BIOPHYSICAL STUDIES OF SPERMATOZOA

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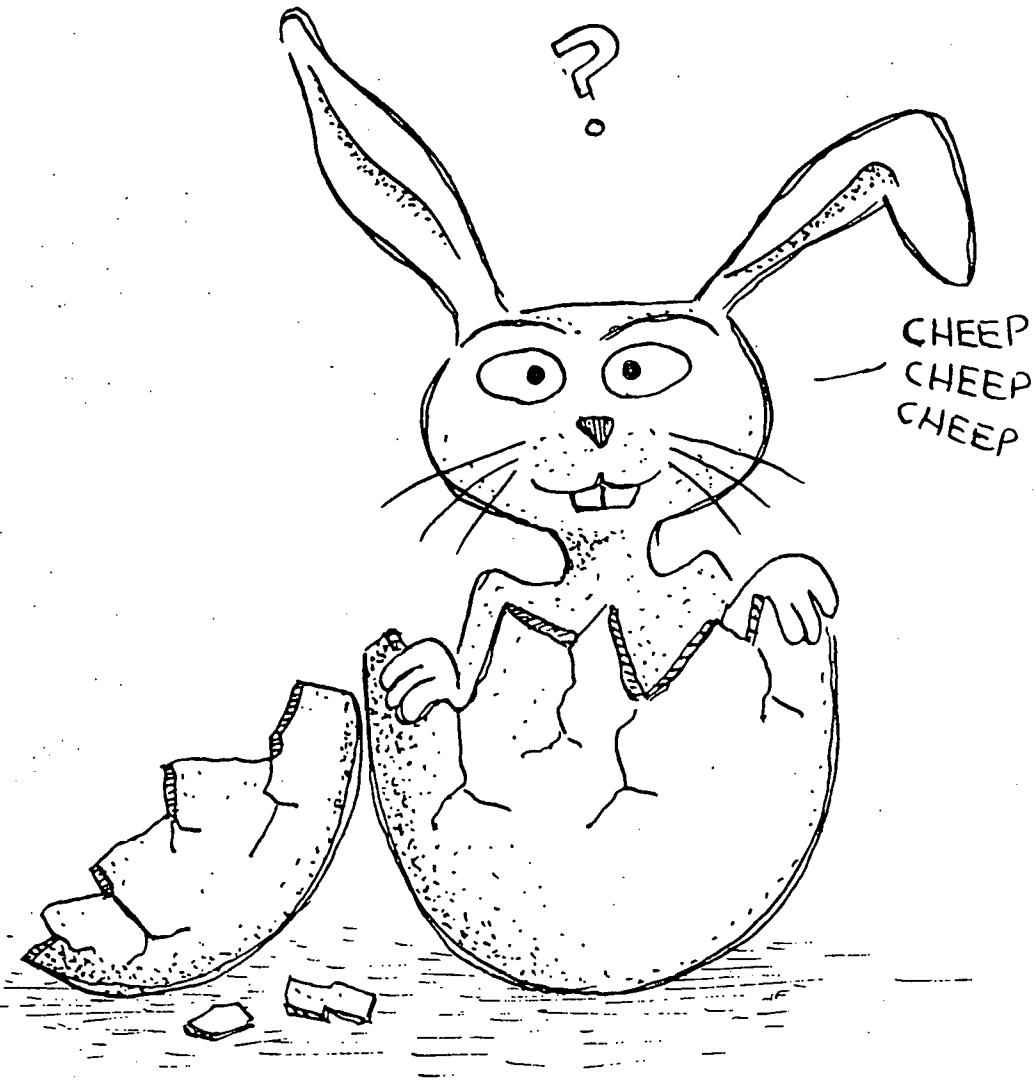
I guess I should have kept a record of which sperm sample was which.

BIOPHYSICAL STUDIES

OF SPERMATOZOA

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ABSTRACT

The objectives of this thesis include characterization of sperm-
atozoa according to several physical properties (morphology, size,
electrophoretic mobility, sedimentation rate and specific gravity),
correlation of these properties with several biological properties
(viability, intrinsic motility, fertilizing capacity, antigenicity and
genetic composition) and an evaluation of interrelationships among
these properties and with selected experimental variables. The major
effort was devoted to stable-flow free-boundary (Staflo) sedimentation
and electrophoresis studies and the use of the intrinsic motility of
the spermatozoa to achieve relative or absolute enrichment of sperm
sub-populations. A special microscopic optical system was developed
to facilitate these studies.

Analytical (theoretical) methods were developed to clarify the
requirements for optimum fractionation of cell populations and to
allow separate determination of two or more of the properties -
sedimentation rate, electrophoretic mobility and intrinsic motility -
when they may all contribute simultaneously to a given migration
process.

Temperatures of $3^{\circ}C$ or lower were found necessary to eliminate
the effects of intrinsic motility. At $3^{\circ}C$ in low density-low viscosity
buffers, the best measured values of physical properties (normalized
to conditions of 1% sucrose) are as follows: Average sedimentation
rate, rabbit: 0.53×10^{-4} cm/sec/cm/sec^{2}. Average electrophoretic
mobility, rabbit: $0.85 \pm 0.10 \times 10^{-4}$ cm/sec/V/cm; fowl: $1.65 \pm
0.10 \times 10^{-4}$ cm/sec/V/cm. At these low temperatures no significant
differences were found among sedimentation or electrophoresis collection

fractions with respect to morphology, motility or viability of sperm-atozoa.

Above $4^{\circ}C$, the relative contribution of intrinsic motility became significant in both sedimentation and electrophoretic studies, and depended upon both the temperature and the initial quality of the ejaculate. The relative (fowl) and absolute (rabbit) enrichment of sperm subpopulations with regard to motility, viability and fertilizing capacity achieved above $4^{\circ}C$ was attributed to the intrinsic motility vector.

A separate study was made on the quality of rabbit ejaculates. This revealed an apparent differential sensitivity of cells in various stages of spermatogenesis to elevated environmental temperatures, with some deleterious effects seen as long as 7-8 weeks after particularly hot days.

In equilibrium density gradient centrifugation experiments using sodium iothalamate gradients, rabbit sperm formed three or more bands ranging from specific gravity 1.19 to 1.37. Absolute enrichment was obtained in the low density band (1.19-1.21) with regard to both motility and viability. Fowl spermatozoa formed a single band ranging from specific gravity 1.19 to 1.21, within which there was a differen-tial distribution of motile sperm (greatest motility at low density border).

In the limited fertility trials, the "low resolution" fractionation of rabbit ejaculates did not give a clear separation of X- and Y-chromosome-bearing spermatozoa. Perhaps more significant, however, the physical properties measured in these experiments are inconsistent with the explanations given in the literature for the partial separation

of rabbit and bull spermatozoa using electrophoretic and sedimentation methods. The present work does show that high resolution experiments are feasible and would give a much better test of the ability to separate X- and Y-sperm according to one or more of the properties enumerated above.

I. INTRODUCTION

Although man's interest in spermatozoa as cells dates from the
initial report by Hamm and Leeuwenhoek in 1677, his interest in the
processes controlling the sex of offspring far precedes their discovery.
Gordon[92] presents an excellent review of the literature of folklore on
the subject. That the folklore is not just ancient history was evident
from the Ann Landers column of the June 17, 1969 Berkeley (California)
Gazette in which a reader recommended a method used successfully to
control the sex of her children - "sleeping on the left side as soon as
pregnancy is suspected yields a boy and vice versa."

