THE NATURE OF SERUM AGGLUTINATION OF BOVINE SPERMATOZOA; A PROPOSED MODE OF ACTION AND ITS METABOLIC EFFECTS

by

John Edward Chandler

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

DOCTOR OF PHILOSOPHY

in

Animal Science

APPROVED:

R. G. Saacke, Chairman

C. E. Polan

J. P. Wightman

J. M. White

T. C. Ward

R. G. Cragle
Department Head

November, 1976

Blacksburg, Virginia

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr. R. G. Cragle, Head of the Department of Dairy Science, for providing the facilities and financial support during my graduate program.

Special recognition is given to Dr. J. P. Wightman, whose guidance along the pathways of the unknown, has opened new horizons for both of us and enkindled an enduring friendship.

My deepest appreciation is extended to Dr. R. G. Saacke, Major Professor, for his assistance, guidance and encouragement throughout my graduate program.

Sincere appreciation is given to Drs. J. M. White, C. E. Polan, and T. C. Ward for their advice and service on my graduate committee.

Recognition is extended to the following individuals:

M. L. O'Connor, for assistance in development and implementation of techniques and for "putting up" with my constant babbling and use of our office as a statistical conference room.

Messrs. Galen, Mercuri and Nos. 85, 64, 53, 78, 13, 73, and 3, for their generous donations of experimental materials.

Ms. B. Golon, for her technical assistance in conducting the experiments and for exhibiting greater enthusiasm for working on Fridays.

Ms. B. Golon, A. Lineen, and J. Mullins, for assistance in the preparation of the graphic portion of this manuscript. Also to

Mrs. Pat Cox, for typing the final draft of this manuscript.

My affectionate thanks go to my wife, Louise, for listening and consoling me during my trials and tribulations. Also, to Edward, who

wants to operate my "water machine" and to Paul, who doesn't know yet.

And lastly, I would like to thank Mr. R. L. Miller, for his assistance in preparing the electrical circuitry and to Drs. L. B. Barnett and H. P. VanKrey for the loan of the Cytopherometer and Warburg apparatus, respectively.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

INTRODUCTION

Numerous past reports have mentioned or have been totally concerned with the significance of spermatozoan agglutination following treatment with blood sera, female genital fluids and other biological and synthetic media. Since semen is a colloid (undissolved particles suspended in a suitable medium such as gas, liquid, solid), spermatozoan agglutination should be at least partially definable by physical chemical measurements and should thereby be predicted and controlled.

In a colloidal system, suspended particles dispersed in an electrolyte solution, may assume a certain charge, while a compensatory charge of opposite sign and equal magnitude stays in the solution in the vicinity of the charged colloidal particles. The compensatory charge extends into the solution and is recognized as having two layers and is referred to as the "double layer".

The double layer consists of an inner region of rigidly attached liquid, the Stern layer, and an outer region, the diffuse layer. The Stern layer consists entirely of compensatory ions oppositely charged from the particle (counterions) and cannot be set in motion by either an electric field or by the motion of the adjacent liquid. The Stern layer partially neutralizes both the charge and the electrostatic repulsion of the individual particles. The diffuse layer contains the remaining counterions required to neutralize the colloid charge, as well as ions of similar charge to those of the colloid. This layer can be

displaced by both of the previously mentioned forces.

Thus, the colloidal system exhibits an electrostatic potential due to the charge difference between the particle surface and the bulk of the solution and is related to the counterion concentration and valency in the bulk. The potential is maximum at the particle surface and zero at the end of the double layer. The total potential of the colloid, the Nernst potential (ψ_o), is the potential from the particle surface to the end of the double layer. The Stern potential (ψ_δ) is that portion of the Nernst potential associated with the Stern layer. The zeta potential (ψ_ζ) is that part of the Nernst potential associated with the diffuse layer.

Colloidal stability (i.e., agglutination, coagglutination or agglomeration versus dispersion, anti-agglutination, etc.) is due to forces of particle-to-particle attraction and particle-to-particle electrostatic repulsion. The attraction forces are mainly those of London-Vander Waal, which remain constant at a given distance from the particle surface, while the repulsion forces are a result of the strength of the zeta potential. The greater the zeta potential (ZP), the more stable is the dispersed state; the closer the ZP is to zero, the greater the instability which is exhibited by clumping or agglutination. Thus, agglutination can be controlled by controlling zeta potential.

ZP control can be accomplished by species and concentration of counterions in the bulk of the solution. Removal or addition of counterions to the bulk will expand or compress the diffuse layer and result in an increase or decrease in ZP, respectively. The counterion

concentration which causes agglutination is called the critical coagulation concentration (CCC) or flocculation value. At this counterion concentration, the repulsive and attractive forces are balanced and a small change in the repulsive force results in a dramatic shift in the colloidal dispersion state.

The counterion species also affects ZP and agglutination through its valency. Divalent ions are about 20 to 80 times more effective in reducing ZP than are monovalent ions; trivalent ions are about 10 to 100 times more effective than divalent ions. This effect is expressed by the empirical Schulze-Hardy rule. These and other aspects of colloid chemistry are reviewed in (1), (2) and (3).

Zeta potential can be measured directly from the electrophoretic mobility of the colloid. Since the electrophoresis of spermatozoa has been well documented, the description of the agglutination phenomenon in spermatozoa should be in part explained by zeta potential and counterion interactions.

Previous work in this laboratory has indicated an association between sperm agglutination, cell quality and sustained motility over an extended incubation. Thus, recognition, and perhaps separation, of healthy cells by physical means is worth pursuing. Also, since semen is a colloid and biological fluids cause sperm agglutination, the understanding of the physical chemical principles involved in a colloidal system should elucidate some basic phenomena involved in the physiology of reproduction.

The overall objectives of these studies were as follows: 1) to elucidate the mechanism of serum-induced head to head agglutination

of bovine spermatozoa; 2) to investigate the relationship of membrane integrity with the colloidal characteristics of diluted bovine semen; 3) to evaluate the metabolic characteristics of agglutinated cells.

LITERATURE REVIEW

Previous workers have indicated that semen from various species exhibit certain properties which are characteristic of a colloidal system. One of these characteristics is an electrokinetic phenomenon, namely electrophoretic mobility (U_e) of the sperm cell.

Electrophoretic Mobility of Spermatozoa

Cooled bull spermatozoa were found to have essentially the same mobilities (-0.88 μ/sec/V/cm) when electrophoresed in seminal plasma; egg yolk-citrate-glycine buffer, or after washing, in unbuffered electrolyte diluent (4). Bey (5) measured the U_e of bull spermatozoa after three washings in low ionic strength buffer and reported an average mobility of -1.30 μ/sec/V/cm. He suggests that the difference in U_e of these two reports is due to differences in ionic strength (μ)[1] of the diluent used.

Bangham (6) electrophoresed cooled ram sperm and reported a mobility of -0.75 μ/sec/V/cm in saline at pH 7 and ionic strength, μ = 0.145. He also showed that, when the saline diluent ionic strength was lowered to μ = 0.100 at pH 7, the cooled spermatozoa U_e increased to an average of -3.25 μ/sec/V/cm for both head and tail anode-oriented cells.

Bey (5) also electrophoresed the sperm cells from cock, man,

---

[1] Ionic strength (μ) equals one-half the summation of the products of the concentration (C) and valency (Z) squared of all ions in solution ($\mu = \frac{1}{2} \Sigma CZ^2$).

rabbit and boar and reported that the mobilities decreased in that order, i.e., -1.54, -1.36, -1.12 and -0.50 μ/sec/V/cm, respectively. He (5) indicated that bull epididymal sperm had a U_e twenty percent greater than ejaculated sperm and that first ejaculate sperm had an eight percent lower mobility than second ejaculate sperm. He (5) also showed that the electrophoretic mobility of ejaculated bull sperm, incubated in their own seminal plasma at 37° C, decreased from -1.25 to -1.07 μ/sec/V/cm within thirty minutes. Bedford (7) found that as rabbit spermatozoa traversed the epididymis, their U_e at 4° C increased from -0.35 to -0.54 μ/sec/V/cm. Vaidya et al. (8) reported U_e of rabbit sperm incubated for six hours in Tyrode's solution, seminal plasma or the estrus uterus. They found that these conditions decreased the spermatozoal U_e in the order given, i.e., Tyrode's, -1.25; seminal plasma, -1.17; uterine, -1.00 μ/sec/V/cm, after washing and electrophoresis in a sorbital-NaCl buffer.

All of these workers reported anodic mobilities, however, Bangham (6) and Bedford (7) indicated both head and tail anode-orientation of the sperm in the same electric field. This is indicative of an asymmetrical charge distribution on the sperm cell surface.

Sperm Surface Charge

Nevo et al. (4) determined the isoelectric point of bull and rabbit sperm to be at pH 3.4 and 3.5, respectively. Therefore, at physiological pH, the cells from both species will be negatively charged. These same workers (4) indicated that separated bull sperm tails had a greater negative charge than did the separated heads and that the

intact cell was intermediate between the two. This was indicated by differences in electrophoretic mobilities, i.e., tails, -1.03; whole sperm, -0.86; heads, -0.60 μ/sec/V/cm.

Bey (5) showed that, after incubating bull sperm with neuraminidase for three hours, the electrophoretic mobility of the cells decreased as free neuraminic acid increased in the supernatant. He thus indicated that the negative excess-charge of the sperm cell may be partially due to neuraminic acid residues in the cell membrane.

Bedford (7) noted that as the rabbit sperm traversed the epididymis, they changed from a head-anode to a tail-anode orientation in the electric field. Cooper and Bedford (9), using electron microscopy, examined the acquisition of the sperm charge in man, Rhesus monkey and rabbit by exposing epididymal cells to colloidal iron and observing the binding sites. They indicated that, in man and monkey, there was no colloidal iron binding to either the head or tail membranes of caput epididymal sperm. However, binding did occur on the cell membrane overlying both the head and tail of sperm from the corpus and cauda epididymis. Binding was greater on the tail than on the head. They (9) and Yanagimachi *et al*. (10) indicated that although binding increased on the sperm midpiece and the tail with epididymal maturation, binding never occurred on the rabbit sperm head.

Yanagimachi *et al*. (10) showed that all surfaces of bull caudal epididymal spermatozoa bound colloidal iron. However, they did find that bull caput epididymal sperm had an uneven charge distribution; the postacrosomal region having the greatest charge, followed by the

acrosomal region and then the sperm tail. They concluded, as did Cooper and Bedford (9) that the overall negative surface charge increased with epididymal maturation.

Thus, it is evident that, as the sperm cell reaches maximum maturity, it will have its greatest electrophoretic mobility; a result of it then possessing its greatest charge. It is also seen that the U_e of spermatozoa differ among species, as well as within species, when placed in different environments. This is undoubtedly, a consequence of differences in sperm surface composition and the interaction of that composition with the cellular environment. Fawcett (11), in a recent review of biochemical and morphological investigations of the sperm cell surface, has confirmed the asymmetrical ordering of the membrane components of rat, mouse, rabbit and hamster spermatozoa.

Sperm Agglutination

Another physical property of a colloid is the tendency of the particles to aggregate. Numerous authors have documented the occurrence of spermatozoal aggregation (agglutination) under a variety of conditions.

Blackshaw (12) noted that the electrolyte environment was involved in spermatozoal agglutination. He observed, in both the rabbit and the bull, that spermatozoa agglutinated head to head after washing them with salt solutions. The salt solutions contained KCl and $CaCl_2$, and ranged in ionic strength from 0.079 to 0.136. This same author (13) reported that electrolyte-induced head agglutination in ram and bull spermatozoa, washed in high ionic strength (μ = 0.125) solution, was

abolished by addition of seminal plasma. Wales and White (14) reported cock sperm agglutination after treatment with calcium ion concentrations of 0.9 and 2.7 mM. These concentrations, in conjunction with the remaining components of the buffer used, represent ionic strengths of about 0.21 and 0.22, respectively.

Edwards (15) noted that, in some samples, rabbit spermatozoa "head-agglutinated" when the semen was diluted with Ringer's, Tyrode's, Baker's solutions or normal saline. Moyer and Almquist(16), while studying ambient temperature semen diluents containing egg yolk, sodium citrate, glucose, skimmed milk, and antibiotics, reported a head to head agglutination of bull sperm that they attributed to the sodium citrate concentrations. Lindahl (17) showed an ATP-dependent head to head agglutination which was activated most efficiently by the addition of calcium ions.

Several authors have noted the agglutinating effect of various biological fluids. Olds and VanDemark (18) noted bull sperm agglutination in samples exposed to cow genital tract fluids. They indicated that the head to head agglutination was greatest in follicular fluid when compared to uterine and oviducal secretions. Lindahl (19) demonstrated that a high molecular weight component of bovine follicular fluid caused head to head agglutination of bull sperm. Senger (20) has reviewed, and Senger and Saacke (21) have further demonstrated the occurrence of serum-induced agglutination of bovine spermatozoa. Edwards (15) also reported that high titers of normal rabbit sperm caused mainly head agglutination, while anti-sperm sera caused tail and head agglutination of rabbit sperm. Yanagimachi (22) noticed a

prominent head to head agglutination of hamster sperm when they were treated with heat-treated normal hamster, rat, guinea pig, rabbit, bull, human and commercially-produced agamma human sera. Padma (23) indicated that normal heterologous sera only agglutinated spermatozoa from members of allied genera. He showed that both bovine and sheep sera agglutinated bovine but not rabbit spermatozoa, and that rabbit and guinea pig sera agglutinated rabbit but not bovine spermatozoa.

Sperm Agglutinating Factor(s) Isolation

Much research has been done in an effort to isolate the agglutinating factor(s), specifically that (those) found in serum. Beck et al. (24) indicated that the agglutinin in sera from guinea pigs, rabbits, mice and men was non-specific in that it reacted with both homologous and heterologous spermatozoa. They speculated that the agglutinin was a gamma globulin, as judged by fluorescent antibody tests. On the other hand, Johnson (25) indicated the presence of a specific gamma globulin, determined by immune fluorescence techniques, in guinea pig sera which reacted with the acrosome of guinea pig spermatozoa. However, neither of these authors (24, 25) measured or tested for sperm agglutination with the isolated agglutinins. Padma (26), on the other hand, extracted from normal rabbit serum a gamma globulin, specifically IgG, which, when dissolved in saline, agglutinated rabbit sperm.

Yanagimachi (22) indicated that the hamster sperm agglutinin in normal hamster serum is associated with the albumin serum fraction, as determined by fractional dialysis. Boettcher et al. (27) indicated that the sperm agglutinins in human sera are proteins with zone electropho-

retic mobilities of beta globulins.

