means for velocity, MOT, PIA, VMOTP and VMOTT for thawed semen in fractions T, 1, 3, 4 and 6 and unseparated semen during post-thaw aging were determined (table 10). Pairwise comparisons indicated differences in several combinations of samples. As expected, separated semen recovered from the top fractions of the column was generally less viable than either unseparated semen or semen recovered from fractions 3, 4 and 6. Similar results with post-thaw semen were reported by Ericsson (1980).

Differences in the velocity of spermatozoa post-thaw samples were not observed. Jeulin (1980) reported that the mean pre-freeze velocities of separated human spermatozoa were greater than the mean velocities of unseparated semen. It seems possible that pre-freeze velocity could have been elevated in separated semen when compared to unseparated semen in this study. Apparently any increase in velocity in pre-freeze separated semen is not retained by the separated spermatozoa after freezing. As a result, velocity does not offer further post-thaw information regarding the quality of the separated spermatozoa isolated in this study.

The proportion of motile sperm cells (MOT) was greater

Table 10

LEAST SQUARES MEANS (+ SE) FOR VELOCITY, VMOTT, VMOTP, MOT AND PIA FOR THAWED SEPARATED AND UNSEPARATED SEMEN<sup>1</sup>

Parameter	Unseparated	Separated					Critical value <sup>2</sup>
		T	1	3	4	6	
Velocity ( $\mu\text{m}/\text{sec}$ )	36.2 +1.2	38.5 + .87	37.9 + .87	39.9 + .87	41.0 + .87	41.4 + .87	53.4
VMOTT (%)	62.8 <sup>d</sup> +2.5	36.7 <sup>a</sup> +1.7	49.0 <sup>b,c</sup> +1.7	67.1 <sup>d</sup> +1.7	69.1 <sup>d</sup> +1.7	70.2 <sup>d</sup> +1.7	10.7
VMOTP (%)	46.4 <sup>b,c,d</sup> +2.6	28.5 <sup>a</sup> +1.8	38.7 <sup>a,b</sup> +1.8	54.0 <sup>c,d</sup> +1.8	58.6 <sup>d</sup> +1.8	57.1 <sup>d</sup> +1.8	11.2
MOT (%)	46.3 <sup>d,e</sup> +1.9	26.3 <sup>a</sup> +1.3	36.0 <sup>b,c</sup> +1.3	51.7 <sup>e,f</sup> +1.3	54.5 <sup>f</sup> +1.3	56.5 <sup>f</sup> +1.3	8.28
Intact Acro. (%)	76.4 <sup>d,e</sup> +1.6	52.1 <sup>a</sup> +1.2	65.0 <sup>b,c</sup> +1.2	81.8 <sup>e</sup> +1.2	81.5 <sup>e</sup> +1.2	83.3 <sup>e</sup> +1.2	7.10

a,b Means in same rows with different superscripts are significantly different ( $P < .05$ ).

c,d Means in same rows with different superscripts are significantly different ( $P < .05$ ).

e,f Means in same rows with different superscripts are significantly different ( $P < .05$ ).

<sup>1</sup>SE - standard error of mean from pooled variance.

<sup>2</sup>Critical value - the value by which samples must differ in order to be significantly different.

in fraction 6 than in the parent semen or the top two fractions but similar to the proportion of motile sperm cells in fractions 3 and 4. The proportions of motile sperm cells in fractions 3 and 4 were similar to each other as well as to the unseparated semen. These results are similar to those regarding the proportion of motile cells recovered from fractions 3 and 6 in Experiment 2. However, Wall et al. (1980) reported an increase in the proportion of motile sperm cells recovered from the bottom of the column over the proportion of sperm cells recovered from the middle fractions. These differing results may have been due to the different types of columns used in each study.