The possibility of sex control has generated interest among
sociologists, scientists and others concerned with genetics, animal
husbandry and reproductive biology in general. Etzioni, in an article
entitled "Sex, Science and Society", suggests that sex control is only
one of many social problems arising from scientific investigation that
should be of concern to everyone[253]. More recently in Life magazine,
Rosenfeld focused on the subject[272]. For the more interested reader,
Rorvik and Shettles have recently published a book presenting their
ideas on sex control[274]. An important obvious consequence of the ability
to control sex would be the possibility of eliminating sex-linked gen-
etic disorders such as color blindness, diabetes insipidus, and hemophilia
A and B from the population. A simple means of selecting the sex of
offspring might also encourage the voluntary reduction of family size
thereby assisting in the limitation of population growth. Many of the
widespread social concerns related to the latter problem are summarized
by Kiefer[255].

Spermatozoa per se are of great scientific interest. Reasons

include their dramatic morphological changes during spermatogenesis, their property of intrinsic motility and its relationship to fertilization and their significance in genetics.

The purpose of this project was to study the physical properties of spermatozoa, and their relationships to biological features, including possible sex control, utilizing some newer experimental methods of characterizing and fractionating cell populations. The stable-flow free-boundary (Staflo) method proved particularly useful in this regard. The rabbit was selected as the donor of mammalian spermatozoa for reasons summarized in Appendix A. For comparison, fowl spermatozoa were later added to the study.

Despite the reputation of rabbits for fecundity, a persistent and perplexing problem encountered was the variable initial quality and durability of ejaculate spermatozoa. Attempts to understand the dependence of this initial quality of spermatozoa on environmental variables, and to interpret the subsequent deterioration of spermatozoa in terms of experimental factors, became an important part of the study and illustrate many of the difficulties encountered in work with spermatozoa. It was eventually possible to develop conditions for media and manipulative procedures such that the "ultimate" biological assay system (obtaining offspring) worked with a high degree of reliability, provided that the initial spermatozoa sample was of high quality.

Organization of Chapters

Chapter II reviews the physical and biological properties of spermatozoa and the sex control literature, and discusses the

interrelationships among these properties and selected experimental variables.

Chapter III describes the basic Staflo method and additional ancillary equipment developed during this study. It also includes a detailed analysis of Staflo migration-fractionation principles which significantly extends the limits of analytical precision for this method and for other related fractionation methods.

Chapters IV through VIII describe the evolution of the experimental work. Chapter IV presents the results of early Staflo-sedimentation experiments during which procedures for handling spermatozoa were developed. The temperature-dependent effects of motility on the sedimentation of rabbit spermatozoa are dramatically illustrated. Chapter V reports on the electrophoretic properties of rabbit and fowl spermatozoa as well as on the effects of temperature and sperm quality on electrophoretic migration. Chapter VI presents the evaluation of media for use with fowl spermatozoa and the results of electrophoresis and electrophoresis-enrichment experiments with these media. Chapter VII is devoted to equilibrium density gradient centrifugation of fowl and rabbit spermatozoa and shows both similarities and differences in the biophysical properties of the two cell types. Chapter VIII presents a series of multi-characterization experiments with rabbit spermatozoa, exploring their separate and combined sedimentation and electrophoretic properties. This work also explicitly demonstrates the importance of the initial sperm quality for eventual fertilization.

Chapter IX discusses differences in measured sperm quality according to the method used for evaluation, and also shows significant prolonged and intermittent effects of elevated environmental tempera-

tures on the initial quality of ejaculate spermatozoa.

The final <u>Chapter X</u> presents overall conclusions and recommendations derived from this study.

II. INTERRELATIONSHIPS AMONG PHYSICAL AND BIOLOGICAL PROPERTIES OF SPERMATOZOA AND IMPORTANT EXPERIMENTAL VARIABLES

Contents

A. Introduction

The great interest in studying mammalian spermatozoa undoubtedly arises from the active role of that cell type in reproduction, especially in the control of sex by virtue of the presence of either an X- or Y-chromosome in the individual spermatozoon. In addition, its property of intrinsic motility makes the spermatozoon an exciting object to study and provides a practical biological assay system.

In reviewing the literature on work with spermatozoa, it is convenient to consider the physical and biological properties separately, even though the purpose of this thesis is to integrate them. The various categories of physical and biological properties, listed in Table I, are discussed in Sections B and C, below. An early attempt to discuss, in a separate section, the interrelationships among these properties and the many experimental variables (also listed in Table I) was abandoned in favor of emphasizing the interrelationships when appropriate in Sections B and C. The importance of these interrelationships is illustrated in many of the subsequent chapters.

Appendix B contains additional information on work with germ cells including 1) the collection and handling of spermatozoa, 2) the normal fertility of the female rabbit, 3) fertilization IN VIVO and IN VITRO, 4) technical considerations in artificial insemination and 5) methods for sexing offspring.

B. Physical Properties

Physical methods used to study spermatozoa include 1) electrophoresis,

Table I. Interrelationships Among Physical and Biological Properties of
Spermatozoa and Important Experimental Variables

Physical Properties	Experimental Variables	Biological Properties
Morphology and Size		Viability
Electrophoretic Mobility		Intrinsic Motility
Sedimentation Rate	Experimental Variables	Fertilizing Capacity
Specific Gravity	Dilution Medium	Antigenicity
	Constituents, pH, ionic strength, osmolarity, viscosity, specific gravity and density gradient.	Genetic Composition
	Spermatozoa Concentration	
	Temperature	
	Time	
	External Forces	
	Light, stress, electric field, gravity, and centrifugation.	

2) sedimentation and froth flotation and 3) differential and equilibrium
density gradient centrifugation. The physical properties upon which these
techniques depend include morphology and size, electrophoretic mobility,
sedimentation rate, and specific gravity. Each of these properties is
considered separately below.

1. <u>Morphology and Size</u>.

Information on spermatogenesis and the morphology of mammalian sperm-
atozoa can be found in the works by Bishop and Walton[42], by Ortavant[183]
and by Austin[13], and on fowl spermatozoa in the works by Lorenz[140] and by
Sturkie[220]. A detailed discussion of the fine structure of the rabbit
spermatozoon is given by Bedford[25]. The discussion in this section is
restricted to considerations of certain aspects of the morphology and
size of fowl and rabbit spermatozoa primarily, and to the relationships of
these features to the physical and biological properties of spermatozoa
and experimental parameters.