Colloidal Stability

Thus, it has been shown that semen, in general, exhibits several characteristic properties common to colloidal systems, namely, electrophoretic mobility and flocculation (agglutination) of spermatozoa which are both affected by inorganic and organic charged species. Napper (28) has reviewed the current theories and mechanisms which govern colloidal stability. He indicated two methods for maintaining or disrupting colloidal stability, namely electrostatic and/or steric treatment of the colloid. The electrostatic control is exerted mainly through control of the double layer and its accompanying electrical potential using different ionic species and/or high molecular weight charged polymers. Thus as the double layer is compressed, the electrical (zeta) potential, and therefore the repulsive energies between particles, is reduced in accordance with the Deryagin, Landau, Verwey and Overbeek (DLVO) theory of colloidal stability. Riddick (29) discusses these same principles and also documents the average zeta potential ranges associated with different stability characteristics. These are seen in Table I.

The steric control of colloid stability is brought about by adsorption of nonionic polymers to uncharged colloid particles. This method of control is primarily involved when the particles are suspended in non-polarizing (non-aqueous) media (28). Thus, this method of stability would presumably be ineffective in most biological systems because they are usually in an aqueous media and possess a charge.

TABLE I. VARIOUS STAGES OF COLLOIDAL STABILITY AND THEIR ASSOCIATED AVERAGE ZETA POTENTIALS.

Stability Characteristics	Average ZP in millivolts
Maximum agglomeration and precipitation	0 to +3
Range of strong agglomeration and precipitation	+5 to -5
Threshold of agglomeration	-10 to -15
Threshold of delicate dispersion	-16 to -30
Moderate stability	-31 to -40
Fairly good stability	-41 to -60
Very good stability	-61 to -80
Extremely good stability	-81 to -100

Agglutinated Sperm Physiology

The head to head agglutination of spermatozoa induced by normal serum or other biological fluids has been noted by several authors to be accompanied by sustained motility and viability of the cell over extended *in vitro* incubation.

Olds and VanDemark (18) measured oxygen consumption and lactic acid production of bovine spermatozoa treated with female genital fluids. These authors noted that follicular and oviducal fluids caused an increase in oxygen consumption and lactic acid production by the cells, when compared to the saline-treated control cells. They also noted that these fluids caused head to head agglutination of the sperm.

Mitall *et al.* (30) showed increased oxygen consumption, fructose utilization and lactic acid production by normal rabbit serum-treated bull sperm, above those of saline-treated controls. These workers noted a pronounced agglutination of the spermatozoa after four hours at 37° C incubation in the serum-treated samples. Also, they reported a definite advantage in percent motile and percent live spermatozoa (negative eosin stain) in the agglutinated samples versus the controls.

Senger (20) noted that homologous serum treated bull spermatozoa retained pregressive motility, after nine hours of 37° C incubation, better than yolk citrate treated cells. The serum treatment caused a substantial amount of head to head agglutination. He suggested that the clumping might be a means of cell conservation, relating to the sperm host gland function in birds and reptiles. O'Connor (31) observed the same conserving effect by both normal and anti-egg yolk immune sera on extended incubation of fresh diluted semen.

Senger (20) also showed a positive correlation between the percentage of cells with intact acrosomal membranes and the percentage of head to head agglutinated cells. This association was more prominent in serum samples from some cows than from others. Senger and Saacke (21) indicated that most of the cells involved in agglutination after extended incubation had intact acrosomal membranes, and thus inferred that the process is selective for healthy cells.

On the other hand, Matousek (32) indicated that bovine spermatozoal fructolysis was unaffected by normal or absorbed immune cow sera compared to saline controls. He indicated that spermatozoal agglutination occurred in both sera types. Peterson (33) found a decrease in oxygen consumption of bovine spermatozoa treated with uterine lavage from sterile, repeat breeder and "easy breeding" cows when compared to saline controls. The lavage from all three cow types caused a low level of sperm agglutination.

Thus, it appears that serum induced head to head spermatozoal agglutination may be conserving, selective or both with respect to the functional life of the spermatozoa. How the dispersion state of the biocolloid influences the cell's physiology is intriguing.

EXPERIMENTAL

The following procedures and materials were used in preliminary work and in the experiments reported herein.

Serum Preparation

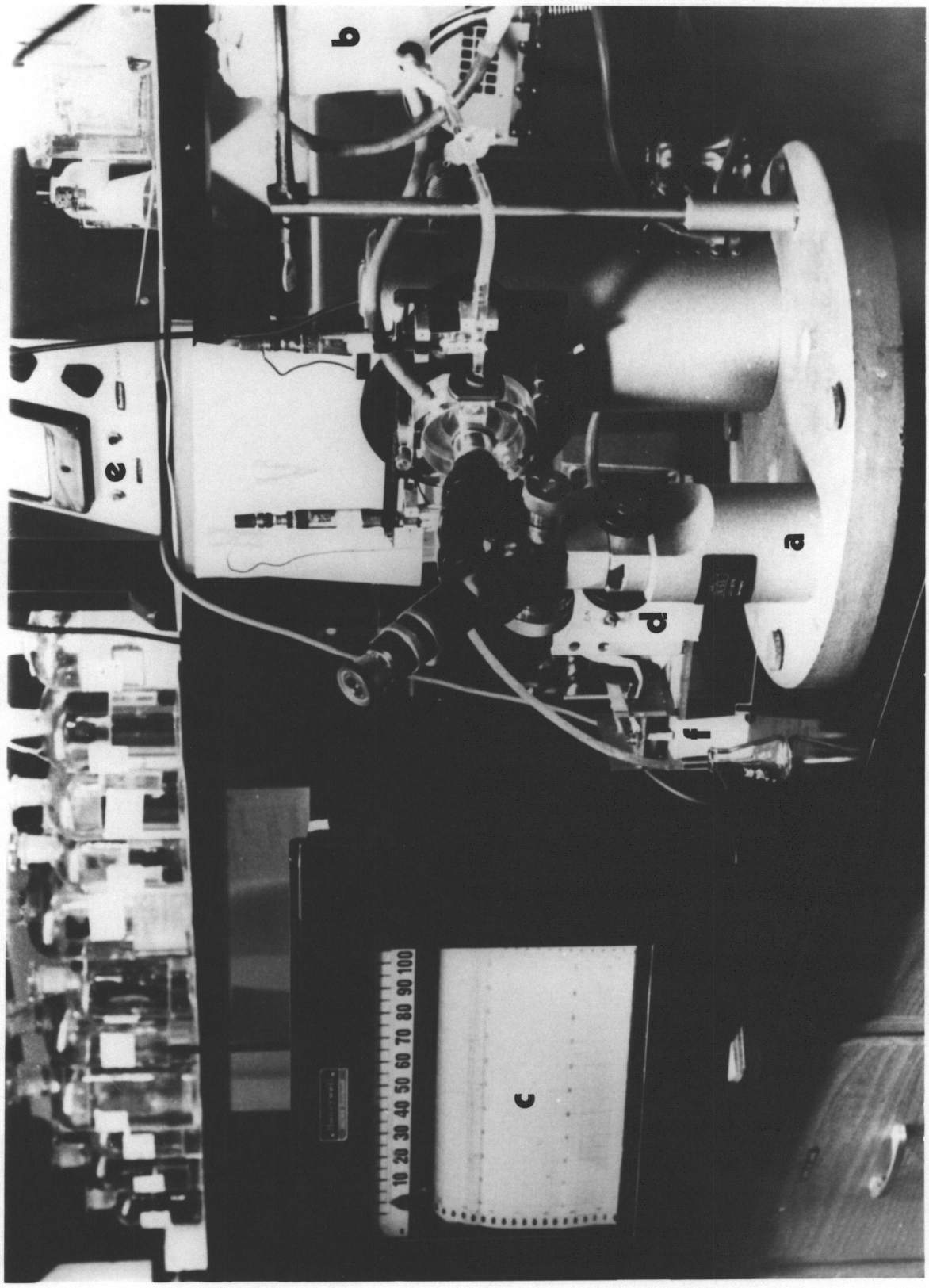
Blood (50 ml/animal) from eight nulliparous heifers was collected via jugular puncture and allowed to coagulate at room temperature for one hour. The samples were then centrifuged at 12,100 g for 10 minutes. Equal volumes of the supernatant (serum) were then pooled and stored at 5° C until use. Prior to use, the pooled serum was compliment-fixed at 56° C for 30 to 60 minutes.

Electrophoretic Apparatus and Measurement

Electrophoretic mobility (U_e) was measured in a Carl Zeiss Cytopherometer designed after Fuhrmann and Ruhenstroth-Bauer (34). Nevo et al. (4) demonstrated that cooling of spermatozoa was necessary to stop their innate motility so that their U_e could be measured. He also emphasized the importance of slow cooling to prevent "cold shock" with its adverse effect on sperm viability. The cytopherometer was thus modified to provide these thermal requirements: the reservoir was enclosed in a water jacket which was connected to the cuvette water jacket so that both would be maintained at the same low operating temperature (7-10° C). Also, a strip-chart recorder was connected to the power switch in order to record the time of exposure of the measured cells to the electric field (Figure 1).

Figure 1. Electrophoretic instrumentation used in determining spermatozoal zeta potential; (a) Carl Zeiss Cytopherometer, (b) low temperature water jacketed reservoir for diluted semen, (c) Honeywell strip chart recorder for recording time was connected to (e) the power supply via the (d) on-off polarizing switch and (f) voltage divider.

16a



The sperm were electrophoresed across a constant distance of 16 microns, equivalent to one division of the optical grid shown in Figure 2, in one direction and then, by reversing the polarity of the electrodes, 16 microns in the other. Electrophoretic mobility was assessed by measuring the time required for the sperm to migrate across the total 32 microns per unit field strength. Field strength (E) was calculated according to Equation [1],

$$E = A \cdot \rho / h \cdot t \qquad [1]$$

which is similar to that given by Shaw (35). In this equation, E is the field strength, A is the applied current, ρ is the specific resistance of the suspending medium, h is the chamber height, and t is the chamber depth.

Electrophoretic mobility (U_e) was calculated according to Shaw (35) and is expressed in Equation [2],

$$U_e = (\mu/sec)/E \qquad [2]$$

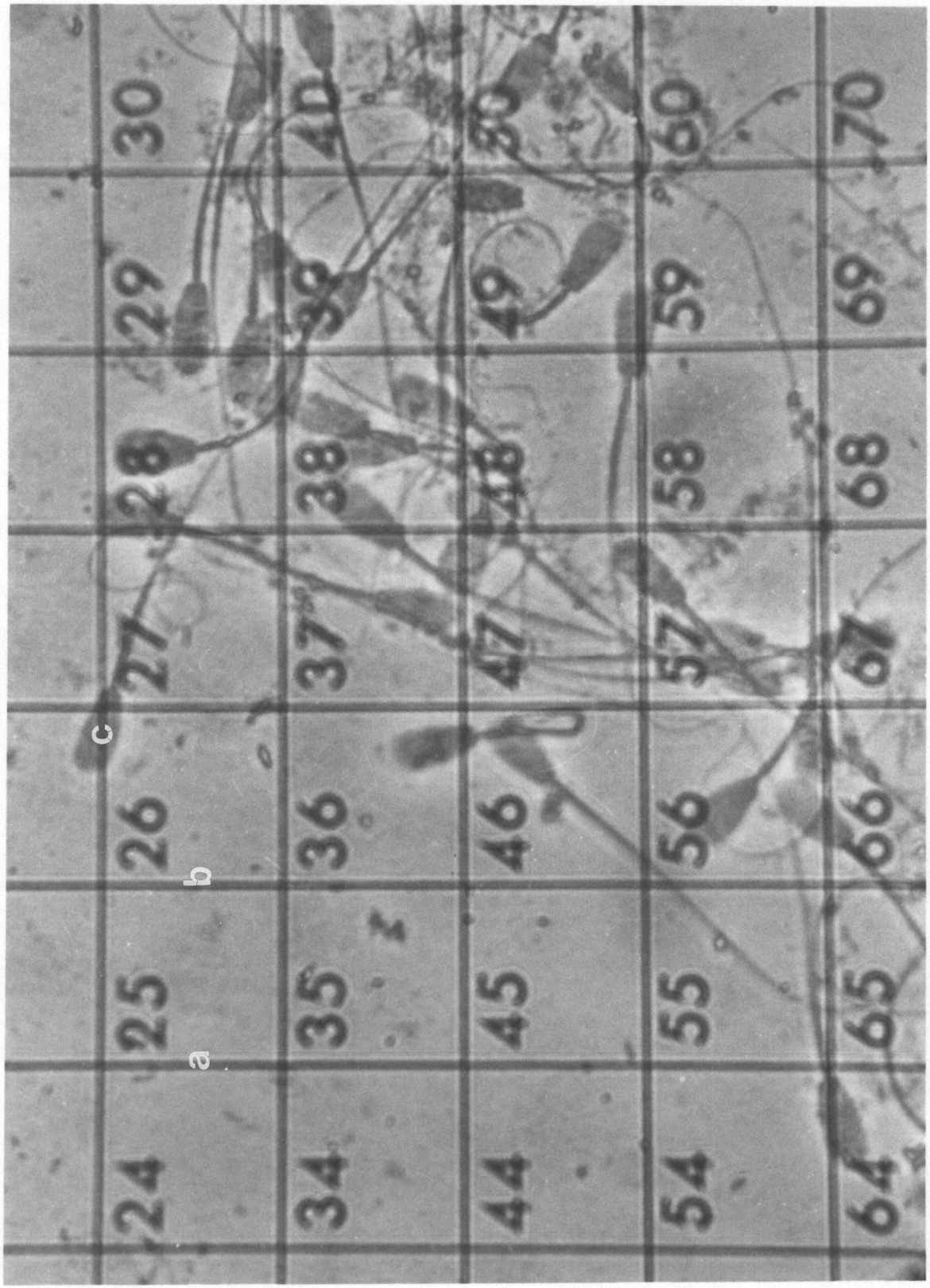
where μ is migration distance, sec is migration time, and E is the field strength as determined in Equation [1].

Zeta potential (ψ_ζ) was calculated according to the Von Smoluchowski Equation [3] for a large non-conducting flat surface (35).

$$\psi_\zeta = U_e \ (\eta/D) \qquad [3]$$

Since diluted semen was used in all determinations, the viscosity (η) and dielectric constant (D) for the suspending medium was considered to be that of water at the measuring temperature.

Figure 2. A typical view of bull spermatozoa (c) as seen through the phase contrast microscope of the Cytopherometer. The distance between any two lines (a-b) is equivalent to 16 microns (1500 x mag).