VMOTP was determined by multiplying the proportion of sperm cells in a sample which moved at a rate of  $\geq 25 \mu\text{m/s}$  by the percentage of moving sperm cells in a sample (VMOTT). As expected, the VMOTP estimates for spermatozoa recovered from fractions 3, 4 and 6 were similar and the VMOTP estimates for spermatozoa recovered from fractions T and 1 were lower than those estimates from unseparated semen. The similar least squares means of MOT and VMOTP indicated that accurate, objective motility estimates, comparable to subjective estimates by a trained observer, were possible using videomicrography (Katz et al., 1981). Differences between sample were not as frequent for estimates of VMOTT as they were for estimates of VMOTP and MOT. Although many of the spermatozoa of a given sample may have been motile,

their motility was not progressive and probably appeared as vibrational motility. Other differences between VMOTT and VMOTP were observed during aging. The total motility of the semen samples, as measured by videomicrography was not different at 0 h post-thaw and 2 h post-thaw. This result indicated that spermatozoa probably become slower moving due to latent injury, as indicated by the significant effect of time on VMOTP, but did not become non-motile in this period of time (table 8).

No differences were observed in the percentage of spermatozoa with intact acrosomes isolated from fractions 3, 4, 6 and the unseparated semen, but the frequency of spermatozoa with intact acrosomes in fractions T and 1 were different from each other as well as all other samples. In contrast, Ericsson (1980) reported that a greater percentage of the spermatozoa recovered from frozen separated semen had intact acrosomes than the spermatozoa recovered from thawed unseparated semen. Results similar to those reported by Ericsson (1980) were reported in Experiment 2. These differing results may be attributed to the differing qualities of the applied semen in each study. The effect of separation on the semen appears to increase as the quality of the semen applied to the gradient decreases. When the original semen sample is highly viable, enhancement of the recovered semen was not as great as enhancement of a sample of poor viability.

The relative change in the percentage of motile spermatozoa before and after freezing and after post-thaw aging was different among the ejaculates collected and among the samples of spermatozoa (table 11). The change in the percentage of spermatozoa with intact acrosomes before and after freezing did not differ among the ejaculates but the change in the percentage of intact acrosomes during post-thaw aging was significantly different among ejaculates. Conversely, differences among samples in the change in intact acrosomes between pre-freeze and post-thaw were significant but acrosomal integrity declined at a similar rate during aging in all samples.

The changes in viability sustained during freezing and thawing and during post-thaw aging are depicted in figure 4. Estimates of the motility of spermatozoa in fractions 3, 4 and 6 and unseparated semen were markedly lower after thawing as compared to prior to freezing (table 12). A greater proportion of motile spermatozoa from fraction 3 became non-motile when frozen than in any other sample. The small changes in the proportion of motile spermatozoa recovered from fractions 3, 4 and 6 and unseparated semen during aging indicated that a greater proportion of the sperm cells of these samples were capable of withstanding the effects of aging than the proportion of cells from fractions T and 1.

Assuming that acrosomal integrity at 2 h post-thaw is

Table 11

RESULTS OF ANALYSIS OF VARIANCE FOR THE CHANGE IN MOTILITY  
AND INTACT ACROSOMES DURING FREEZING, THAWING AND POST-THAW AGING

Source		Prefreeze to 0 hr post- thaw		0 hr-Post- thaw to 2 hr post-thaw		Prefreeze to 2 hr post-thaw	
		PIA	Motil.	PIA	Motil.	PIA	Motil.
Ejaculate	14	264.2	966.5**	270.6**	843.1**	616.9**	1019.2***
Sample	5	604.8**	962.4**	119.7	3691.0***	279.6	751.9*
Error	145	186.9	303.6	123.3	413.3	192.5	357.3