<u>Rabbit Spermatozoa</u>. Figure 1 is a composite drawing of a rabbit spermato-
zoon prepared from both light and electron microscopy observations. The
overall length of the rabbit spermatozoon varies from 60-70 microns with
head and midpiece lengths of approximately 8-10 and 10 microns, respect-
ively. The head is flat with a width of 4-5 microns and a thickness of
approximately 1 micron. The average head lengths of spermatozoa from the
vas deferens and the ejaculate are about the same[22]. The breadth and area
measurements of heads of spermatozoa vary little among males of the same
litter, but vary markedly among the means of litters[22]. The weight of
the rabbit and the environment have little influence on these parameters[170].
Among males of a litter, the head length, percent capless, and percent
stained spermatozoa all vary markedly, while the variations among the litter
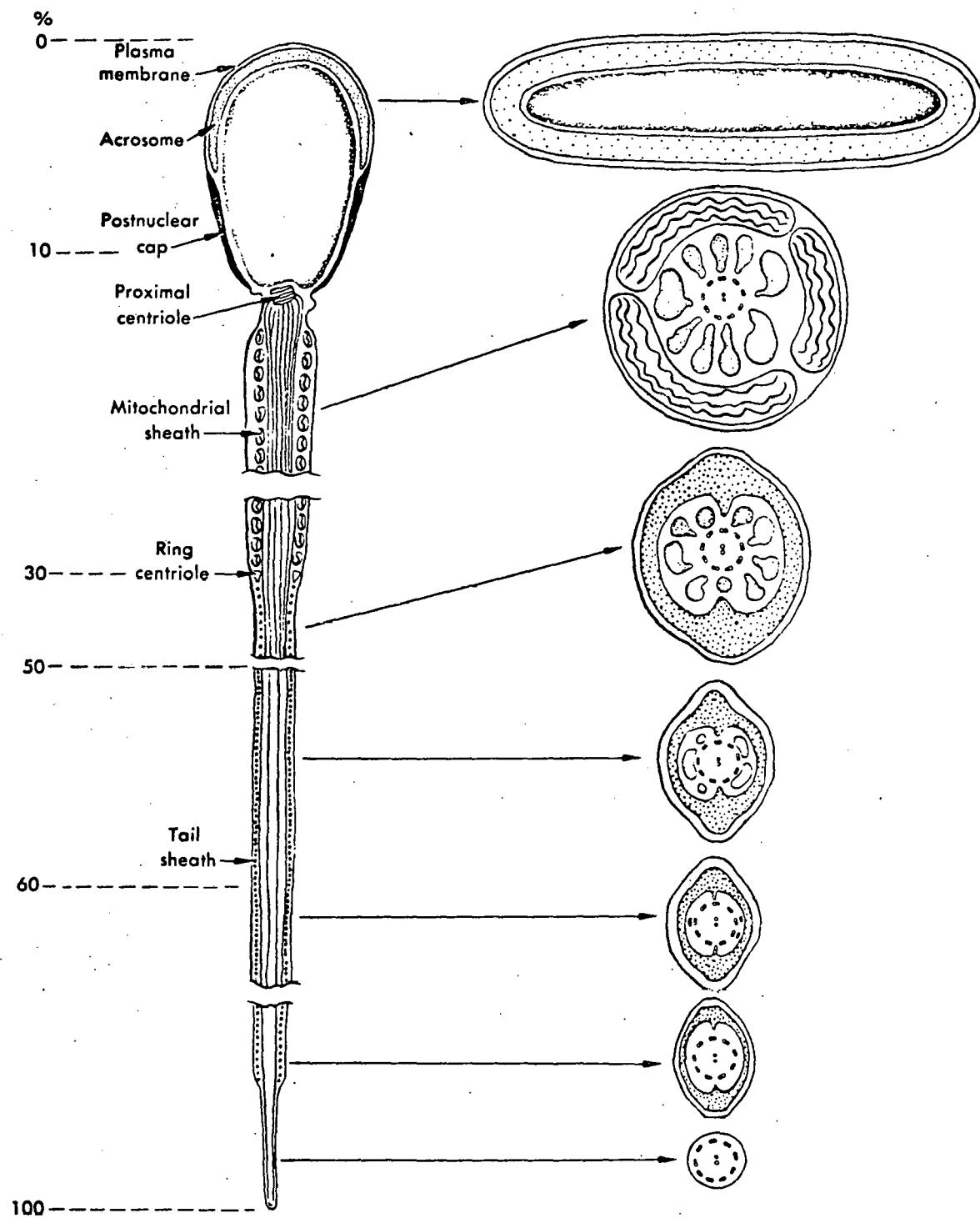
Fig. 1. Structure of the rabbit spermatozoon.

means is small[22]. There is a positive association between the head length

of normal, unstained spermatozoa and fertility[170].

Shettles[213] describes 2 populations of human spermatozoa distinguished

by elliptical and concentric configurations of light diffraction patterns

in small and large heads, respectively, under phase contrast microscopy.

He hypothesizes that the Y-chromosome is found in the smaller head and

the X-chromosome in the larger head. This observation is questioned by

Rothschild[197] and by Bishop[41].

Gordon observed head length dimorphism of the rabbit spermatozoa

populations he studied but found no correlation of the size dimorphism

with the sex ratio of offspring from the insemination of 2 groups of

spermatozoa obtained by electrophoretic fractionation[92]. Bhattacharya

reports that the length and breadth measurements of the heads are less

for spermatozoa obtained from the top than from the bottom fractions of

his gravity sedimentation device.[35]

Iversen describes a surface reflection interference microscopy tech-

nique which may provide more accurate measurements of the size of sperm-

atozoa[121]. Bahr and Zeitler describe a method of quantitative electron

microscopy permitting the recording of the mass cross-section (total mass

per unit length) of an object, making possible determinations of the dis-

tribution of the total mass of very long and narrow structures[17]. They

estimate that the distribution of mass in the bull spermatozoon among the

head, tail and midpiece are 63.7 $\pm$ 3.1%, 36.3 $\pm$ 1.0% and 17.3 $\pm$ .05%,

respectively. Table 4 of their article presents a summary of measurements

of bull spermatozoa structures from the literature[17].

Referring to Figure 1, the spermatozoon head is surrounded by a plasma

membrane which appears to be continuous over the midpiece and over most of the length of the tail. The plasma membrane appears to be applied more loosely and to have greater stickiness over the acrosomal cap region than over the post-nuclear cap region[25]. The continuity of the plasma membrane over most of the spermatozoon is an important consideration with regard to surface charge, which is considered in another section. The anterior portion of the nuclear material is surrounded by a dense acrosomal cap which has its own inner and outer cytoplasmic membranes. The integrity of the acrosomal cap appears to be essential for the maintenance of fertilizing capacity and is a key morphological feature in assessing viability of spermatozoa. Hereditary abnormalities of the acrosomal cap are associated with sterility in bulls[273].

Bedford has shown that during passage of spermatozoa through the epididymis, there is a decrease in acrosome width and length, which is correlated with the movement of the cytoplasmic droplet from the base of the sperm head to the center or distal portion of the midpiece[26]. This reduction in acrosome size appears to be related to the acquisition of fertilizing ability, as shown by a significantly higher conception rate with the insemination of spermatozoa from the cauda epididymis than from the caput epididymis[26]. The histology of the epididymis is described very well by Nicander[175].

Austin found no morphological difference between epididymal spermatozoa and sperm recovered from the fallopian tube[13]. Bedford suggests that the plasma membrane may be separated more easily from the anterior and lateral edges of the underlying acrosomal cap in uterine sperm than in spermatozoa from the ejaculate[25]. Uterine spermatozoa also showed the presence of small particles apposed to, and probably adherent to, the plasma membrane over the

acrosome. He suggest that this adhesiveness may help to keep the sperm-
atozoa attached to the ovum[25]. In disintegrating spermatozoa, the inner

acrosomal membrane and the apical body always remained intact. The apical

body was observed to persist in spermatozoa penetrating the zona pellucida

at which time the material of the acrosomal cap had almost disappeared[13].