24
25
26
27
28
29
30
34
35
36
37
38
39
40
44
45
46
47
48
49
50
54
55
56
57
58
59
60
64
65
66
67
68
69
70
a
b
c

Spermatozoan Viability Evaluation

Percent progressive motility of sperm was estimated from live sperm smears in using a phase contrast microscope equipped with a warm stage (37°). Percent head to head agglutination was obtained as the average of duplicate differential counts of one hundred cells from live smears, using a differential interference contrast (DIC) microscope (20). Percent intact acrosomes, noted as the presence of the apical ridge, was determined as the average of duplicate direct counts of one hundred cells in random fields of a live smear using DIC optics (36).

Experiment I. The effect of graded serum levels on zeta potential, percent head to head agglutination, percent intact acrosomes and motility of bull spermatozoa

A randomized complete block (ejaculates) design was used to evaluate the effect of graded levels of serum on the zeta potential, head to head agglutination, motility and acrosomal integrity of bovine spermatozoa. The relationship between zeta potential, percent head to head agglutination, intact acrosomes and motility were also studied under these conditions.

Four ejaculates were collected via artificial vagina, two ejaculates from one bull and one ejaculate each from two other bulls. Initial motility was estimated with a phase contrast microscope, and sperm numbers were determined photometrically using a Bausch and Lomb Spec 20 colorimeter. Each ejaculate was then diluted to 50×10^6 cells/ml in a 20% (v/v) egg yolk-sodium citrate (2.9%) diluent and split into nine 25-ml aliquots. Aliquots were then cooled to 4° C in two hours. Prior to semen dilution, the yolk-citrate diluent was clarified by centrifuga-

tion at 9150 g for 30 minutes. Five hundred units/ml of potassium penicillin G and 500 µg/ml of dihydrostreptomycin sulphate were added at this time.

After cooling the diluted semen, 25 ml of pre-cooled (4° C) serum-sodium citrate solutions (0-16%[v/v]) were added to the respective treatment flask. The final serum concentrations studied, ranged from zero to eight percent (v/v) in 1% increments. The final volume per flask was 50 ml; final egg yolk concentration - 10% (v/v) and the final cell concentration was 25 x 10^6 cells/ml. The mixtures were refrigerated at 4° C overnight. Viability and electrophoretic measurements were made the following day.

Five ml aliquots of each mixture were warmed to 35° C, and the cells were evaluated for motility, percent agglutination and percent intact acrosomes. The remaining volume was placed in the cytopherometer reservoir and maintained at 7-10° C during electrophoresis. Four replicates of 25 sperm per treatment were electrophoresed at an average field strength of 1.77 (volts/cm). The field strength and the calculated zeta potentials were corrected for operating temperatures in each replication and treatment.

Experiment II. Evaluation of the zeta potential of live versus killed bull spermatozoa

A randomized complete block (ejaculates) design was utilized to study the effect of rapid freezing and death of the sperm on the zeta potential of diluted bovine semen.

Four ejaculates were diluted to 25 x 10^6 cells/ml with 10% clarified egg yolk-sodium citrate-antibiotic (cf. Experiment I) diluent

to a final volume of 25 ml per treatment. Initial motility and cell concentration were measured as previously described.

The live treatment aliquots were cooled to 5° C in a water bath over a 90-minute period and kept at that temperature overnight. The killed treatment aliquots were obtained by subjecting the cells to rapid freezing in liquid nitrogen vapor, thawing in 37° C water and refreezing in nitrogen vapor. The cells were then allowed to thaw to 5° C and kept at that temperature overnight.

An aliquot of each ejaculate-treatment combination was warmed to 35° C and evaluated for motility and percent intact acrosomes. The remaining volume was then placed in the cooled (7-10° C) cytopherometer reservoir and sampled for U_e measurements. Fifty random cells per ejaculate-treatment combination were electrophoresed at an average field strength of 2.71 (v/cm). The calculated zeta potential was corrected for operating temperature.

Experiment III. Serum-treated live and dead bull spermatozoa

A randomized complete block (ejaculates) design with a factorial arrangement of treatment with subsampling was used to investigate the effect of 10% (v/v) serum and the particle geometry (single vs clumped cells) on the zeta potential of live and dead sperm.

Two ejaculates, one from each of two bulls, were collected via an artificial vagina. Each ejaculate was evaluated for initial motility and cell concentration as described previously. Two samples from each ejaculate were then diluted to 25×10^6 cells/ml in a final volume of 25 ml with 10% (v/v) clarified egg yolk-sodium citrate-antibiotic diluent (cf. Experiment I). One aliquot from each ejaculate (live

treatments) was cooled to 5° C in a water bath over a 90-minute period. The remaining two samples (dead treatments) were subjected to rapid freezing in nitrogen vapor, thawing in 37° C water and refreezing. After thawing to 5° C, 10% serum was added to each of the four treatment flasks and the samples were allowed to stand at 5° C overnight.

One hundred electrophoretic measurements at 7° C were made per treatment as previously described. However, for each treatment, the 100 measurements were composed of fifty single cells and fifty cell aggregates. The number of cells in an aggregate were noted as 2, 3, 4 or greater than 4. The field strength was held constant (5.4 volts/cm) for all measurements by adjusting the amperage according to the operating temperature and pre-measured specific resistance.

Experiment IV. The effect of buffer ionic strength and serum level on colloidal and biological aspects of diluted bull semen

A randomized complete block design with a factorial arrangement of treatments was used to investigate the effect of buffer ionic strength and serum on the zeta potential, head to head agglutination and acrosomal integrity of diluted bovine semen. Also studied was the relationship of zeta potential to head to head agglutination under these conditions.

Each replicate was obtained by pooling four to five ejaculates from several bulls. The pooled semen was slowly cooled without dilution to 5° C, in a water bath, over a 5-hour period and kept at thattemperature overnight. The semen was then allocated across eight treatment buffers at 5° C. The final cell concentration in each treatment buffer was 25×10^6 cells/ml in a volume of 30 ml.

The diluent compositions for the eight factorial treatment combinations are listed in Table II. There were two serum levels and four ionic strength levels. Sodium citrate and magnesium citrate were used in isomolar quantities so as to test not only the concentration aspect of ionic strength but also the valency component. Egg yolk was not used because it denatured at low ionic strength.

Electrophoretic mobility, head to head agglutination and acrosomal integrity were measured at 5° C beginning 15 minutes after each treatment dilution. This insured sufficient time for agglutination to occur yet provided a large enough single cell population to measure, i.e., agglutination would not have maximized in this interval. These measurements were performed as previously described except, aliquots were not warmed to measure percent head to head agglutination and intact acrosomes. Twenty-five cells per replicate-treatment combination were electrophoresed to obtain the zeta potential measurement. Also, the specific resistance was measured at 5° C in the presence of the cells. The amperage was then adjusted accordingly to maintain a constant field strength of 7.0 volts per cm.

Experiment V. The effect of serum treatment on the aerobic metabolism of diluted bull semen

A randomized complete block design with a factorial arrangement of serum and non-serum treatments with time was used to compare the oxygen consumption rates of bovine spermatozoa, selected for their agglutination. The linear, quadratic and cubic regression components across time were partitioned within each treatment. Three replicates of two treatments (0 and 20% [v/v]) were obtained by collecting three or four ejaculates

TABLE II. COMPOSITION AND IONIC STRENGTH OF THE BUFFERS USED TO STUDY THE BIOCOLLOIDAL ASPECTS OF DILUTED BOVINE SEMEN.

Treatment Number	Serum %(v/v)	Component (Molar x 10^{-3})				Ionic Strength (μ)
		Citric Acid	Tris	Sodium Citrate	Magnesium Citrate	
1	0.2	7.4	23.1	---	---	0.045
2	0.2	0.2	---	14.8	---	0.090
3	0.2	---	53.2	---	14.8	0.180
4	0.2	---	52.6	14.8	14.8	0.337
5	10.0	7.4	23.1	---	---	0.045
6	10.0	0.2	---	14.8	---	0.090
7	10.0	---	53.2	---	14.8	0.180
8	10.0	---	52.6	14.8	14.8	0.337

All treatments were adjusted to isotonicity with the required amount of sucrose and ranged from pH 7.0 to 7.2.

from several bulls each day (replicate) and pooling them to eliminate bull and ejaculate variation. The pooled semen was evaluated for initial motility and cell concentration as previously described.

The pooled semen for each replicate was diluted to a concentration of 2.5×10^8 cells per ml with a 10% egg yolk-2.9% sodium citrate-antibiotic (cf. Experiment I) diluent. The diluted semen was divided into three aliquots and treatments were applied as follows: 1) 20% (v/v) serum; 2) equivalent volume of the diluent; 3) the same as #2. For each replicate, the treated semen was allowed to stand at room temperature for one hour and then sampled for head to head agglutination. Serum-treated samples contained greater than 50% agglutination; controls were not agglutinated.

Two and one-half ml of each treated solution were placed in the main chamber of four Warburg flasks. The side arm of treatment 1 and 2 flasks contained 0.5 ml of 7.5% fructose solution, while that of the treatment 3 flasks contained 0.5 ml of a saturated $HgCl_2$ solution. The treatment 3 flasks served as the thermobarometers. The center cell of every flask contained 0.2 ml of 20% potassium hydroxide solution on filter paper. The final cell concentration in each flask was 2×10^8 cells per ml.

All flasks were allowed to equilibrate in the bath at 37° C with agitation for 30 minutes prior to beginning the reaction. Pressure changes were recorded at 30-minute intervals for a 2-hour period. The rate of oxygen consumption per 10^8 cells per minute was calculated and analyzed.

Experiment VI. The effect of serum and head to head agglutination on aerobic (methylene blue reduction rate [MeBR]) and anaerobic (lactic acid production rate [LAPR]) metabolism

A randomized complete block design with a covariate was used to investigate the effect of level of serum or of head to head agglutination on aerobic and anaerobic metabolic rates of diluted semen. Percent head to head agglutination was used as the covariate across eight replicates of two serum levels.

Eight ejaculates (replicates) were collected by artificial vagina from the same bull over a 14-day period, 2 ejaculates per day for a total of four test days. Each ejaculate was evaluated for initial motility and cell concentration as described before, and then, diluted to 7.5×10^8 cells per ml with 2.9% sodium citrate-antibiotic (cf. Experiment I) buffer.

The methylene blue reduction test, a modification of Beck and Salisbury (37), and lactic acid production test were performed on each ejaculate according to the following sequence in 10 x 45 mm test tubes.

1) Tube 1 - 0.50 ml 2.9% sodium citrate buffer;
   tube 2 - 0.59 ml 2.9% sodium citrate buffer;
   tube 3 - 0.60 ml 2.9% sodium citrate buffer;
   tube 4 - 0.69 ml 2.9% sodium citrate buffer.
2) Tubes 1, 2, 3, and 4 - 0.2 ml diluted semen.
3) Tubes 2 and 4 - 0.01 ml saturated $HgCl_2$, mix and record initial lactic acid production time.
4) Tubes 1 and 2 - 0.2 ml serum (20% [v/v]) and mix;
   tubes 3 and 4 - 0.1 ml serum (10% [v/v]) and mix.

5) Tubes 2 and 4 - 0.01 ml 50 mg% methylene blue (MeB) solution and mix.

6) Tubes 1 and 3 - 0.10 ml MeB solution, mix and record initial MeBR time.

7) Place all tubes in transparent water bath (46° C) and observe until the color of tubes 1 and 3 match that of tubes 2 and 4, respectively, then record final MeBR time.

8) A wet smear was obtained from each tube and evaluated for head to head agglutination.

9) After a lapse of about 50 minutes, 0.01 ml of saturated $HgCl_2$ was added to tubes 1 and 3 and final lactic acid production time recorded.

Tubes 2 and 4 represented not only initial lactic acid concentration but also 90% methylene blue reduction. The MeB solution was 50 mg MeB in 100 ml of 2.9% sodium citrate; the final concentration in tubes 1 and 3 was 1.56×10^{-7} moles and in tubes 2 and 4, 0.16×10^{-7} moles. Thus, methylene blue reduction rate (MeBR) was the time required to reduce 1.4×10^{-7} moles (90% reduction) of methylene blue to leucomethylene blue, i.e. when the color of tubes 1 and 3 matched that of tubes 2 and 4, respectively.

Lactic acid production rate (LAPR) was expressed as the difference in lactic acid concentration in tubes 1 and 3 versus that of tubes 2 and 4 per minute of incubation time for each level of serum, respectively. Lactic acid concentration was determined enzymatically in duplicate on every tube (38).

Statistical Methods and Analyses

All percentage data were arcsin square root transformed to insure homogeneity of variances, and zero percentages were corrected for discontinuity (39). All zeta potential data are averages of the number of spermatozoa sampled in each experiment, except those of Experiments II and III, where the data are those of the individual cells.

Univariate statistical analysis (39, 40) and multivariate analysis of variance (41) were obtained by least squares methods and applied to the data as the nature of the observations and design of each experiment warranted.

Differences between treatment means were tested by F-test or Wilks Test for those experiments which had only two treatments or where treatment variation was partitioned into single degree of freedom contrast. Duncan's New Multiple Range Test (39) was used to test mean differences in multiple comparisons between means of equal numbers. Kramer's modification of Duncan's test (42) was used to make comparisons when the means contained unequal numbers.

The maximum F value (Max F) is the largest F value that can be obtained by any linear function of the individual dependent parameters. The comparison of Max F to the individual (univariate) F values of the dependent parameters reveals which parameter contributes mostly to the rejection of the null hypothesis concerning each variation source, i.e., a significant Wilks Test.

The linear combination means are formed from the vector of dependent parameter means for each treatment and weighting coefficients calculated from the variance-covariance matrices according to Kramer

(43). The linear combination means are response indices used to delineate the treatment effects on the system of dependent parameters studied. The linear combination mean differences were tested according to Kramer (43).

RESULTS

Experiment I. The effect of adding graded levels of serum on spermatozoal zeta potential, the percentage of agglutinated spermatozoa, the maintenance of spermatozoal acrosomal membranes and motility are shown in Table III. Also shown in Table III are the linear combination means generated from the multivariate analysis of variance. As the serum percentage was increased, there was an overall reduction in zeta potential with a corresponding increase in percent head to head agglutination. There was no consistent relationship between changing serum levels and cell viability, as indicated by acrosomal integrity and motility (Table III). The linear combination means (L_i, Table III) indicate that the total system response was high at low and high serum levels and minimal, at intermediate levels.

By comparison of the maximum F value (Table IV) for treatment and linear and quadratic regression on serum level with the individual F values of each parameter, it is seen that the differences in the significant ($P<0.01$) treatment response of the total system is due mainly to the serum effect on agglutination. However, there was also a significant serum effect on zeta potential, especially when the change in zeta potential was expressed as a function of changing serum levels (ZP linear regression F value, Table IV). Table IV also shows that zeta potential contributed mostly to the significant ($P<0.01$) replicate effect. The multivariate analysis of variance is shown in Appendix Table I.