\*P<.1

\*\*P<.05

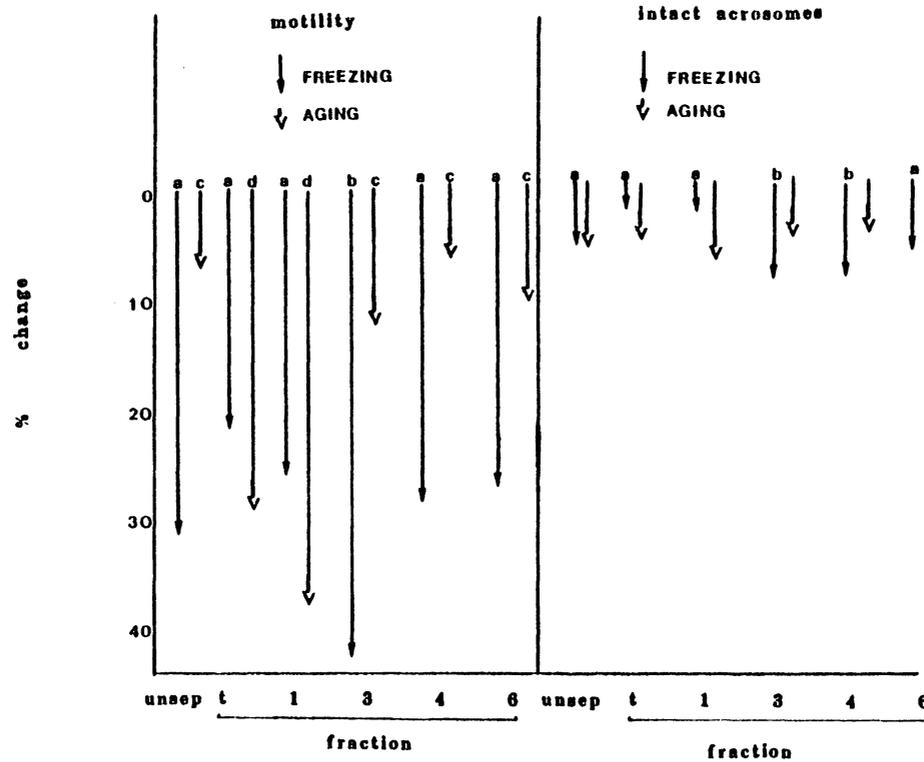
\*\*\*P<.001

Table 12

MEANS FOR PERCENTAGES OF MOTILE SPERMATOZOA AND SPERMATOZOA  
WITH INTACT ACROSOMES BEFORE FREEZING AND AT 0 AND 2 HOUR  
POST-THAW

<u>Sample</u>	<u>Prefreeze (%)</u>		<u>0 hr Post-thaw (%)</u>		<u>2 hr Post-thaw (%)</u>	
	<u>Motility</u>	<u>Intact Acro.</u>	<u>Motility</u>	<u>Intact Acro.</u>	<u>Motility</u>	<u>Intact Acro.</u>
Unsep	70.3	84.0	48.0	78.3	44.7	74.5
Frac T	40.7	55.9	31.0	54.6	21.7	49.7
Frac 1	54.3	67.4	41.7	68.7	30.3	61.3
Frac 3	88.2	93.0	55.3	84.3	48.0	79.3
Frac 4	85.8	93.6	57.0	85.4	54.3	77.5
Frac 6	90.3	93.4	60.3	87.1	52.6	79.4

CHANGES IN MOTILITY AND INTACT ACROSOMES OF SEPARATED AND UNSEPARATED SPERMATOZOA DURING FREEZING AND AGING



a, b  
Arrows with different superscripts are significantly different (P < .05)

c, d  
Arrows with different superscripts are significantly different (P < .05)

Figure 4. Percent change in motility and intact acrosomes during freezing and aging.

more highly correlated with potential fertility than motility (Saacke and White, 1972), fraction 6 and the unseparated semen would be considered the most potentially fertile samples in this study. Fraction 6 acquired a high percentage of motile spermatozoa and a high proportion of sperm cells with acrosomal integrity. The sperm cells of this fraction retained their viability during freezing and aging better than other separated fractions.

These results indicate that post-thaw viability is generally greater for separated spermatozoa recovered from fraction 6 of the BSA gradient than for separated spermatozoa recovered from the other fractions of the column. However, sperm cells from fraction 6 and spermatozoa from unseparated semen appear similar in their ability to maintain their acrosomal integrity but differ in their ability to retain their motility during freezing and post-thaw aging. Nevertheless, separated spermatozoa recovered from fraction 6 were highly motile and could be used commercially for artificial insemination. According to Roberts' (1972) theory, fraction 6 may possess a greater proportion of Y-chromosome-bearing spermatozoa than X-chromosome-bearing spermatozoa.