The molecular structure of spermatozoa is being elucidated by studies

such as those concerning (1) the distribution of metabolic activity, phos-

pholipid and hyaluronidase between the heads and tails of bull spermatozoa[147],

(2) the distribution of ATP activity in ram and bull spermatozoa[191], (3)

the phospholipid composition of rat and ram epididymal spermatozoa[62], (4)

the chemical composition of the acrosomes of ram spermatozoa[111], (5) the

chemical composition of bull spermatozoa[33], (6) free amino acids in the

semen and spermatozoa of the fowl and turkey[6], and (7) free carbohydrates

in the seminal plasma and sperm of fowl[7]. Microspectrophotometric tech-

niques have been used to determine the DNA composition of rabbit[45] and

bull[241, 242] spermatozoa and biochemical techniques have been used to study

the DNA composition of fowl spermatozoa[100].

Welch, Hanly and Guest found a significant correlation between the

DNA content of spermatozoa measured microspectrophotometrically and the

motility and sperm count, and, to a lesser extent, the morphology of the

semen spermatozoa from bulls[242]. Compared to bulls showing the greatest

variation in DNA content, bulls showing the least variation had higher

quality spermatozoa samples judged by the degree of motility, morphology

and sperm count[242]. Baker and Salisbury found that cytophotometrically

determined Feulgen DNA values in fresh bovine spermatozoa were distributed

normally[268]. Spermatozoa stored for 11 days at 5°C in an egg yolk-citrate

buffer had a normal distribution of DNA values, but had a lower mean value

and a larger standard deviation than the fresh spermatozoa. Assuming an equal contribution of individual chromosomes to the Feulgen stained material and an equal probability of nondisjunction among all chromosomal pairs, 10% nondisjunction is needed to explain all of the observed within-sample Feulgen DNA biological variance. Because 10% nondisjunction is unacceptably high, they concluded that aneuploidy contributes little to normal Feulgen-DNA variance in spermatozoa and that the major sources of biological DNA variance among spermatozoa in single, normal ejaculates are related to variations in sperm age and to the extent of autolysis of dead and dying cells[268].

Bouters and coworkers attributed the variable Feulgen-DNA content of individual ejaculated spermatozoa to varying percentages of older sperm cells in the ejaculates[45]. They found no significant differences among 3 consecutive ejaculates, with respect to Feulgen-DNA content, in 15 experimental rabbits. Aging appeared to have no influence on the UV-DNA of spermatozoa. They suggest that the decrease in Feulgen-positive material which occurs in spermatozoa during the passage along the male tract results from structural changes in DNA[45].

Fowl Spermatozoa. Fowl spermatozoa, illustrated in Figure 2, are approximately 140 microns long. The cylindrical sperm head is approximately 14 microns in length and 0.5 microns in diameter, while the midpiece is approximately 4 microns long.

According to Lorenz, the cytoplasmic membrane over the head is continuous over about two-thirds of the midpiece[140]. The acrosomal cap is present but less conspicuous in fowl than in rabbit spermatozoa. The tail is covered by an amorphous sheath which gradually becomes thinner distally and ends about 2 microns from the tip of the tail[140]. As shown in Figure 2,
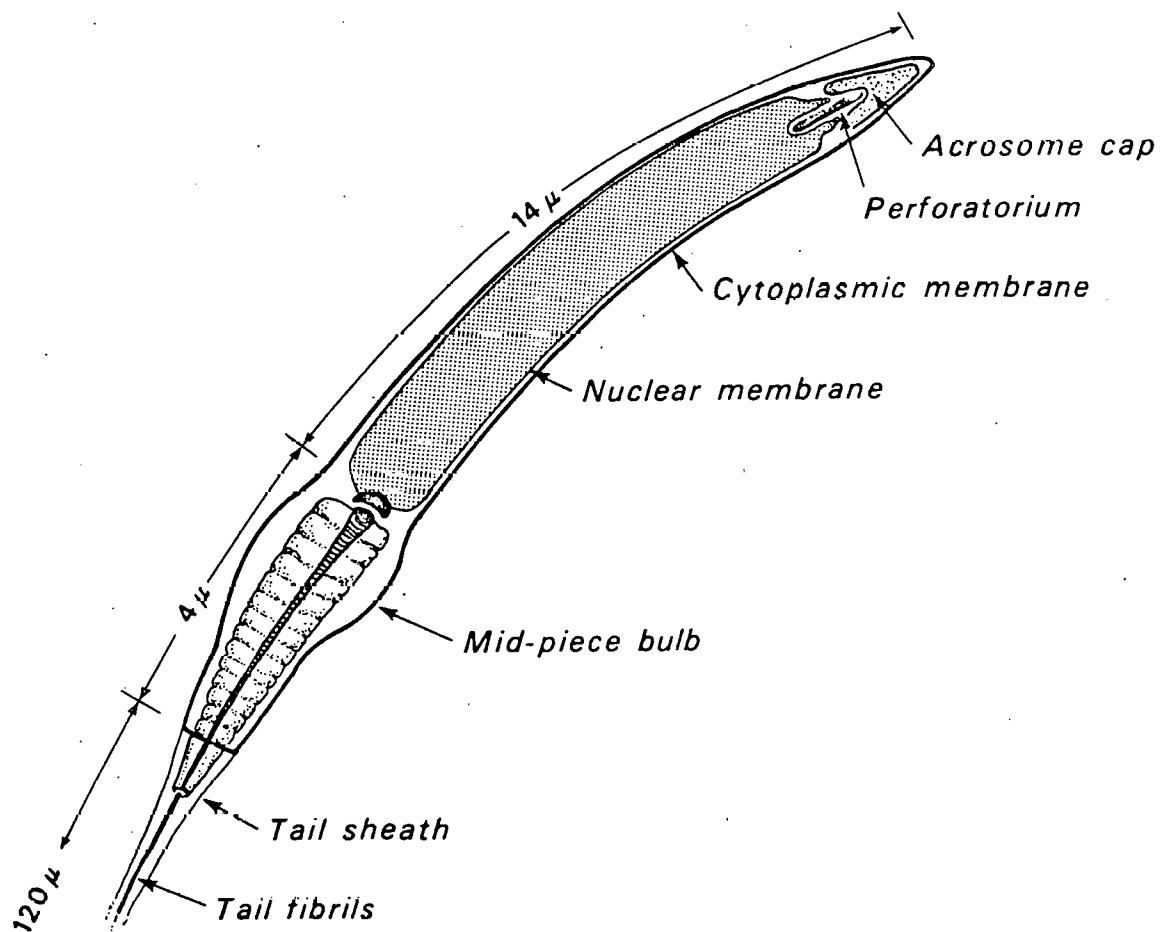
Fig. 2. Structure of the fowl spermatozoon.

fowl spermatozoa have a small acrosomal cap. Trypsin completely removes
the cytoplasmic membrane around the head, permitting disorganization and
at least partial digestion of the contents, while the head is little
affected by pepsin treatment[140].