TABLE III (EXPERIMENT I). THE EFFECT OF GRADED LEVELS OF SERUM ON THE ZETA POTENTIAL (ZP), PERCENT HEAD TO HEAD AGGLUTINATION (HHAgg), PERCENT INTACT ACROSOMES (IA), AND PERCENT MOTILITY (MOT) AND THEIR LINEAR COMBINATION (L_i) MEANS OF BULL SPERMATOZOA.

Serum %(v/v)	ZP (-mV)	HHAgg (%)	IA (%)	MOT (%)	L_i
0	15.48	0.2	80.2	42.5	97.32^{a}
1	16.20	7.6	76.1	34.7	68.25^{b}
2	13.19	21.3	78.6	45.5	30.59^{de}
3	13.08	41.5	81.0	42.5	00.01^{f}
4	15.66	53.5	78.6	39.5	17.11^{ef}
5	13.36	59.4	79.4	42.5	29.55^{de}
6	12.49	66.2	76.1	47.5	38.28^{cde}
7	12.38	71.7	81.0	45.5	45.53^{bcd}
8	12.87	84.0	83.3	52.4	61.23^{bc}

$^{a-f}$Any two combination means followed by the same superscript are not significantly different ($P<0.05$).

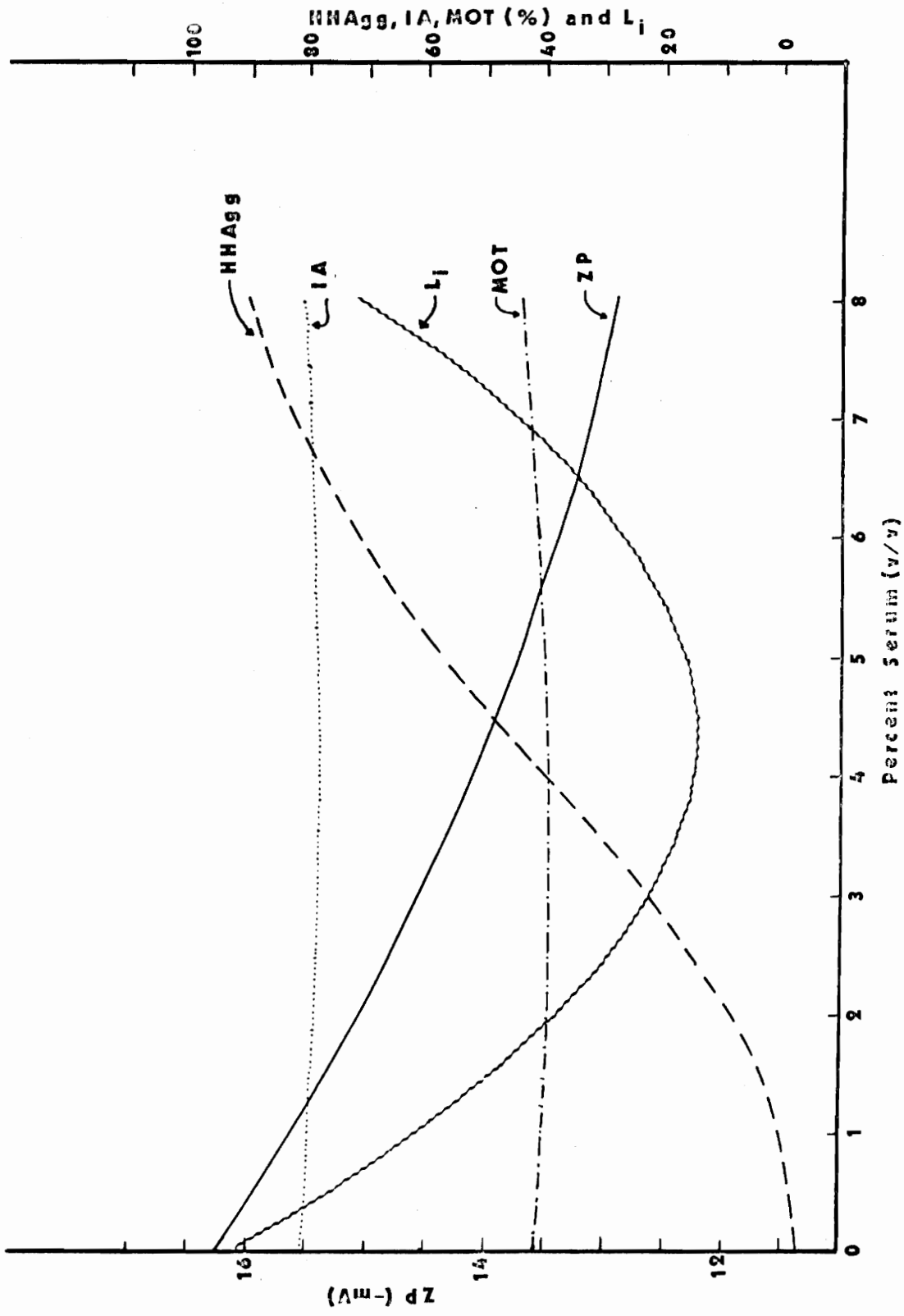
TABLE IV (EXPERIMENT I). RESULTS OF MULTIVARIATE ANALYSIS OF VARIANCE OF THE EFFECTS OF GRADED LEVELS OF SERUM ON ZETA POTENTIAL (ZP), HEAD TO HEAD AGGLUTINATION (HHAgg), ACROSOMAL INTEGRITY (IA) AND MOTILITY (MOT) OF DILUTED BOVINE SEMEN.

Source	df	Wilks Test	MAX F	ZP-F	HHAgg-F	IA-F	MOT-F
Total	35						
Replicate	3	0.036**	46.72	42.00†	5.42	20.12	3.36
Treatment	8	0.008**	88.26	3.78	80.24†	2.94	1.39
Linear Regression	1	0.035**	656.36	15.08	601.74†	3.58	5.71
Quadratic Regression	1	0.310**	53.48	0.41	32.66†	4.60	1.04
Lack of Fit	6	0.256	22.10	2.46	1.29	3.00	0.71
Error Variance	24			2.343	0.007	0.001	0.007

**P<0.01.

†Indicates which dependent parameter contributes most to the significance of the Wilks Test.

Figure 3. (Experiment I). The predicted response curves of zeta potential (ZP), head to head agglutination (HHAgg), intact acrosomes (IA), motility (MOT), and linear combination expressed as a function of changing serum levels.


HHAgg, IA, MOT (%) and L_i
100
80
60
40
20
0
HHAgg
IA
L_i
MOT
ZP
16
14
12
ZP (-mV)
0
1
2
3
4
5
6
7
8
Percent Serum (v/v)

system is not apparent from the individual predicted responses in Figure 3. Nevertheless, as seen in Figure 3, when a diluted semen sample (25 x 10^6 cells/ml) with 40% motile and 78% intact cells is treated with this serum level (4.3%), the spermatozoal zeta potential would be -13.96 mV and sufficiently reduced to allow 46% of the cells to agglutinate.

Colloid theory indicates that as the zeta potential of the particle is reduced, the colloid becomes less stable and flocculation (agglutination) occurs. In order to test this relationship, the mean head to head agglutination was expressed as a function of mean zeta potential. Figure 4 depicts the significant ($P<0.05$) negative relationship between mean percent agglutination and mean spermatozoal zeta potential. Table V illustrates the regression analysis of this relationship. Thus, colloidal theory is implicated in serum-induced spermatozoal agglutination phenomenon.

The overall correlations between zeta potential and the other dependent parameters were, -0.18, 0.39 ($P<0.02$) and -0.06 for head to head agglutination, acrosomal integrity and motility, respectively. The overall correlations between agglutination and the remaining parameters were 0.21 and 0.63 ($P<0.03$) for intact acrosomes and motility, respectively; that between acrosomal integrity and motility was 0.42 ($P<0.01$).

Experiment II. Killed spermatozoa (freeze-thawed without cryprotection) have lower zeta potentials than live spermatozoa that were cooled slowly (Table VI). The inspection of each rapid frozen

Figure 4. (Experiment I). Head to head agglutination (HHAgg) expressed as a function of zeta potential (ZP) of bull spermatozoa under changing serum level.

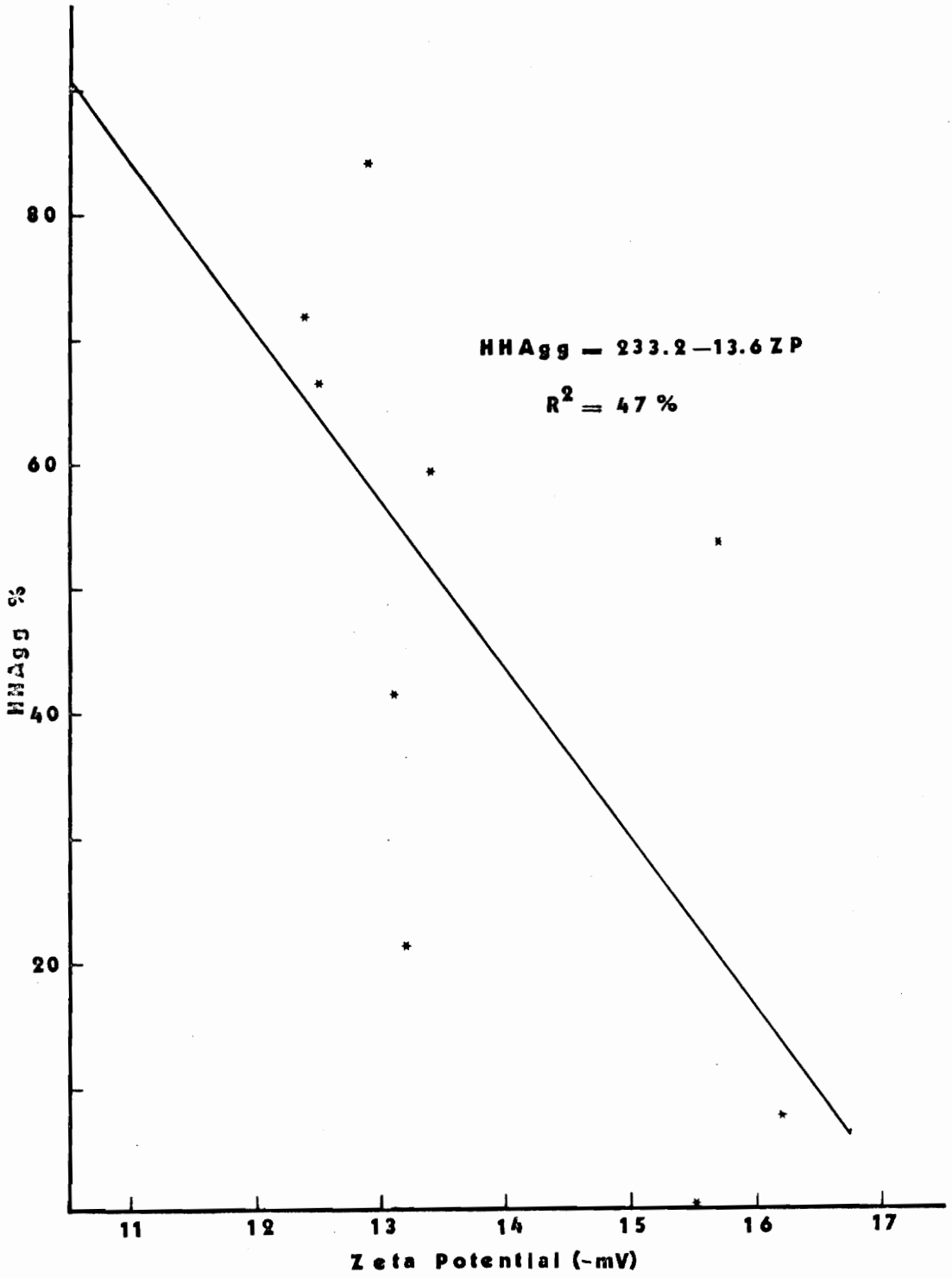

HHAgg = 233.2 −13.6 ZP
R^2 = 47 %
HHAgg %
80
60
40
20
11
12
13
14
15
16
17
Zeta Potential (-mV)

TABLE V (EXPERIMENT I). REGRESSION ANALYSIS OF THE RELATIONSHIP OF PERCENT HEAD TO HEAD AGGLUTINATION TO THE MEAN ZETA POTENTIAL OF BULL SPERMATOZOA UNDER CHANGING SERUM LEVELS.

Source	df	Sum of Squares	F Ratio
Total	8	6942.66	
Regression on zeta potential	1	3270.19	6.23*
Residual	7	3672.47	

*P<0.05.

TABLE VI (EXPERIMENT II). THE EFFECT OF RAPID FREEZING VERSUS SLOW COOLING ON THE AVERAGE ZETA POTENTIAL (ZP) OF BOVINE SPERMATOZOA.

Ejaculate	Mean ZP (-mV) Per Ejaculate-Treatment	
	Rapid Frozen	Slow Cooled
1	12.21	12.41
2	10.52	15.28
3	12.32	12.75
4	14.08	15.16
Mean	12.30[a]	13.91[b]

[a-b]Treatment means are significantly different ($P<0.01$).

sample showed that these cells had no intact acrosomal membranes as compared to 77% intact membranes in the slow cooled treated cells. Thus, it appears that the loss of the plasma and acrosomal membranes results in a reduction of the net charge on the surface of the spermatozoa. The effect was more dramatic in some ejaculates than in others (Table VI).

Table VII illustrates the analysis of variance of the zeta potential data from Experiment II. Since four ejaculates are not an adequate random sample of all ejaculates, and the experimental hypothesis concerned the cells and not ejaculates primarily, the linear model was considered fixed and thus the cell-to-cell variation was used to test the null hypothesis of no treatment effect. Thus as seen in TAble VII, in addition to the significant ($P<0.01$) treatment effect, the effects due to ejaculates and ejaculate by treatment interaction were also significant ($P<0.01$).

Experiment III. The rapid freezing of bull spermatozoa significantly ($P<0.01$) reduced their zeta potential (Table VIII) as compared to slow cooling of the cells. This is presumably a result of the loss of the plasma and acrosomal membranes as was observed in Experiment II. However, since acrosomal integrity of the cells was not assessed, this is not a certainty.

Since a single spermatozoan, and, most certainly, large aggregates of spermatozoa, could approach the upper limit of colloidal dimensions ($\approx 10^4 A°$) and possibly violate the electrokinetic theory with respect to frictional forces, the effect of particle size on spermatozoal zeta potential was investigated. Serum-induced head to head agglutination

TABLE VII (EXPERIMENT II). ANALYSIS OF VARIANCE OF THE ZETA POTENTIAL OF RAPID FROZEN AND SLOW COOLED BULL SPERMATOZOA.