Experiments 4 and 5: An attempt to isolate Y-chromosome-bearing spermatozoa using a BSA gradient

Roberts (1972) suggested that the separation of X- and

Y-chromosome-bearing spermatozoa was possible based on the differing motility rates between the two types of sperm cells. Ericsson et al. (1973) and Quinlivan et al. (1981) confirmed this hypothesis by applying human semen to a discontinuous BSA gradient and isolating a population of spermatozoa with a high proportion of Y-chromosomes as indicated by quinacrine staining. In contrast to these results, attempts to isolate a population of Y-chromosome-bearing spermatozoa in this study have not been successful.

Thirty-nine cows inseminated with separated semen (fraction 6) gave birth to a similar proportion of male and female calves. Furthermore, the sex-ratio did not differ between cows inseminated with separated and unseparated semen ( $P < .2$ ; table 13). Cows bred with separated semen gave birth to 17 male and 22 female calves whereas cows inseminated with unseparated semen gave birth to 12 male and 8 female calves. An additional 135 pregnant cows bred with unseparated and separated semen are expected to calve before January 1983. The results of this study to date are limited by the number of calves born, however, and the current trend does not suggest successful isolation of Y-chromosome-bearing spermatozoa. Results of the additional calvings are expected to confirm the current conclusions. The inability to alter the sex ratio of calves born to cows inseminated with semen separated using a BSA gradient was also reported by Ross et al. (1975), Evans et al. (1975) and Ericsson

(1980).

Measurement of the relative amount of the DNA in the sperm cells of separated semen, unseparated semen, and unseparated semen exposed to BSA confirm the results of the breeding study. All semen samples contained similar proportions of X- and Y-chromatin material (table 14). X-chromosome-bearing spermatozoa possessed more chromatin material than Y-chromosome-bearing spermatozoa, as expected. The relative differences in the DNA content for X- and Y-chromosome-bearing spermatozoa were 3.8% and 4.0% for separated spermatozoa recovered from the 4% and 10% BSA, 3.8% for the spermatozoa exposed to 10% BSA but not separated, and 3.6% for unseparated semen with no exposure to BSA. Coefficients of variation were less than 2% for all samples.

The results of this study and the work reported by other researchers indicate that Roberts' theory on separation may apply to human spermatozoa but not to bovine spermatozoa. The shape and motility pattern of the spermatozoa of each species differs and may account for these differing results. The shape differences between bovine X- and Y-chromosome-bearing spermatozoa may not be of great enough magnitude or their motility rates may not differ when applied to a BSA gradient. These are possible explanations, however, it seems more reasonable to assume that the differing results are more likely due to other

Table 13

SEX AND FREQUENCY OF CALVES BORN TO COWS INSEMINATED  
WITH EITHER SEPARATED OR UNSEPARATED SEMEN

Sex	Treatment		Total
	Separated	Unseparated	
Male	17	12	29
Female	22	8	30
TOTAL	39	20	59

Chi-square = 1.424

Prob = .2316

Table 14

THE FREQUENCY OF SPERMATOZOA POSSESSING X- OR Y-  
CHROMOSOMES IN SEPARATED AND UNSEPARATED SEMEN

Item	Sample (% of sperm)			
	Unseparated	BSA (unsep)	4% BSA	10% BSA
Ratio of chromosomes (Y:X)	50:50	53:47	51:49	52:48

physical factors or unknown species differences.

Although, the larger column used in this study is similar in principle to the column designed by Ericsson et al. (1973), there are differences in the shape, volume and surface area between the two columns. Ericsson et al. (1973) used a BSA gradient which could only separate a small number of sperm cells in contrast to the larger column used in this study. Perhaps the smaller column was more precise in its ability to separate X- and Y-chromosome-bearing spermatozoa.

Nearly all results regarding the successful separation of human X- and Y- chromosome bearing spermatozoa have been obtained through the use of quinacrine staining. Only a small number of children have been born to women inseminated with separated semen. Dmowski et al. (1979) reported that that 5 male and 2 female children have been born to women inseminated with separated semen. All other claims for successful separation of X- and Y-chromosome-bearing spermatozoa using a BSA gradient have been based on the quinacrine staining technique which in itself has been questioned (Beatty, 1971).