2. Electrophoretic Mobility.

Schröder reported electrophoretic separation of rabbit spermatozoa
into two populations using a sperm dilution of 1:15 in a glycine-Ringer
buffer at pH 7.10 in a U-shaped electrophoresis cell[206]. Insemination
of spermatozoa which migrated toward the anode gave predominantly female
offspring, whereas cathode spermatozoa gave predominantly male offspring.
The number of Y-chromosome bearing spermatozoa obtained from the cathode
was a maximum at 10°C[206]. Biochemical and immunological studies tended
to confirm the biological results[207].

Gordon tested Schröder's experiments, using a similar U-shaped electro-
phoresis cell with a glycine-Ringer buffer at pH 7.10 and 15°C[91,92]. The
electrophoresis time was 45-50 minutes, and, for any one experiment, sperm-
atozoa could be obtained only from one electrode. Insemination of sperm-
atozoa recovered from the anode and cathode gave predominantly female and
male offspring, respectively. Upon applying the electric field, he ob-
served immediate and passive orientation of previously active and randomly
oriented spermatozoa such that they moved tail first toward the anode[92].
Lewin also reported two-way electrophoretic migration of rabbit spermato-
zoa[135], but other investigators have shown that, at pH 5-8, all bull,
ram and rabbit spermatozoa have a net negative charge[18,174,233].

Vesselinovitch reported that immotile bovine spermatozoa, when sus-
pended in Krebs-Henseleit-Ringer buffer or modified Schröder buffer
always migrated electrophoretically towards the anode[233]. He concluded

that bovine semen does not represent a mixture of spermatozoa of two
distinct electrical densities which would migrate to opposite poles when
suspended in buffer solution of a pH intermediate to their isoelectric
points. He observed also that with an electric field of the proper mag-
nitude, spermatozoa are recovered from both the anode and the cathode due
to: 1) electrophoretic movement of immotile spermatozoa toward the anode,
2) active swimming of spermatozoa of medium motility toward the cathode
after undergoing galvanotaxis and 3) active swimming of highly motile
spermatozoa toward both electrodes at random. Variations in the strength
of the electric field would be reflected primarily in the distribution
of the second group of spermatozoa. Because of the random contribution
of highly motile spermatozoa to the sperm population obtained from both
electrodes, and the equal probability of their being X- or Y-chromosome
bearing spermatozoa, Vesselinovitch believes that the sex ratio will
depend upon the ratio of X- to Y-chromosome bearing spermatozoa in the
initial sample[233]. MacPherson and Vesselinovitch obtained no significant
difference in the sex ratio of offspring from cattle inseminated with
spermatozoa from anodic and cathodic electrophoretic fractions[146].

Nevo, Michaeli and Schindler stressed the importance of immobilizing
spermatozoa prior to making electrophoretic measurements[174]. They inac-
tivated spermatozoa by the use of low temperature (4-5°C) or by the use
of 5% CO_2 at room temperature. Neither method had adverse effects on the
spermatozoa. Using a Northrop-Kunitz microelectrophoresis apparatus, they
found that the electrophoretic mobilities of bull spermatozoa were essen-
tially the same whether the spermatozoa were in seminal plasma, egg yolk-
citrate-glycine buffer with diluent or had been washed up to four times
in diluent. They concluded that no substances which contributed to the

surface charge were removed from the surface of the cells by the washings or were adsorbed from the egg yolk-glycine-citrate buffer. Their observations showed that both before and after dilution and washing, originally immotile spermatozoa and those inactivated by the low temperature were all negatively charged and that they had essentially the same electrophoretic properties. The isoelectric point was approximately pH 3.5 for both immotile and reversibly immobilized spermatozoa. Additional work with rabbit spermatozoa showed an isoelectric point of approximately pH 3.5. They saw no two-way migration with either species between pH 2.5 and pH 8.5 in any of the buffers used. They obtained electrophoretic mobilities for bull spermatozoa suspended in seminal plasma and in diluent, after 4 washings, of 0.89 and 0.88 x 10^{-4}(cm/sec)/(volt/cm), respectively, with an electric field strength of 5.4 V/cm at 4-5°C[174].

Bangham studied the electrophoretic characteristics of ram and rabbit spermatozoa over a wide range of pH and ionic strength[18]. In a solution consisting of 1 mM NaCl and 277 mM fructose at an electric field strength of approximately 12 V/cm, he observed (1) that motionless (dead) ram spermatozoa showed no particular orientation in the electric field and moved as though they had a uniform negative charge over their entire surface, (2) that certain spermatozoa had a greater negative charge density on their tails than on their heads (which oriented their tails toward the anode), and, depending upon the magnitude of the electrophoretic component relative to their intrinsic forward velocity, the spermatozoa swam toward the cathode or were pulled toward the anode, and (3) that head anode spermatozoa had a greater negative charge density on their heads than on their tails and, therefore, moved toward the anode at a rate determined by their intrinsic forward velocity and the electrophoretic

component in the same direction. In this 1 mM NaCl medium from pH 4.8-7.5, the percentage of head anode spermatozoa was 75% or greater, whereas an increase in NaCl concentration to 10 mM led to a reduction in the percentage of head anode spermatozoa from 70% at pH 4.6 to less than 10% at pH 5.8 In the 10 mM NaCl solution at pH 4.9, approximately equal numbers of head anode and tail anode spermatozoa were present. In 145 mM NaCl all spermatozoa were tail anode and the net electrophoretic charge on the spermatozoa varied only slightly over the range pH 4 to 7. In a glycine-Ringer buffer or in 145 mM NaCl over this pH range, no tail cathode spermatozoa were observed with samples of ram or rabbit spermatozoa at intermediate temperatures. At 8°C when spermatozoa are reversibly immobilized, all spermatozoa were tail anode type. At 25°C, the sperm appeared to be of two types, but Bangham explains this by the relative effects of the electric field and the intrinsic movement of the spermatozoa[18].

Bangham concluded that separation of cooled, motionless spermatozoa would be impractical because of the small difference in the magnitude of the negative charge on the tails and the heads, but that separation would be enhanced if swimming spermatozoa were used and the contrasting effect of the electrophoretic component on the tail and head exploited. Thus, by a suitable choice of electric field strength, those spermatozoa with a net negative charge on their tails could be held stationary or allowed to progress toward the cathode, while those with the net negative charge on their heads would move rapidly toward the anode[18].

Work by Joel and coworkers showed no significant difference in electrophoretic mobility of spermatozoa from normal and pathological human semen. The electrophoretic mobility of normal human spermatozoa ranged from 0.61-0.86 x 10^{-4} (cm/sec)/(V/cm) at 20°C and pH 7.8[122].

Laird studied the electrophoretic properties of bull spermatozoa using a phosphate diluent and buffer at pH 7.0 with a microelectrophoresis chamber similar to that used by Gordon[129]. At the end of an 8-hour electrophoresis run, he observed the distribution of spermatozoa characterized morphologically by a collar at the base of the head to be approximately 100% at the cathode and only 5% at the anode. The initial percentage of spermatozoa with collars was approximately 50%. The viability of the spermatozoa at the end of the experiment was extremely poor at both poles of the electrophoresis chamber. Spermatozoa recovered from the anode were longer than those recovered from the cathode, but both categories of separated spermatozoa were shorter than the unseparated sperm. He concluded that the spermatozoa migrated in two different directions in the electric field[129].