Source	df	Sum of Squares	F Ratio
Total	394	2128.67	
Ejaculate	3	329.30	35.07**
Treatment	1	257.74	82.35**
Ejaculate x treatment	3	333.21	35.49**
Error	387	1211.13	

**P<0.01.

TABLE VIII (EXPERIMENT III). THE MEAN ZETA POTENTIAL OF RAPID FROZEN (RF) AND SLOW COOLED (SC) SERUM TREATED (10% [v/v]) SPERMATOZOA, AS SINGLE CELLS AND IN AGGREGATES OF DIFFERENT SIZES.

# Cells Per Aggregate	Mean Zeta Potential (-mV)		
	Rapid Frozen	Slow Cooled	Aggregate Means
Single Cell	12.75(50)[1]	16.03(50)	14.39[a]
2	15.88(23)	17.99(10)	16.56[bc]
3	15.31(8)	15.74(12)	15.57[abc]
4	18.34(4)	17.61(7)	17.88[c]
>4	13.84(15)	15.93(21)	15.06[ab]
Means	14.06[a]	16.28[b]	

[1]Number of cells measured per mean.

[a-c]Any two means, within a classification, followed by the same superscript are not significantly different ($P<0.05$).

was not assessed as such in this experiment, however, serum was added to all samples and the aggregates formed were assumed to be of that type. As deduced from Table VIII, differences in zeta potential were not a function of increasing particle size or cells in any one aggregate. Thus, there was not divergence from electrokinetic behavior. The zeta potential was greater for one ejaculate across both factors than that of the other.

The analysis of variance of the data from Experiment III is shown in Table IX. There was, in addition to the significant ($P<0.01$) treatment effect, a significant ejaculate by treatment interaction. The cell-to-cell variation was used to test the null hypothesis concerning the ejaculate, treatment and interaction effects because the linear model was considered fixed and the experimental hypothesis concerned the cells not the ejaculates.

Experiment IV. The effects of serum and buffer ionic strength on zeta potential, head to head agglutination and acrosomal integrity of bull spermatozoa are shown in Table X. As the serum levels were increased, there was a significant ($P<0.01$) zeta potential reduction along with a significant ($P<0.01$) increase in agglutination. Acrosomal integrity was also improved significantly ($P<0.01$) by the higher serum level.

Also, as seen in Table X, as the buffer ionic strength was increased, zeta potential decreased and intact acrosomes increased significantly ($P<0.01$). Agglutination also increased with increasing ionic strength. These differences were more pronounced at the low serum level for all three parameters; the percentage change between the highest and lowest value of any parameter was greater at 0.2% serum

TABLE IX (EXPERIMENT III). ANALYSIS OF VARIANCE OF THE ZETA POTENTIAL OF SERUM-TREATED, RAPID FROZEN (RF) OR SLOW COOLED (SC), SINGLE AND AGGLUTINATED (PARTICLE SIZE) BOVINE SPERMATOZOA.

Source	df	Sum of Squares	F Ratio
Total	199	3001.79	
Ejaculate	1	340.22	33.66**
Treatment	9		
RF vs SC	1	53.73	5.73**
Particle size	4	194.28	5.18**
(RF-SC) x Particle size	4	71.46	1.91
Ejaculate x treatment	9	679.42	8.06**
Residual	180	1685.64	

**P<0.01.

TABLE X (EXPERIMENT IV). THE EFFECT OF SERUM AND BUFFER IONIC STRENGTH ON SPERMATOZOAL ZETA POTENTIAL (ZP), HEAD TO HEAD AGGLUTINATION (HHAgg), INTACT ACROSOMES (IA) AND THE LINEAR COMBINATION (L_i) OF THESE PARAMETERS.

	Serum (%)	Buffer Ionic Strength 0.045	0.090	0.180	0.337	Parameter Means Per Serum Level
ZP (-mV)	0.2	15.07	14.30	11.74	10.74	12.96^{a}
	10.0	11.97	9.87	9.34	10.33	10.38^{b}
	Means	13.52	12.09	10.54	10.54	
HHAgg (%)	0.2	3.6	4.8	4.8	6.1	4.8^{a}
	10.0	35.7	39.5	48.5	31.9	38.9^{b}
	Means	19.6	22.1	26.6	19.0	
IA (%)	0.2	39.5	50.5	57.4	56.4	51.0^{a}
	10.0	60.4	66.2	70.4	67.1	66.1^{b}
	Means	49.5	58.4	64.2	61.2	
L_i		-1.88^{a}	-5.00^{b}	-7.76^{c}	-7.16^{bc}	

$^{a-c}$Any two means, within a parameter or classification, followed by the same superscript are not significantly different ($P>0.05$).

than that at 10%. All of the parameters showed a curvilinear response to changing ionic strength which is illustrated by the least squares prediction curves in Figure 5.

The linear combination means (L_i, Table X), averaged across serum levels, showed a significant ($P<0.01$) decline in response to increasing ionic strength levels. The combined response (L_i, Figure 5) showed a significant ($P<0.01$) negative quadratic response to increasing ionic strength.

The multivariate analysis of variance for Experiment IV is shown in Appendix Table II. The results of the analysis are shown in Table XI along with the comparison of the univariate F ratios and the maximum F value for each variance component of the model. All of the partitioned sources of variance were significant ($P<0.01$) except the factorial interaction (serum by μ) and the lack of fit component of the regression on ionic strength (Wilks Test, Table XI). In accordance with the definition of the maximum F value, acrosomal integrity contributed mostly to the replicate effect, while head to head agglutination and zeta potential contributed mostly to the serum and ionic strength effects, respectively. Zeta potential and intact acrosomes had nearly equal contributions to linear combination regression on ionic strength.

Therefore, the linear combination values are essentially a summation of the influence of the bulk ionic environment on the integrity of the plasma membrane and the ionic double layer adjacent to it. A minimum, calculated to be at $\mu = 0.30$ ($dL/dX = 0$, Figure 5), in this combined response would indicate an optimum environment that would allow the colloid particle to remain viable. The spermatozoa behaved

Figure 5. (Experiment IV). The least square predicted responses of zeta potential (ZP), head to head agglutination (HHAgg), intact acrosomes (IA), and linear combination of these (L_i) to changing buffer ionic strength.

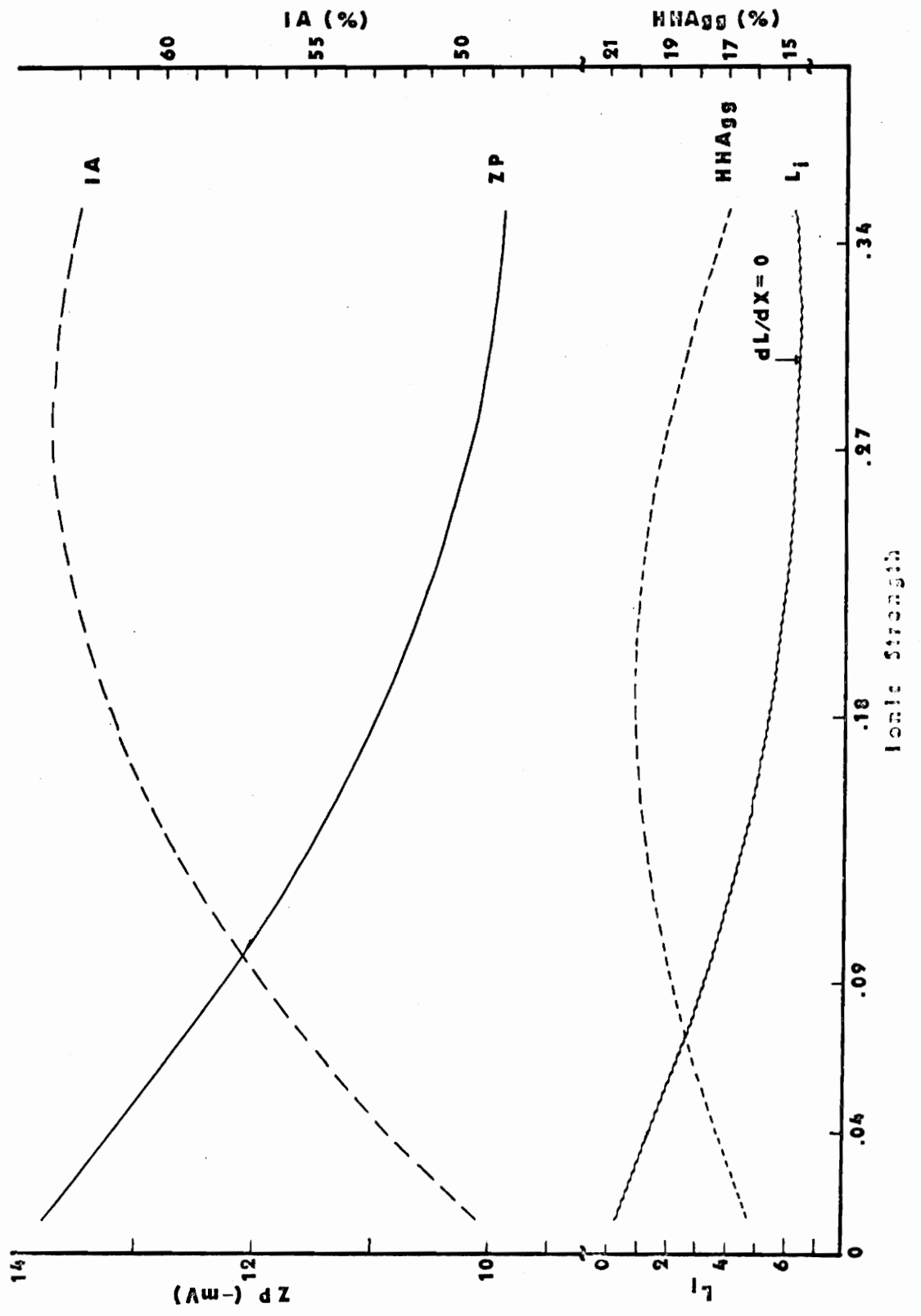

IA (%)
60
55
50
HHAgg (%)
21
19
17
15
IA
ZP
HHAgg
L_i
dL/dX = 0
.34
.27
.18
.09
.04
0
Ionic Strength
14
12
10
ZP (-mV)
0
2
4
6
L_i

TABLE XI (EXPERIMENT IV). RESULTS OF THE MULTIVARIATE ANALYSIS OF VARIANCE OF THE EFFECTS OF CHANGING SERUM AND BUFFER IONIC STRENGTH (μ) ON THE ZETA POTENTIAL (ZP), HEAD TO HEAD AGGLUTINATION (HHAgg), AND INTACT ACROSOMES (IA) OF DILUTED BOVINE SEMEN.

Source	df	Wilks Test	Max F	ZP-F	HHAgg-F	IA-F
Total	39					
Replicate	4	0.39**	5.54	1.12	2.66	4.42†
Treatment	7					
Serum	1	0.17**	133.74	18.04	87.59†	25.86
μ	3	0.42**	11.96	5.54†	0.52	4.66
Regression on μ	2	0.49**	13.77	6.91†	0.42	5.04
Lack of Fit	1	0.76	8.84	2.80	0.85	4.48
Serum x μ	3	0.72	2.66	1.89	0.67	0.51
Error Variance	28			3.71	0.02	0.09

**P<0.01.

†Indicates which dependent parameter contributes most to the significant Wilks Test.

much like an inert particle in terms of zeta potential yet deteriorated in terms of viability below this optimum ionic strength.

As in Experiment I, and as prescribed by colloidal theory, mean head to head agglutination was again expressed as a function of mean zeta potential under these conditions. Figure 6 depicts the significant ($P<0.05$) linear relstionship of the mean agglutination to the mean spermatozoal zeta potential. The regression analysis of this relationship is presented in Table XII.

The total correlations between the dependent parameters under these conditions were; zeta potential to head to head agglutination and intact acrosomes, -0.44 ($P<0.01$) and -0.47 ($P<0.01$), respectively; head to head agglutination to intact acrosomes, 0.57 ($P<0.01$).

Therefore, the response of the spermatozoal zeta potential to the buffer ionic strength demonstrates the colloidal nature of spermatozoa. This response and the functional relationship seen between head to head agglutination and zeta potential further reinforces the biocolloidal nature of diluted semen and thus the description of the agglutination phenomenon in terms of colloidal principles.

Experiment V. Oxygen consumption of serum and non-serum treated bovine semen during a 2-hour incubation period is presented in Table XIII. A decrease in oxygen consumption was observed at each 30-minute time interval throughout the incubation for both treatments. However, serum treated cells consumed significantly ($P<0.01$) more oxygen than the controls, with the greatest difference being apparent in early stages of the incubation.

Figure 6. (Experiment IV). Head to head agglutination (HHAgg[%]) expressed as a function of zeta potential (ZP) of bull spermatozoa under changing serum and ionic strength conditions.