Finally, it is possible that those factors influencing sedimentation as a method of separation may also influence separation procedures based on motility. As a result, the theoretical differences between X- and Y-chromosome-bearing bovine spermatozoa may become obscured, rendering the BSA

column separation unsuccessful as a method of separating X- and Y-chromosome-bearing spermatozoa.

## SUMMARY

This study was conducted to characterize the motility, morphology, and sex chromosome content of spermatozoa isolated using a discontinuous BSA gradient. The BSA column was capable of isolating a greater proportion of the motile sperm cells from the applied sample when  $1 \times 10^9$  sperm cells were applied to the column when 3, 5, 7, 14 or  $21 \times 10^9$  sperm cells were applied. The column excluded more spermatozoa with tail abnormalities from fraction 3 of the gradient, when compared to unseparated semen, and further excluded spermatozoa with tail or midpiece abnormalities from fraction 6 of the gradient. As a result, a greater frequency of morphologically normal spermatozoa were identified in fraction 6 of the column than fraction 3 or the unseparated semen. Spermatozoa with head abnormalities were not excluded from the gradient.

The post-thaw viability of separated and unseparated semen and the ability of the two populations of spermatozoa to resist damage from freezing and aging were evaluated in a subsequent experiment. Semen from fraction 3, 4 and 6 were similar in their post-thaw viability as measured by PIA, VMOTP and VMOTT, but fraction 6 had a greater proportion of motile spermatozoa as determined by MOT. The proportion of viable cells in fractions 3, 4 and 6 and in unseparated semen was similar when measured by VMOTT and PIA. Fractions

T and 1 frequently had a lower proportion of viable cells than unseparated semen or semen from fractions 3, 4 and 6. Of all the samples evaluated, spermatozoa from fraction 6 and spermatozoa from unseparated semen withstood freezing and aging better than all other samples.

Although the BSA column was not capable of separating X- and Y-chromosomebearing spermatozoa, it could isolate a potentially highly fertile population of spermatozoa which would offer economic benefits to the livestock industry. Spermatozoa isolated from fraction 6 were more motile, more morphologically normal, and retained their viability better than spermatozoa from any other sample evaluated. Therefore, inseminating cows with spermatozoa isolated from fraction 6 of the column could potentially increase conception rates. In addition, because of the viability and homogeneity of the population of cells isolated in fraction 6, fewer spermatozoa per insemination unit would be needed to insure fertility. The separation of semen could therefore further extend the semen from a genetically superior sire.

In order for this method of separation to be employed commercially, a larger, more efficient column must be developed. Application of an entire ejaculate to a BSA gradient with a large proportion of the potentially fertile sperm cells recovered are necessary criteria for commercial acceptance of this separation procedure.

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ATTEMPTS TO ISOLATE HIGHLY VIABLE, MORPHOLOGICALLY  
NORMAL,  
Y-CHROMOSOME-BEARING BOVINE SPERMATOZOA

by

Lydia Margaret White

(ABSTRACT)

Five experiments were conducted to characterize a discontinuous bovine serum albumin (BSA) gradient and to determine its ability to isolate highly viable, morphologically normal, Y-chromosome-bearing bovine spermatozoa. The column consisted of a 500 ml separatory funnel in which 26 ml of extended semen was layered on top of a discontinuous gradient of 4% BSA (60 ml) over 10% BSA (60 ml). Results indicated that the greatest percentage of the applied semen was recovered from the bottom 20 ml of the gradient (fraction 6) when  $1 \times 10^9$  sperm cells were applied to the column. The column was able to exclude spermatozoa with midpiece and tail abnormalities but sperm cells with head abnormalities were similar in frequency in separated and unseparated semen. Semen isolated from

fraction 6 was more viable and more resistant to damage by freezing and aging than semen from the top fractions of the column. The proportion of Y-chromosomebearing spermatozoa isolated from fraction 6 did not differ from the proportion in unseparated semen. Cows inseminated with separated semen and unseparated semen gave birth to similar proportions of male and female calves.