The change in electrophoretic mobility of spermatozoa when passing through the epididymis was measured by Bedford with a microelectrophoresis apparatus using a spermatozoa concentration of 2×10^6/ml in a 0.145 M NaCl solution (pH not given) at $4°C$[24]. He obtained mobilities of 0.3468 $\times 10^{-4}$ and 0.5363 $\times 10^{-4}$(cm/sec)/(V/cm) for spermatozoa from the caput and corpus epididymis, respectively, and 0.5371 $\times 10^{-4}$(cm/sec)/(V/cm) for spermatozoa from the cauda epididymis, vas deferens or washed, mature sperm from an ejaculate. Spermatozoa from the caput epididymis were primarily head anode with some random orientation and less than 10% tail anode. Spermatozoa from the corpus epididymis were primarily tail anode; however, there was some random orientation and, occasionally, head anode spermatozoa. The orientation of spermatozoa from the cauda epididymis and the ejaculate was primarily tail anode. With reversal of the direction of the electric field, the reorientation of spermatozoa from the caput epididymis took 3-4 minutes for individual spermatozoa and from 5-6 minutes for the entire

field of spermatozoa. The reorientation of spermatozoa from the corpus epididymis took 2-4 minutes for individuals and 3-4 minutes for the entire field. Both individual and the entire field of spermatozoa from the cauda epididymis, vas deferens and ejaculate took only 1-1.5 minutes to reorient[24].

In a solution of 6% fructose, cytoplasmic droplets appear to possess electrophoretic properties similar to those of spermatozoa heads, both of which are more strongly attracted to the anode than is the distal part of the tail[24].

Bey reports that, after washing fresh spermatozoa 3 times with isotonic NaCl at 4°C, most bull, boar, rabbit and cock spermatozoa had lost their intrinsic motility[251]. In a low ionic strength buffer in a rectangular electrophoresis chamber, she obtained electrophoretic mobilities as follows: (units of 10^{-4} cm/sec/V/cm) bull, 1.302 $\pm$ 0.031; rabbit, 1.121 $\pm$ 0.022 and cock, 1.545 $\pm$ 0.020[251].

Sevinç (1968) observed two-way migration of rabbit spermatozoa using an intermittent electric field and motile spermatozoa at temperatures of 29 and 30°C at pH 7.1 in 3 and 7 chambered electrophoretic cells[210]. In one experiment using 3 different buffers, the insemination of spermatozoa recovered from the anode gave 19 offspring, 15 (79%) of which were male, while insemination of spermatozoa recovered from the cathode gave 68 offspring, 26 (38%) of which were males. The difference between the expected normal sex ratio and the sex ratios of the offspring obtained in this experiment was highly significant. However, in 3 subsequent experiments, only a slightly higher percentage of male offspring was born following the insemination of spermatozoa which migrated both to the anode and to the cathode[210]. In view of the conflicting results of the several experiments, no conclusions could be drawn with regard to the possibility of separating the X- and Y-chromosome bearing spermatozoa by this technique.

From the description of the apparatus given, it does not appear that observations of the spermatozoa in the electric field could be made. With the media and conditions he used, he achieved excellent fertility and a high offspring per litter ratio.

3. Sedimentation Rate.

Using a sedimentation apparatus consisting of a 10 ml burette with a 1.1 cm diameter, Bhattacharya observed that nearly 30% of the spermatozoa placed at the top of the burette sedimented a distance of 10 cm in 24 hours at 0°C through a medium of specific gravity 1.0012 and viscosity 17.956 centipoises (cp)[36]. Approximately 70% of the spermatozoa sedimented the same distance in 24 hours at 0°C through a medium of specific gravity 1.0025 and viscosity 13.436 cp. The specific gravities and viscosities of the media were determined at 21°C. The "light" and "heavy" media contained 5% glycine solution plus egg yolk in ratios of 1:2 and 1.75:2, respectively. At the end of a 24-hour sedimentation time, the bottom 1-2 ml of the column of high density medium contained from 9.7% to 14.4% and the top 2 ml contained 5% to 16.5% of the total number of spermatozoa. Insemination of the spermatozoa from the bottom 2 ml resulted in 22 offspring, 17 (77.3%) of which were females and spermatozoa from the top 2 ml gave a total of 14 offspring, 12 (85.7%) of which were males. Insemination of spermatozoa recovered near the middle of the sedimentation column gave approximately 50% of each sex[36].

Bhattacharya repeated his sedimentation experiment, using a medium consisting of 42.5% egg yolk in a glycine-citrate buffer with a specific gravity of 1.0304 and a viscosity of 75 cp[35]. Approximately 200 x 10^6 spermatozoa were placed upon a column of medium 10 cm high, and, after 12 hours at 0°C, spermatozoa were distributed over the top 6 cm of the column.

Insemination of 1-20 x 10^6 spermatozoa from the top 2 fractions gave 77.4%
male offspring, and spermatozoa from the bottom 2 fractions gave 28.2%
male offspring. Insemination of spermatozoa from the intermediate fractions
gave near normal sex ratios. All inseminations resulted in a low percen-
age fertilization. The dimorphism observed by Bhattacharya suggested that
the spermatozoa with the greater volume sedimented at a faster rate and
gave a predominance of female offspring[35].

Bhattacharya[3] reported a partial bimodal distribution of rabbit and
bull spermatozoa and a unimodal distribution of fowl spermatozoa in the
sedimentation apparatus[35]. The sedimentation rate of fowl spermatozoa
was significantly less than that of the rabbit or bull spermatozoa. He
had not yet investigated the relationship of sedimentation rate to specific
gravity or to the DNA content of the spermatozoa[35].

Bhattacharya, Bangham, Cro, Keynes and Rowson applied Bhattacharya's
sedimentation technique to the separation of bull spermatozoa. Eighty-four
of 173 (48.6%) offspring obtained in 3 groups of inseminations were males,
or nearly a 1:1 sex ratio[37].

Schilling fractionated bull spermatozoa using equipment similar to
that used by Bhattacharya and a medium consisting of skimmed milk, salt
solutions and egg yolk with a specific gravity ranging from 1.037-1.044
and a viscosity ranging from 7-10 cp[205]. Sixty minutes after being layered
on top of the column at 0°C, the spermatozoa appeared to be distributed in
a single peak over 10-12 fractions. No additional migration was observed
after 60 minutes. The spermatozoa in the different fractions were concen-
trated by centrifuging at 4,000 rpm for 15 minutes to make possible insem-
ination of a high number of spermatozoa in a small volume. Insemination

of spermatozoa from the lowest fractions resulted in 86 calves, 60 (69.8%) of which were females compared to the normal ratio of cattle of 123 males to 100 females (44.8%) in artificial insemination[205].