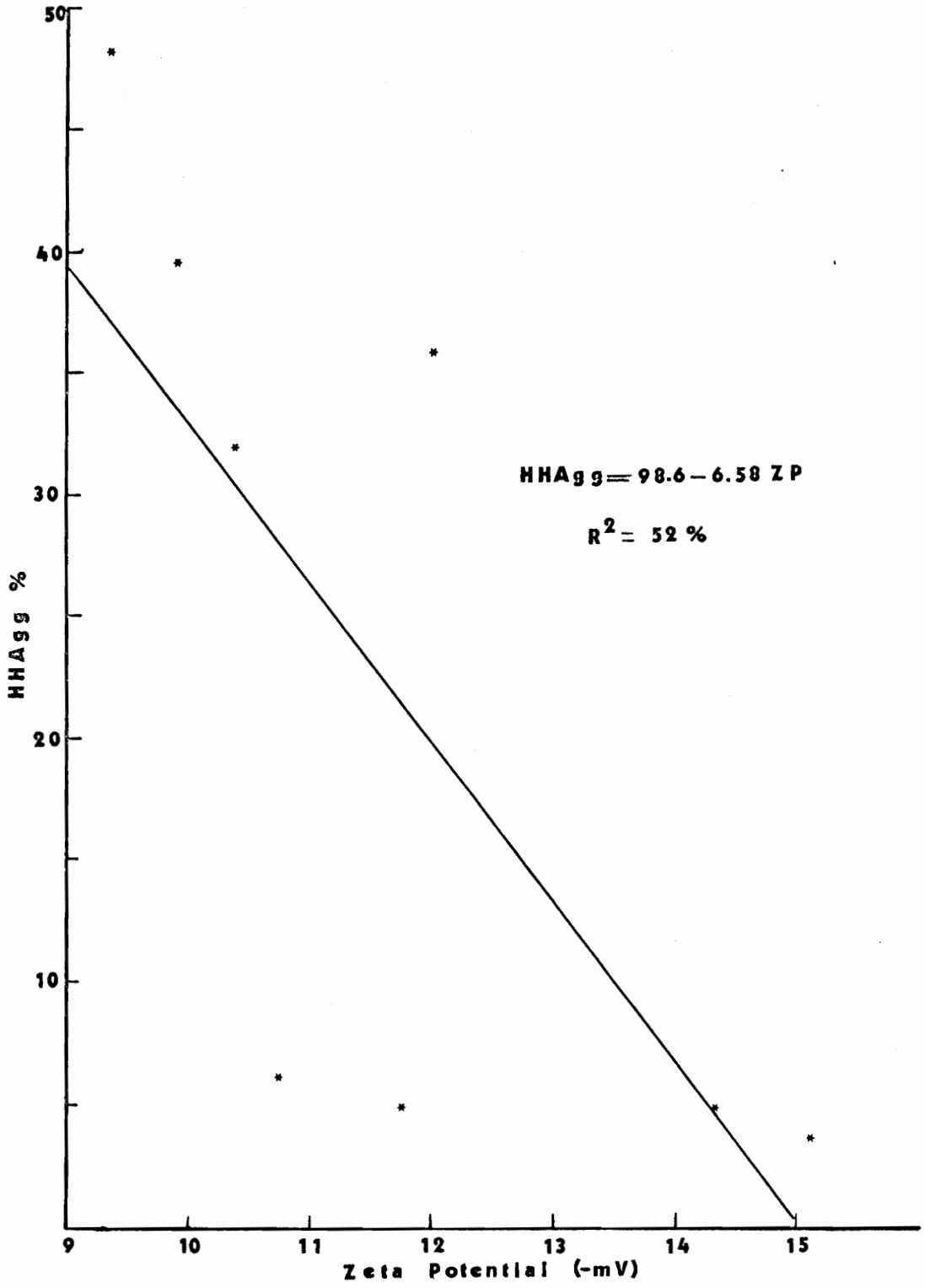

50
40
30
20
10
HHAgg %
HHAgg = 98.6 – 6.58 ZP
R2 = 52 %
9
10
11
12
13
14
15
Zeta Potential (-mV)

TABLE XII (EXPERIMENT IV). REGRESSION ANALYSIS OF THE MEAN HEAD TO HEAD AGGLUTINATION ON THE MEAN ZETA POTENTIAL OF BULL SPERMATOZOA UNDER CHANGING SERUM AND BUFFER IONIC STRENGTH CONDITIONS.

Source	df	Sum of Squares	F Ratio
Total	7	2477.10	
Regression on Zeta Potential	1	1292.83	6.55*
Residual	6	1184.27	

*P<0.05.

TABLE XIII (EXPERIMENT V). THE MEAN OXYGEN CONSUMPTION ($\mu l/10^8$ CELLS) OF SERUM TREATED (ST) AND NON-SERUM TREATED (NS) BOVINE SPERMATOZOA PER THIRTY MINUTE INTERVAL AND OVERALL.

Treatment	Time (Min.)				Overall $\bar{X}$
	30	60	90	120	
ST	10.92	9.41	7.16	6.34	33.83[a]
NS	9.68	8.40	6.73	6.36	31.17[b]

[a-b]Total consumption per treatment was significantly different ($P<0.01$).

The changing consumptions with time resulted in a significant ($P<0.01$) quadratic response for the serum treated cells, while the non-treated cells had only a significant ($P<0.01$) linear response throughout incubation. The control quadratic component only approached significance ($P<0.10$). These responses are depicted in Figure 7.

The analysis of variance of the oxygen consumption data is shown in Table XIV. There was a significant ($P<0.01$) replicate effect. The seven treatment degrees of freedom were partitioned into individual degrees of freedom contrast, one for the difference due to serum versus no-serum treatment and the remaining six as time regressions within each serum treatment. There was a highly significant ($P<0.01$) serum versus non-serum effect. Also, there were highly significant ($P<0.01$) linear time effects within each treatment and a significant ($P<0.05$) quadratic effect for the serum-treated cells.

Experiment VI. The mean methylene blue reduction rate, lactic acid production rate and their linear combination response for each serum level, pre- and post covariate adjustment for head to head agglutination are shown in Table XV. Spermatozoa treated with 20% serum reduced the available methylene blue at a significantly ($P<0.025$) faster rate than those treated with 10% serum. They also produced lactic acid significantly ($P<0.01$) faster than 10% serum treated cells. Treatment of spermatozoa with 20% serum increased the level of head to head agglutination, above that of 10% serum treatment, by an average of 32.9% (HHAgg, Table XV).

Figure 7. (Experiment V). Oxygen consumed ($\mu l/10^8$ cell) versus incubation time of serum treated (ST) and non-serum (NS) treated bovine spermatozoa.

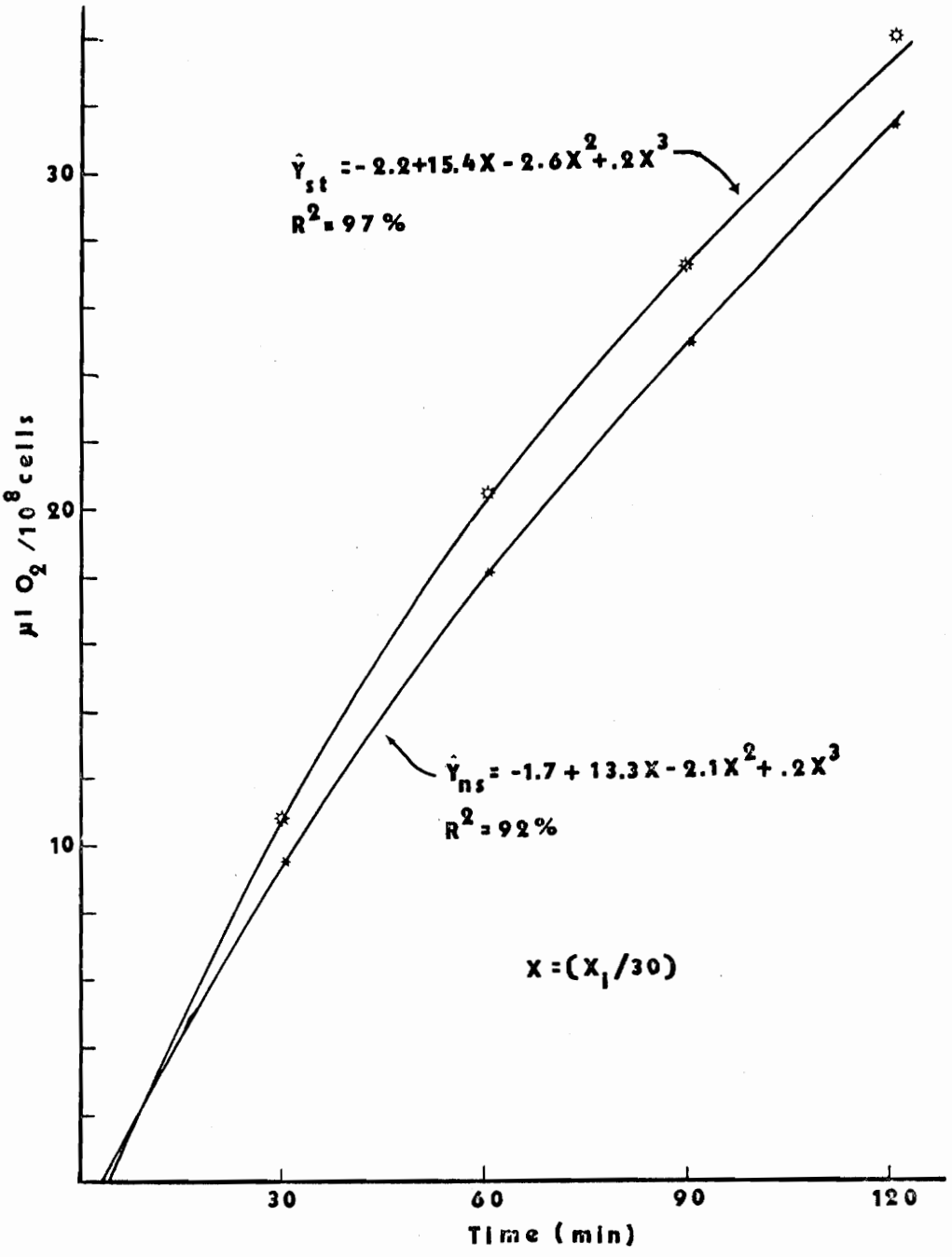

$\hat{Y}_{st} = -2.2 + 15.4X - 2.6X^2 + .2X^3$
$R^2 = 97\%$
$\hat{Y}_{ns} = -1.7 + 13.3X - 2.1X^2 + .2X^3$
$R^2 = 92\%$
$X = (X_1/30)$
30
20
10
μl O_2 /10^8 cells
30
60
90
120
Time (min)

TABLE XIV (EXPERIMENT V). ANALYSIS OF VARIANCE OF THE OXYGEN CONSUMPTION OF SERUM TREATED (ST) AND NON-SERUM TREATED (NS) BOVINE SPERMATOZOA.

Source	df	Sum of Squares	F Ratio
Total	95	7162.46	
Replicate	2	104.15	15.07
Treatment [1]	7		
ST vs NS	1	117.04	33.88**
Time	6		
$Time_l$ x ST	1	3455.90	1000.26**
$Time_q$ x ST	1	28.45	8.23*
$Time_c$ x ST	1	1.23	0.36
$Time_l$ x NS	1	3040.78	880.11**
$Time_q$ x NS	1	12.43	3.60
$Time_c$ x NS	1	1.01	0.29
Replicate x treatment	14	48.37	0.70
Sampling	72	353.08	

*P<0.05, **P<0.01.

[1]All partitioned treatment variance components were tested with the interaction term.

[1,q,c]Linear, quadratic or cubic components of the per treatment time regression.

TABLE XV (EXPERIMENT VI). MEAN RATES OF METHYLENE BLUE REDUCTION (MeBR) AND LACTIC ACID PRODUCTION (LAPR) AND THEIR LINEAR COMBINATION (L_i), PRE- AND POST HEAD TO HEAD AGGLUTINATION (HHAgg) ADJUSTMENT.

Serum %(v/v)	HHAgg (%)	MeBR (moles x 10^{-8}/min.)	LAPR	L_i
		PRE-ADJUSTMENT		
10	27.5	1.94[a]	1.33[a]	10.72[a]
20	60.4	2.18[b]	2.51[b]	18.81[b]
		POST ADJUSTMENT		
10	44.4	2.07	1.46	-0.38[c]
20	44.4	2.04	2.38	-1.96[c]

[a-c]Prior to adjustment means were significantly different ($P<0.01$), afterward, they were not ($P>0.05$).

The linear combination means (L_i, Table XV), which are indices of total metabolic rate, reflect the significant ($P<0.01$) differences seen in the methylene blue reduction and lactic acid production rates before and after adjustment for level of agglutination. Prior to adjustment, there was a significant ($P<0.01$) difference between the combination means. However, after adjusting the combined response and the individual responses to a common level of agglutination, the effects due to the 20% serum treatment were not different from those due to the 10% serum treatment (post adjusted means, Table XV). These results indicate that the metabolic differences initially observed were due to the level of head to head agglutination rather than the serum levels, per se.

The mole ratio of methylene blue reduction to lactic acid production rates serves as an indicator of preferred metabolic route. Aerobic metabolism would be favored when the ratio is greater than 1. Anaerobic metabolism would be favored when the ratio is less than 1. Both of the metabolic routes would be proceeding at equal rates when the ratio equals 1. Table XVI shows a ranking of the mole ratio data within and across serum levels according to the level of agglutination. It can be seen, that, as agglutination level increased both within and across serum levels, there was a significant ($P<0.01$) shift in the preferred metabolic route from aerobiosis to anaerobiosis.

Head to head agglutination was correlated to the metabolic parameters in this study as follows: methylene blue reduction rate, 0.50 ($P<0.05$); lactic acid production rate, 0.79 ($P<0.01$); mole ratio, -0.60 ($P<0.01$). The correlations between the metabolic parameters were: mole ratio to methylene blue reduction rate, -0.14 and to lactic

TABLE XVI (EXPERIMENT VI). MOLE RATIO RANKED WITHIN SERUM LEVELS ACCORDING TO LEVEL OF HEAD TO HEAD AGGLUTINATION (HHAgg).

	Serum (%)			
	10		20	
	HHAgg (%)	Ratio (mole/mole)	HHAgg (%)	Ratio (mole/mole)
	10.0	1.29	42.5	1.85
	23.0	3.51	55.0	1.16
	23.5	3.09	55.0	0.81
	27.0	1.01	61.0	0.78
	27.5	1.54	61.5	0.90
	36.0	1.21	62.5	0.64
	36.5	1.49	69.5	0.76
	42.0	0.94	77.5	0.84
$\bar{X}$	28.3	1.76[a]	60.3	0.97[b]

[a-b] Ratio means prior to adjustment for level of HHAgg were significantly different ($P<0.01$).

acid production rate, -0.74 ($P<0.01$); methylene blue reduction rate to lactic acid production rate, 0.63 ($P<0.01$).

The multivariate analysis of covariance of the data from Experiment VI is shown in Appendix Table III. As can be seen, the sums of squares and cross products are reduced by 91.1%, 101.8% and 99.6% for lactic acid production rate, the cross products and methylene blue reduction, respectively after covariate adjustment. This reduction is calculated as follows: 100 [1.0 - (adjust SS/unadjust SS)]. The significant ($P<0.01$) unadjusted serum level effect was negated by these reductions due to covariate adjustment. This indicates that the differences between serum treatments was due to the induced agglutination level and not the serum level.

Table XVII shows the results of the analysis of covariance of this study. The comparison of the maximum F value with the univariate values indicates that methylene blue reduction rate contributes mostly to the significant ejaculate (replicate) effect and lactic acid production rate to the significant pre-adjusted treatment (serum level) effect.

The analysis of covariance of the mole ratio data with agglutination is shown in Table XVIII. The F test indicates significant differences in the mole ratio due to ejaculates and to serum level prior to covariate adjustment. However, the serum level variance (Table XVII, row 3) was reduced by 78% when adjusted for agglutination level (Table XVIII, row 6). Again, this reduction in serum level variance indicates that the observed average mole ratio differences were a function of agglutination level.

TABLE XVII (EXPERIMENT VI). RESULTS OF MULTIVARIATE ANALYSIS OF COVARIANCE OF THE EFFECTS OF SERUM AND OF HEAD TO HEAD AGGLUTINATION ON METHYLENE BLUE REDUCTION RATE (MeBR) AND LACTIC ACID PRODUCTION RATE (LAPR).

Source	df	Wilks Test	Max F	MeBR F	LAPR F
Total	15				
Ejaculate	7	0.007**	12.12	11.75†	6.70
Treatment	1	0.101**	62.20	9.15*	61.86†**
Treatment, adj. for HHAgg	1	0.522ns	5.50	0.02	5.09

*P<0.025. **P<0.01.