Bedford and Bibeau found no difference in the sex ratio of rabbits resulting from the insemination of spermatozoa fractionated by gravity sedimentation using a device and medium similar to those used by Schilling[29]. The specific gravity and viscosity of the medium at 2°C were 1.048 and 6.7 cp, respectively. The total height of the medium in the burette was 19 cm. Spermatozoa in 1.0 ml of the medium were layered on top of the column and twenty fractions of 1 ml each were recovered from the column at the conclusion of the sedimentation time. In one series of experiments with a 2-hour sedimentation time, spermatozoa inseminated from the bottom 4 fractions of the sedimentation column resulted in 35 male and 27 female offspring. In another series of experiments with a 1-hour sedimentation time, insemination of the bottom 2 fractions resulted in 22 male and 21 female offspring. Insemination of the spermatozoa from the top 2 fractions recovered after 2 hours of sedimentation resulted in 15 male and 19 female offspring[29]. In a figure showing the distribution of spermatozoa throughout the sedimentation column in 8 trials, one profile suggested a unimodal distribution, 1 or 2 a bimodal distribution and the remainder were more representative of uniform distributions.

4. Specific Gravity.

In 1925, Lush investigated the possibility of sex control by artificial insemination of centrifuged spermatozoa employing a differential centrifugation technique[143]. His observations showed no significant difference in the sex of offspring obtained by insemination of spermatozoa samples fractionated by this technique. His results suggested a possible

dimorphism in measurements of head lengths. However, the difference was

very small and he could not show a relationship between differential mi-

gration rate and head length[143].

Lindahl and Kihlström studied alterations in the specific gravity

of bull spermatozoa during ripening[138]. Spermatozoa from 3 ejaculates

collected from each bull in immediate succession were centrifuged for 10

minutes at 2,300 g in hematocrit tubes over solutions of different concen-

trations of the methylglucamine salt of umbradil (umbradilic acid). The

percentage of unripe spermatozoa increased while that of overripe sperma-

tozoa decreased from the first to the third ejaculate in each series. The

percentage of ripe spermatozoa was nearly equal in each of the 3 ejaculates

constituting a series. The specific gravity of the spermatozoa ranged from

about 1.24-1.35 and the mean specific gravities of the first, second and

third ejaculates were 1.2867 $\pm$ 0.0022, 1.2897 $\pm$ 0.0040, and 1.2668 $\pm$ 0.0048,

respectively. The distribution of specific gravities appeared to be uni-

modal around the mean. From the distribution of the unripe, ripe and over-

ripe spermatozoa, the authors concluded that the density of spermatozoa

increases during ripening[138].

Using test solutions of umbradilic acid in water, Kihlstrom found

the specific gravity of the tail and middle pieces of bull spermatozoa

to be from 1.035-1.056 and that of the heads to be greater than 1.276[124].

Lindahl attempted to separate the X- and Y-chromosome bearing sperm-

atozoa in bull ejaculates by counter-streaming centrifugation[137]. Insem-

ination of "heavy" spermatozoa gave a total of 63 offspring, 27 (42.9%)

of which were male. The fertility from spermatozoa subjected to a rota-

tional speed of 1,000 rpm was 53.6% compared to 35.6% at 1,100 rpm. He

obtained 24.3% males from pooled samples giving greater than 50% fertility

compared to 69.2% males from those giving less than 50% fertility. From these data, he suggested that it may be possible to select viable female determining spermatozoa by running the counter-streaming centrifuge at moderate speed (1,000 rpm)[137].

Insemination of the "light" spermatozoa gave 27 males (58.7%) out of a total of 46 offspring. This series of inseminations was divided into pooled samples with more or less than 40% fertility for analysis. The group of inseminations resulting in an average fertility rate of 47.4% gave 40.7% males, while the group with an average fertility rate of 29.7% gave 84.2% male offspring. The average sex ratio of 58.7 for the 2 groups did not differ significantly from the control sex ratio of 59.8[137].

Lindahl and Thunqvist centrifuged intact epididymal and ejaculate spermatozoa and their component parts for 12-15 minutes at 1,000 g in specific weight gradients composed of the synthetic polysaccharide Ficoll[139]. They observed specific gravities of 1.10-1.125 for unfragmented epididymal spermatozoa, 1.240-1.334 for ejaculate spermatozoa, 1.045 for midpieces with tails and 1.276 for heads from ejaculate spermatozoa not treated with ultrasonics. After ultrasonic treatment, specific gravities observed were as follows: unfragmented epididymal and ejaculate spermatozoa, 1.100-1.120 and 1.210-1.330, respectively; midpieces with tails from both type of spermatozoa, 1.040-1.070; heads of epididymal spermatozoa, 1.12-1.140; and heads of ejaculate spermatozoa, 1.250-1.350. The authors concluded that the marked difference in specific gravity of epididymal and ejaculate spermatozoa appears to be localized to the head[139].

Beatty evaluated the use of bovine plasma albumin, colloidal silica and dextran as major constituents in media for equilibrium density gradient centrifugation of mammalian spermatozoa[20]. Rabbit spermatozoa remained

fertile after exposure for up to 2 hours in bovine plasma albumin (25%

in either 0.45 or 0.90% NaCl) and in dextran (Ficoll, 37.9%) but not in

colloidal silica. A unimodal distribution of specific gravities of rabbit

spermatozoa was obtained with a colloidal silica gradient. About 95% of

the spermatozoa were in the range 1.09-1.18, and 50% were in the range

1.11-1.14. The mean was 1.132[20].

Benedict, Schumaker and Davies[32] evaluated sodium chloride, sodium

nitrate, potassium tartrate, potassium iodide, cesium chloride, glycerol,

sucrose, Ficoll, bovine serum albumin, a colloidal silica solution (Ludox),

and the methyl-glucamine salt of 3,5-di-iodo-4-pyridone-N-acetic acid (MGU)

as gradient materials for equilibrium centrifugation studies. With all

solutions except Ficoll, glycerol and potassium iodide, they observed a

low density class of bull spermatozoa with a buoyant density of 1.16-1.19

g/ml and, depending upon the solute, a high density group ranging from

1.25-1.32 g/ml, which, on many occasions, divided into subclasses over

a narrow range. Motility was preserved in the bovine serum albumin, Fi-

coll, Ludox and MGU solutions. Because of the undesirable effects of the

high viscosity of albumin and Ficoll solutions, and because of gelation

and lowered motility in the Ludox solution, they primarily used MGU sol-

utions to prepare the linear density gradients. They used a Beckman Model

L Ultra-centrifuge with a SW 39 swinging-bucket rotor[32].

Benedict, et al, centrifuged spermatozoa from the same bull sample

for 5, 15 and 30 minutes at rotor speeds of 5,000, 15,000 and 30,000 rpm

at temperatures of 5, 25 and 37°C. Under all conditions of time, centri-

fugal force and temperature, apparent buoyant densities were 1.170 $\pm$ 0.025

and 1.290 $\pm$ 0.040 g/ml for the upper and lower bands, respectively. They

noted increased numbers of spermatozoa in the lower band with increasing

temperature and time of exposure in the medium. Recentrifugation of the

upper band gave both an upper band and a lower band, the development of which appeared to be related to time. Recentrifugation of the lower bands produced diffuse bands in the corresponding density range. They observed a splitting of the low density band into 2 sub-bands only at 5°C and a rotor speed of 30,000 rpm. No significant changes in the size or shape of the spermatozoa were observed. Similar results were obtained with rabbit and bull spermatozoa[32].