†The dependent parameter which contributes most to the significance of the Wilks Test.

TABLE XVII (EXPERIMENT VI). ANALYSIS OF COVARIANCE OF THE EFFECT OF SERUM AND HEAD TO HEAD AGGLUTINATION (HHAgg) ON THE MOLE RATIO OF BOVINE SPERMATOZOA.

Source	df	Mean Squares	F Ratio
Total	15		
Ejaculate	7	0.85	3.36*
Serum level	1	2.50	9.90**
Error	7	0.25	
Error adjust for HHAgg	6	0.29	
Serum level adjust for HHAgg	1	0.55	1.93ns

*P<0.05; **P<0.01; ns = nonsignificant (P>0.05).

DISCUSSION

Colloidal Aspects of Diluted Semen

The biocolloidal properties of spermatozoa and other cell suspensions have been eluded to and associated with physiological phenomenon by several authors. Bedford (7) mentioned the Verwery-Overbeek theory of lyophobic colloidal stability as a possible explanation of "auto-agglutination" in rabbit spermatozoa as they traverse the epididymis. Riddick (29) makes reference to a number of biocolloids, including milk and blood, in his treatise on zeta potential control of colloidal stability. However, he did not attempt to quantitate the amount, but only the occurrence, of flocculation in conjunction with his zeta potential measurements on these colloid systems. Sachtleben (44) measured the electrophoretic mobilities and amount of agglutination of B-type human erythrocytes suspended in graded dilutions of incompatible A-type serum. His zeta potential values, calculated from his electrophoretic mobilities, decreased from -14 to -9 mV and agglutination increased from zero to 4 with increasing serum concentrations.

Several investigators have measured electrophoretic mobility of spermatozoa from various species. If one takes the liberty of calculating the zeta potentials from these electrophoretic measurements (using the von Smoluchowski equation), the values generated, in millivolts, are: ram, -13.9, (6); rabbit, -11.0, (7); rabbit, cock, boar, and human, -14.9, -20.6, -6.6, and -18.2, respectively, (5); bull,

-18.3, (4) and -17.4 (5). Control zeta potentials of -15.5 mV (Table III) and -13.9 mV (Table VI) in the present study are in reasonable agreement with those of Nevo (4) and Bey (5) for the bull. A major source of variation in electrophoretic mobility and therefore zeta potential among reported studies is the composition of the cellular ionic environment. The cells were washed and diluted in some studies and they were simply diluted in others; however, the buffer compositions were quite variable, thus influencing their ionic strength.

In the present study, the spermatozoa were never washed free of seminal plasma. Semen was diluted with egg yolk-sodium citrate diluent except when the experimental treatments dictated specific changes in cellular environment; e.g., Experiments IV and VI. The rationale for these restrictions was to maintain, as close as possible, spermatozoal environments conducive for maintenance of their metabolic, structural and functional life as developed for artificial insemination.

The data presented from Experiment I (Table III) show that, by increasing serum concentration, spermatozoal zeta potential is reduced with a concomitant increase in head to head agglutination. The data from Experiment IV (Table X) further support the hypothesis of zeta potential involvement in the serum-induced head to head agglutination phenomenon. Thus, these data in conjunction with those of Sachtleben (44) and Riddick (29) illustrate the zeta potential control of biocolloidal stability.

The results of Experiment IV (Table X) also illustrate that spermatozoal zeta potential can be significantly influenced by environmental counterion concentration and valence (buffer ionic

strength) similar to the zeta potential of non-biological colloids (29). Also shown in these data is an example of the empirical Schulze-Hardy rule (cf. p. 3). The reduction in the sperm zeta potential by the divalent cation Mg^{++} (0.18 μ treatment) is about twice that of the monovalent cation Na^{+} (0.09 μ treatment) from the basal level of the 0.045 μ treatment. These cations were isomolar in concentration. A preliminary study using lanthanum chloride, a trivalent cation (La^{+++}), in low concentrations (10^{-5}M) caused extensive agglutination of diluted semen. This La^{+++} treatment resulted in not only clumping of the cells but also precipitation of the particulate seminal plasma and egg yolk components as well. This apparently highly effective counterion was not used further because of the incompatibility with the maintenance of a viable sample after agglutination was induced.

Shaw (35) states, "The exact value of the flocculation concentration depends upon the criterion which is set for judging whether the sol is flocculated or not, ---". Riddick (29) has shown the critical coagulation concentration (CCC) for various inert and biological systems under a number of conditions. Mysel (1) described the CCC as the counterion concentration at which the repulsive and attractive forces are balanced and a small change in concentration can change the repulsive force enough to result in a dramatic shift in the state of dispersion. Thus, the critical point calculated from the total system response ($L_{4.3}$, Figure 5) is analogous to the CCC characteristic of colloid systems, because at this percent serum, zeta potential and head to head agglutination are balanced.

Riddick (29) has shown the effect of increasing colloid

concentration on the critical coagulation concentration. His data illustrate that as the number of particles are increased in a system, a higher concentration of counterion is required. Saacke et al. (45) reported a decrease in head to head agglutination at constant serum level (10% [v/v]) when sperm concentration was increased from 10 to 160 x 10^6 cells/ml. The data from Experiment VI also illustrate this characteristic. The sperm concentration, which was 80 times that in Experiment I, required a fourfold increase in serum concentration (5 vs 20%) to bring about a comparable amount of agglutination, 59.3 vs 60.4%, respectively.

Abramson et al. (46) indicated that for particles the size of cells suspended in electrolyte solutions, the zeta potential depends on the cell surface, not on their size or shape. It was thought that, by increasing the particle size through agglutination, the increased mass would be affected electrophoretically by greater viscous drag and thereby decrease the calculated zeta potential. The particle size aspect of Experiment III addressed this problem. The zeta potentials of different size aggregates were different (Table VIII). However, there was not significant relationship found when aggregate zeta potential was expressed as a function of size. Thus, it seems that increased viscous drag had no effect on zeta potential estimation, but that the particle with the greater charge density or quantity moved at a greater velocity, a result of the higher potential.

Padma (26) isolated the normal rabbit serum spermagglutinin and identified it as gamma globulin, specifically IgG. However, the procedures used by this author concentrated serum IgG to approximately

50% (v/v) in his agglutination test using 5×10^8 cells/ml. Boettcher et al. (27) identified the spermagglutinin in human sera as a beta globulin (IgM) in one instance and a gamma globulin in another. They used extremely high serum levels in their agglutination test. As noted in the literature survey, others have observed spermatozoal agglutination in the presence of specific inorganic salts and various other media as well.

The agglutination in response to ionic strength variation (Experiment IV, Table X) indicates that this phenomenon is not due simply to an inorganic mono- or divalent component of serum. Although agglutination did increase with increasing buffer ionic strength, the increase was not as dramatic when compared to the difference in agglutination between serum levels.

The net protein charge is influenced by the position of the protein isoelectric point (pI) in reference to the pH of the medium. Gamma-2 globulin (IgG) is the only serum globulin with a pI (7.8, White, Handler and Smith [47]), above physiological pH, and at that pH it is net positively charged. Napper (28) indicated that charged macromolecules could flocculate electrostatically stabilized particles by adsorbing to the particle surface and thus reducing the surface charge density through charge neutralization. Thus, IgG could act as a polyvalent cation, which in addition to the already present cations in solution, would be required in small amounts to cause zeta potential reduction and flocculation (head to head agglutination).

The role of IgG as a polyvalent cation would be enhanced if it was adsorbed on selective areas of the cell surface. The possibility of

this phenomenon is evidenced from the works of several authors. Cooper and Bedford (9) and Yanagimachi et al. (10) have demonstrated by colloidal iron binding techniques that the spermatozoal surface charge of various species including the bull was asymmetrically distributed which changes with epididymal maturation. Yanagimachi et al. (10) also indicates that the binding of colloidal iron is generally more dense in the tail regions of the spermatozoa than in the head. He also noted that, while the charge density is rather uniform within a particular anatomical segment, there were abrupt changes in charge density at the morphological junctions between segments. Nevo et al. (4) demonstrated that severed bull sperm heads had the slowest electrophoretic mobility (lowest zeta potential) when compared to intact cells and severed tails. Fawcett (11) has shown that the cell membrane overlying the acrosomal region of rat and guinea pig spermatozoa displayed highly ordered plaque-like areas, whereas the post acrosomal and neck regions did not. The association of these plaque-like areas with the source of the surface charge was not ascertained, however, it seems reasonable to assume that the surface charge density is not homogeneous over the entire cell membrane. Nicolson and Yanagimachi (48) showed by specific agglutination that sialic acid residues are the major source of the surface charge of rabbit and hamster spermatozoa. Bey (5) showed a reduction in electrophoretic mobility and a simultaneous increase in free neuraminic acid after incubation of bull spermatozoa with neuraminidase for three hours.

Experiment II revealed that the spermatozoal zeta potential of dead cells (destroyed acrosomal caps), was less than that of live cells

(possessing acrosomal caps). Although acrosomal integrity was not measured in Experiment III, the killed treatment resulted in a significant reduction in zeta potential, when compared to the live treatment. The data from Experiment IV indicated that serum treatment was a effective in reducing zeta potential as was buffer ionic strength, yet serum was much more effective in inducing head to head agglutination. Therefore, with these and previous data, it seems feasible to describe serum-induced head to head agglutination as resulting from a specific differential reduction of the already asymmetrical zeta potential of the sperm head.

Cellular Viability and Its Association to Semen Colloidal Characteristics

Senger and Saacke (21) and O'Connor (31) have both noted sustenance of diluted spermatozoal motility associated with the agglutinated state following a 9 hour - 37° C incubation. Their agglutination was a result of exposing diluted semen to 10% (v/v) homologous serum. In Experiment II, there was a significant reduction in spermatozoal zeta potential (Table VI) when the cells were intentionally killed and had no intact acrosomal membranes. The data from Experiment IV revealed the interdependency of zeta potential, agglutination and intact acrosomes in that, as the serum level was increased (Table X), the zeta potential was sufficiently reduced to allow a substantial increase in agglutination. This serum effect was seen in conjunction with a significant improvement of acrosomal integrity at 10% serum level over that seen at 0.2%. However, in terms of the ionic strength effect (Experiment IV, Table X), the relationship between zeta potential and its effect on

agglutination was confounded by the determential effect of ionic strength on the integrity of the cell membrane (intact acrosomes).

The Metabolic Consequence of the Dispersion State of the Colloid

Previous investigators have shown an increased oxygen consumption and lactic acid production when spermatozoa were treated with serum or other biological fluids (cf. Literature Review). Many of these authors have also noted that these treatments caused spermatozoal agglutination. Senger (20) suggested that the agglutination could be a conserving process since he observed a maintenance of motility and acrosomal integrity when semen was exposed to certain serum samples which had greater agglutinating quality than others.

The Experiment V data indicate that those cells, which were treated with 20% serum and greater than 50% agglutinated, consumed more oxygen on the average than non-serum treated (non-agglutinated) cells. The initial consumption rate of the serum-treated cells was greater than that of the controls. However, the consumption rate of the agglutinated cells was less than that of the controls as incubation time increased. This is indicated by the siginificant ($P<0.05$) quadratic response of the serum-treated cells with time versus the non-significant quadratic response of the controls (Table XIV). Thus, serum-treatment of spermatozoa reduced their oxygen requirement over an extended period, which appeared to be associated with the agglutination phenomenon.

As indicated by the results of Experiment VI, spermatozoa treated with 20% serum reduced methylene blue and produced lactic acid faster and thus had a greater combined response (L_i, Table XV) than did cells

treated with 10% serum. However, it was seen that, after adjustment to a common level of agglutination, the differences across both parameters and their combined response due to serum level, was not significant. Thus, the initial metabolic differences merely reflected differences due to the level of agglutination and not to the serum levels, per se. The metabolic response of the cells to agglutination was mediated more through the changes in lactic acid production rate than through changes in methylene blue reduction rate.

The aerobic metabolic rate (MeBR) was greater than the anaerobic rate (LAPR) in the lesser agglutinated samples as evidenced by the mole ratio values in Table XVI. However, as the level of agglutination increased the opposite was true. These observations are supported by the conversion of oxygen consumption and lactic acid production data of previous workers to rates and forming mole ratios. Olds and Van Demark (18) indicated the highest degree of agglutination resulted from the treatment of the cells with follicular fluid, the mole ratio was 1.35 compared to 2.71 for the saline control samples. Mittal et al. (30) noted a pronounced agglutination of bovine spermatozoa treated with 33% normal rabbit serum. Their mole ratio was 1.21 versus that of 1.45 for the non-agglutinated saline controls.

Thus, as the incidence of head to head agglutination increased, the spermatozoa increased their overall metabolic rate and anaerobic metabolism is favored over aerobic. This increase in lactic acid production may provide the cells with an easily assessible metabolite for the sustenance of motility through later energy production or for the synthesis of phospholipids to maintain the integrity of their

membranes. The feasibility of phospholipid synthesis is demonstrated by Terner and Korsh (49) where they showed that bull spermatozoa incorporated Carbon-14 from labeled glucose into the glycerol moiety of phospholipids. It seems noteworthy that the predicted level of agglutination at the critical point of the system (4.3% serum, Figure 5) was virtually the same as that which was predicted when the change in preferred metabolic route occurred (post adjusted HHAgg, Table XV).

In conclusion, diluted bovine semen has been shown to have colloidal characteristics which are governed by the cellular environment and the integrity of cell membranes. The introduction of blood serum into the spermatozoal environment significantly reduced the spermatozoal zeta potential without adversely affecting cell viability. This zeta potential reduction has been shown to significantly influence the level of head to head sperm agglutination and that this biocolloidal system has an analogue to the critical coagulation concentration. It has also been demonstrated that live spermatozoa had greater zeta potentials than did dead cells,and the size of the cell aggregates did not influence their estimated potentials.

The spermatozoal zeta potential has been shown to respond to buffer ionic strength in accordance with the Schulze-Hardy rule, yet head to head agglutination was relatively unaffected. However, cell viability was dramatically reduced under these conditions.

Metabolic measurements have indicated that the occurrence of head to head agglutination is related to an overall increase in metabolic rate, which is mostly due to a greater increase in anaerobic metabolic rate than to the increase in aerobiosis.

REFERENCES

1. Mysel, K. J., "Introduction to Colloid Chemistry", Interscience Publ. Co., New York, 1959.

2. Overbeek, J. Th. G., in "Advances in Colloid Science", (H. Mark and E. J. W. Verwey, Eds.), Vol. 3, p. 97, Interscience Publ. Co., New York, 1950.

3. Williams, V. R., and Williams, H. B., "Basic Physical Chemistry for the Life Sciences", Freeman and Co., San Francisco, 1967.

4. Nevo, A. C., Michaeli, I., and Schindler, H., Exp. Cell Res. 23, 69 (1961).

5. Bey, E., in "Cell Electrophoresis", (E. J. Ambrose, Ed.), p. 142, Little. Brown and Co., Boston, 1965.

6. Bangham, A. D., Proc. Roy. Soc. London, Ser. B. 155, 292 (1961).

7. Bedford, J. M., Nature, 200, 1178 (1963).

8. Vaidya, R. A., Glass, R. H., Dandekar, P., and Johnson, K., J. Reprod. Fert. 24, 299 (1971).

9. Cooper, G. W., and Bedford, J. M., Anat. Rec. 169, 300 (1971).

10. Yanagimachi, R., Noda, Y. D., Fujimoto, M., and Nicolson, G. L., Amer. J. Anat. 135, 497 (1972).

11. Fawcett, D. W., Devel. Biol. 44, 394 (1975).

12. Blackshaw, A. W., J. Physiol. 120, 465 (1953).

13. Blackshaw, A. W., J. Gen. Physiol. 36, 449 (1953).

14. Wales, R. G., and White, I. G., Aust. J. Biol. Sci. 11, 589 (1958).

15. Edwards, R. G., J. Reprod. Fert. 1, 268 (1960).

16. Moyer, R. F., and Almquist, J. O., J. Dairy Sci. 45, 383 (1962).

17. Lindahl, P. E., Exp. Cell Res. 81, 413 (1973).

18. Olds, D., and Van Demark, N. L., Amer. J. Vet. Res. 18, 603 (1957).

19. Lindahl, P. E., Int. J. Fert. 11, 297 (1966).

20. Senger, P. L., Ph.D. Thesis, Virginia Polytechnic Institute and State University, 1974.

21. Senger, P. L., and Saacke, R. G., J. Reprod. Fert. 47, 215 (1976).

22. Yanagimachi, R., Biol. Reprod. 3, 147 (1970).

23. Padma, M. C., J. Reprod. Fert. 31, 119 (1972).

24. Beck, J. S., Edwards, R. G., and Young, M. R., J. Reprod. Fert. 4, 103 (1962).

25. Johnson, M. H., J. Reprod. Fert. 16, 503 (1968).

26. Padma, M. C., Nature, New Biol. 238, 25 (1972).

27. Boettcher, B., Kay, D. J., Rumke, P., and Wright, L. E., Biol. Reprod. 5, 236 (1971).

28. Napper, D. H., Ind. Eng. Chem. Prod. Res. Develop. 9, 467 (1970).

29. Riddick, T. M., "Control of Colloid Stability through Zeta Potential", Livingston Publ. Co., Wynnewood, Pa., 1968.

30. Mittal, K. K., Salisbury, G. W., Graves, C. N., and Rasmaslu, B. A., J. Reprod. Fert. 10, 29 (1965).

31. O'Connor, M. L., M. S. Thesis, Virginia Polytechnic Institute and State University, 1974.

32. Matousek, J., J. Reprod. Fert. 8, 13 (1964).

33. Peterson, H. P., Amer. J. Vet. Res. 26, 873 (1965).

34. Fuhrmann, G. F., and Ruhenstroth-Bauer, G., in "Cell Electrophoresis", (E. J. Ambrose, Ed.), p. 22, Little, Brown and Co., Boston, 1965.

35. Shaw, D. J., "Introduction to Colloid and Surface Chemistry", Butterworth and Co., London, 1970.

36. Saacke, R. G., and White, J. M., Proc. Fourth Tech. Conf. NAAB, 1972.

37. Beck, G. H., and Salisbury, G. W., J. Dairy Sci. 26, 483 (1943).

38. Gutmann, I., and Wahlefeld, A. W., in "Methods of Enzymatic Analysis", (H. U. Bergmeyer, Ed.), p. 1464, Acad. Press, New York, 1974.

39. Steel, R. G. D., and Torrie, J. H., "Principles and Procedures of Statistics", McGraw-Hill Book Co., New York, 1960.

40. Snedecor, G. W., and Cochran, W. G., "Statistical Methods", The Iowa State University Press, Ames, Iowa, 1967.

41. Kramer, C. Y., "A First Course in Methods of Multivariate Analysis", Edwards Bros., Ann Arbor, Michigan, 1972.

42. Kramer, C. Y., Biometrics. 12, 307 (1956).

43. Kramer, C. Y., J. Qual. Tech. (In Press).

44. Sachtleben, P., in "Cell Electrophoresis", (E. J. Ambrose, Ed.), p. 100, Little, Brown and Co., Boston, 1965.

45. Saacke, R. G., O'Connor, M. L., and Chandler, J. E., J. Anim. Sci. 43, 302 (1976).

46. Abramson, H. A., Moyer, L. S., and Gorin, M. H., "Electrophoresis of Proteins and the Chemistry of Cell Surfaces", Reinhold, New York, 1942.

47. White, A., Handler, P., and Smith, E. L., "Principle of Biochemistry", 4th Ed., p. 711, McGraw-Hill Book Co., New York, 1968.

48. Nicolson, G. L., and Yanagimachi, R., Science 177, 276 (1972).

49. Terner, C., and Korsh, G., Biochem. 1, 367 (1962).

APPENDIX

APPENDIX TABLE I. MULTIVARIATE ANALYSIS OF VARIANCE OF THE EFFECTS OF GRADED LEVELS OF SERUM ON ZETA POTENTIAL (ZP), HEAD TO HEAD AGGLUTINATION (HHAgg), ACROSOMAL INTEGRITY (IA) AND MOTILITY OF DILUTED BOVINE SEMEN.

		SUMS OF SQUARES AND CROSS PRODUCTS										
Source	df	ZP^2	ZP* HHAgg	ZP*IA	ZP*MOT	$HHAgg^2$	HHAgg* IA	HHAgg* MOT	IA^2	IA*MOT	MOT^2	U-Test
Ejaculate	3	295.15	4.26	2.91	1.08	0.11	0.02	0.03	0.06	0.04	0.07	0.036**
Treatment	8	70.80	-12.13	-0.49	-1.84	4.31	0.11	0.37	0.03	0.03	0.08	0.008**
Linear Regression	1	35.34	-11.95	-0.36	-1.20	4.04	0.12	0.41	0.00	0.01	0.04	0.035**
Quadratic Regression	1	0.95	-0.46	0.07	0.08	0.22	-0.03	-0.04	0.01	0.01	0.01	0.310**
Lack of Fit	6	34.51	0.28	-0.06	-0.56	0.05	0.02	0.01	0.02	0.01	0.03	0.256ns
Error	24	56.23	0.10	0.25	0.06	0.16	0.02	0.04	0.03	0.01	0.17	
Total	35	422.18	-7.77	2.67	-0.71	4.59	0.15	0.45	0.11	0.08	0.32	

**P<0.01.

ns = nonsignificant (P>0.05).

APPENDIX TABLE II. MULTIVARIATE ANALYSIS OF VARIANCE OF THE EFFECT OF SERUM AND IONIC STRENGTH ON ZETA POTENTIAL (ZP), HEAD TO HEAD AGGLUTINATION (HHAgg) AND ACROSOMAL INTEGRITY (IA).

		SUMS OF SQUARES AND CROSS PRODUCTS						
Source	df	ZP^2	ZP*HHAgg	ZP*IA	$HHAgg^2$	HHAgg*IA	IA^2	U-Test
Total	39	270.22	-12.66	-6.84	3.04	0.87	0.77	
Replicate	4	16.63	-00.07	-0.66	0.25	0.09	0.16	0.39**
Treatment	7							
Serum	1	66.95	-11.71	-3.92	2.05	0.68	0.23	0.17**
Ionic strength	3	61.65	-00.79	-2.68	0.04	0.05	0.12	0.42**
Polynomial regression	2	51.26	-00.34	-2.07	0.02	0.02	0.09	0.49**
Lack of fit	1	10.40	-00.45	-0.62	0.02	0.03	0.04	0.76ns
Serum*Ionic strength	3	21.06	-00.53	-0.36	0.05	0.01	0.01	0.72ns
Error	28	103.93	00.45	0.78	0.66	0.04	0.25	

**P<0.01.

ns = nonsignificant (P>0.05).

APPENDIX TABLE III. MULTIVARIATE ANALYSIS OF COVARIANCE OF THE EFFECTS OF SERUM AND HEAD TO HEAD AGGLUTINATION (HHAgg) ON METHYLENE BLUE REDUCTION RATE (MeBR) AND LACTIC ACID PRODUCTION RATE (LAPR) OF DILUTED BOVINE SPERMATOZOA.

		Sums of Squares and Cross Products			
Source	df	$LAPR^2$	LAPR*MeBR	$MeBR^2$	U-Test
Total	15	14.18	3.91	2.72	
Ejaculate	7	7.92	2.67	2.32	0.007**
Serum level	1	5.63	1.14	0.23	0.101**
Error	7	0.64	0.11	0.18	
Adjusted error	6	0.59	0.06	0.13	
Serum level adjusted for HHAgg	1	0.50	-0.02	0.00+	0.522ns

**P<0.01.

ns = nonsignificant (P>0.05).

VITA

Name: John Edward Chandler

Birth: February 26, 1941, Knoxville, Tennessee

High School Education: Knoxville Catholic High School

College Education: St. Bernard College, St. Bernard, Alabama, 1959-1964, A.B. in Biology

University of Tennessee, Knoxville, Tennessee, 1966-1969, M.S. in Animal Husbandry-Reproductive Physiology

Experience: Southern Research Institute, Birmingham, Alabama, 1964-1966, Cancer Chemotherapy

UT-AEC Comparative Animal Research Laboratory, Oak Ridge, Tennessee, 1967-1969, Reproductive Physiology

Smith Kline Corporation, West Chester, Pennsylvania, 1969-1973, Nutritional Biochemistry

Publications:

J. E. Chandler and K. M. Barth. 1967. Effect of cutting frequency on total digestible nutrients of Buffalo Alfalfa. Tennessee Farm and Home Science. July.

J. E. Chandler and B. H. Erickson. 1969. Effect of dose and dose-rate (γ-radiation) on follicular development and reproductive capacity in the prepuberal rat. (Abst.). Radiation Research. May.

J. E. Chandler, W. V. Chalupa, and R. E. Brown. 1972. Methionine requirements for growing lambs fed a natural diet. (Abst.). J. Anim. Sci. 35:262.

J. E. Chandler, M. L. O'Connor, and R. G. Saacke. 1976. Metabolism of serum agglutinated spermatozoa. (Abst.). J. Anim. Sci. 43:278.

Professional Organizations: American Society of Animal Science
Who's Who in the East

Signed John E Chandler

THE NATURE OF SERUM AGGLUTINATION OF BOVINE SPERMATOZOA: A PROPOSED MODE OF ACTION AND ITS METABOLIC EFFECTS

by

John E. Chandler

(ABSTRACT)

A series of studies were designed to determine if colloidal properties are involved in serum-induced head to head agglutination (HHAgg) and the effect of HHAgg on sperm metabolism. Zero to 8% (v/v) serum was added to diluted bull semen and the zeta potential (ZP), HHAgg, motility (MOT) and intact acrosomes (IA) of sperm were measured. ZP was determined by microelectrophoretic methods. Percent HHAgg and % IA were determined from wet smears using differential interference contrast optics and motility, using phase contrast optics. With increasing serum levels, a significant ($P<0.01$) linear ZP decrease and quadratic % HHAgg increase occurred, while MOT and IA were relatively unaffected. As serum was raised from 0 to 8%, ZP decreased from -15.5 to -12.9 mV., HHAgg increased from 0 to 84%, and MOT and IA averaged 43.6 and 79.3%, respectively. In a second experiment, semen was exposed to two serum levels (0.2 and 10% [v/v]) each at four ionic strengths. ZP, HHAgg and IA were measured. Again, increasing serum levels depressed ZP and increased HHAgg and IA. Increasing ionic strength levels depressed ZP from -13.5 to -10.5 mV and increased IA from 49.5 to 642.%, yet did not significantly affect HHAgg. IA was greatest at the higher serum-ionic strength levels. The negative mean HHAgg regression on mean ZP was

significant ($P<0.05$) for both studies. Additional studies showed that live sperm had significantly ($P<0.05$) greater ZP values than dead sperm (-15.1 vs -13.2 mV) and that the size of cell aggregates did not cause a deviation from electrokinetic theory.

Oxygen consumption of serum-treated bovine spermatozoa was measured in a Warburg apparatus. Ten % egg yolk, 90%-2.9% sodium citrate diluted semen (2×10^8 cells/ml) with and without 20% (v/v) complement-fixed serum was compared in three replicates. Fructose and antibiotics were added to all flasks. Serum-induced HHAgg was greater than 50% in each replicate. Serum-treated sperm consumed oxygen at a significantly ($P<0.05$) higher rate than controls (23.0 vs 19.8 nmoles/10^8 cells/min) in a 2-hr period. In a second experiment, methylene blue reduction rate (MeBR), lactic acid production rate (LAPR) and % HHAgg were measured on eight ejaculates which were split and treated with 10 or 20% (v/v) complement-fixed serum. Twenty % serum significantly ($P<0.01$) increased MeBR LAPR and HHAgg above those of 10% serum (MeBR, 21.8 vs 19.4 nmoles/min; LAPR, 25.1 vs 13.3 nmoles/min; and HHAgg, 60.6 vs 28.2%, respectively). After covariate adjustment of the MeBR and LAPR responses for level of HHAgg, differences in MeBR and LAPR due to serum level were no longer significant. The mole ratio, an indicator of preferred metabolic route formed from MeBR and LAPR, showed that an increase in anaerobiosis was more closely related to an increase in HHAgg than to an increase in the level of serum. Thus, serum-induced HHAgg is indeed partly explained by spermatozoal ZP manipulation and that HHAgg is closely related to a transition from aerobic to anaerobic metabolism.

In conclusion, diluted bovine semen has been shown to exhibit characteristics common to colloidal systems. These are: 1) the introduction of blood serum into the spermatozoal environment reduced the sperm ZP and increased % HHAgg without affecting cell viability, 2) the magnitude of the sperm ZP was significantly related to % HHAgg and the system had a critical point at a serum level which was analogous to the critical coagulation concentration of colloidal systems, 3) increasing buffer ionic strength reduced sperm ZP according to the Schulze-Hardy rule, yet had little influence on % HHAgg. There was a significant increase in cell viability with increasing ionic strength. Live sperm were shown to have a greater ZP than did dead cells and the size of the cell aggregates did not influence the ZP estimates. Percent HHAgg was shown to be related to an overall metabolic rate increase which was the result of greater anaerobiosis than aerobiosis.