Using a nigrosin-eosin staining technique, they observed a lower percentage of eosin-positive cells in the low density fraction than in the high density fraction in all experiments[32]. With fresh spermatozoa, the low density (top) fraction had a slightly higher percentage of eosin-positive cells than did uncentrifuged spermatozoa from the same sample. However, when using aged and mixed (aged and fresh) spermatozoa, the low density (top) fraction had a lower percentage of eosin-positive cells than did the uncentrifuged sample[32].

Insemination of rabbit spermatozoa from the different bands gave fertility rates of 36% from the upper band, 11% from the middle band, 4% from each of 2 bottom bands, and 6% from combined bottom bands compared to 58% fertility with uncentrifuged (control) spermatozoa[32]. However, no conclusions could be drawn with regard to the sex ratio of the offspring.

Lavon and co-workers determined the specific gravity of bull spermatozoa from different parts of the reproductive tract using non-water-miscible phthalate ester mixtures of predetermined specific gravity in micro-hematocrit tubes[133]. The average specific gravities of spermatozoa from the mediastinum testis, caput epididymis and cauda epididymis were estimated to be 1.0440 ± 0.002, $1.0583 \pm .002$ and $1.0817 \pm .002$, respectively[133]. Thus, the specific gravity increases during maturation as suggested by Lindahl. In another report, the specific gravity of bull

spermatozoa in the first ejaculate was 1.0760 ± 0.003[132]. These values are considerably lower than those reported by Lindahl and Thunqvist.

Morton used a colloid mill to disrupt bovine epididymal spermatozoa and used a sucrose linear density gradient to determine the buoyant density of parts of spermatozoa[164]. The apparent buoyant densities of components ranged from approximately 1.25 g/ml for tails to greater than 1.347 g/ml for heads only. Midpieces were slightly more dense than tails and the apparent buoyant density of combinations of heads with midpieces were less for the fragments with the larger midpieces[164].

C. Biological Properties

Because of the close relationships among the biological properties of spermatozoa, any arbitrary division of them is artificial. However, for convenience of discussion, they will be divided into 5 main categories as follows: (1) viability, (2) intrinsic motility, (3) fertilizing capacity, (4) antigenicity and (5) genetic composition.

1. Viability.

Among the criteria by which the viability of spermatozoa is judged are the staining properties and their relationship to morphology, motility, fertilizing capacity, and metabolic rate. In addition, one is interested in the effects of the age and breed of the rabbit, the frequency of ejaculation and the season of the year on the quality of the spermatozoa.

a. Vital Staining. Beatty and Napier found a great difference between the percent capless and the percent stained spermatozoa in ejaculates[22]. For instance, in ejaculates with 25.4, 34.2 and 32.6 percent stained spermatozoa, the percentages of capless spermatozoa were 13.3, 18.6 and 16.9 percent, respectively. They stated that scoring of stained spermatozoa is difficult[22]. Napier suggests that the percentage of stained spermatozoa

using a nigrosin-eosin technique is one of the best guides for predicting fertilizing capacity, which is inversely proportional to the percentage of stained spermatozoa[169]. He found the mean litter size to be related to the mean spermatozoan head length of the sire[171]. Among strains the incidence of stained spermatozoa varies markedly, indicating the possibility of hereditary differences in fertility; however, the variation in the percentage of capless spermatozoa is not significant[23].

In studies with bull spermatozoa, Munro saw no difference among samples with visual observation of motility, but found a 10% difference with vital staining[165]. Conception rates were approximately 65% for samples with over 70% live spermatozoa by staining. Fertility was reduced an unusual degree when either the incidence of live spermatozoa fell below 70% or the incidence of primary and secondary abnormal spermatozoa rose above 23%. Head defects appeared to be the most important abnormalities[165]. Napier reports that, in samples containing a high percentage of stained or capless spermatozoa, the unstained spermatozoa are of low viability[169].

The percentages of stained and capless spermatozoa increased significantly after incubation at 38.5°C for 5 hours in 0.9% saline, while incubation for the same period of time in a suspension of 50% seminal fluid and 50% saline did not significantly change the mean percentage of stained or capless spermatozoa[169]. Neither suspending a sample from 1 male in the seminal plasma from a different male nor mixing of spermatozoa from 2 different males affects the viability of the spermatozoa[169].

Stress, such as centrifugation, causes the same absolute change in the percentage of stained or capless spermatozoa in all samples, regardless of the initial viability. Thus, with initially low viability, the percentage change is greater than with high viability spermatozoa, indicating

that the apparently normal spermatozoa in a large population of abnormal "low quality" sperm are less viable[169].

b. <u>Metabolism</u>. Work by Murdoch and White has shown that rabbit spermatozoa oxidize glucose faster than acetate and oxidize lactate at a faster rate than glucose, fructose, pyruvate or acetate[166]. Fructose is the predominant sugar found in the semen of most mammalian species, including the rabbit, while glucose is the sugar found in the semen of the domestic cock[166]. Salisbury reported that the optimum oxygen uptake by ejaculated bull spermatozoa in semen occurred only when CO_2 in amounts normally respired (about 5%) was present[201]. Wales and O'Shea reported that the presence of respired CO_2 during incubation of bull spermatozoa had no effect on oxygen uptake or substrate oxidation, but increased lactate accumulation[237]. Hamner and Williams reported that 2.36×10^{-3} M $NaHCO_3$ in equilibrium with 2% CO_2 at pH 7.0 is the optimum CO_2 level for maximum metabolic activity of rabbit spermatozoa[107].

Salisbury states that, during a 3 hour period of incubation at 37°C, both optimum oxygen consumption and optimum aerobic glycolysis by bull spermatozoa occurred in a NaCl solution of approximately 280 mosm[201]. (The osmolarity of seminal plasma is approximately 285 mosm). The effect of osmolarity was less obvious when a potassium chloride solution was used. He suggests that the optimum osmolarity for the preservation of spermatozoa and conservation of intracellular reserves would be provided by a medium with an osmolarity of 350 mosm, which is approximately that found in the epididymis[201].

Wales and Wallace studied the effects of potassium, magnesium, calcium and phosphate ions on the metabolism of bull, dog, rabbit and fowl spermatozoa[236]. Fowl spermatozoa had the lowest rate of metabolism, and

dog spermatozoa had the highest respiratory activity. Washing bull, dog and rabbit spermatozoa greatly reduced the oxidation of substrates other than fructose, while washing had little effect on fowl spermatozoa. Phosphate ions generally depressed the respiration of bull spermatozoa and stimulated their aerobic fructolysis. It stimulated the respiration of unwashed dog spermatozoa, but had no important influence on the metabolism of rabbit and fowl spermatozoa. Potassium stimulated some aspects of metabolism of sperm of all species, but had its greatest effect upon the respiration of dog spermatozoa. It stimulated the oxidation of fructose by rabbit spermatozoa. Magnesium depressed the respiration of bull and fowl spermatozoa. Calcium apparently had no effect on any of the spermatozoa. They observed few significant interactions among the ions tested[236]. The injurious effects of high concentrations of phosphate on the oxidative metabolism of bull spermatozoa has also been reported by other investigators[193].

Nevo studied the dependence of motility and respiration of bull, ram and cock spermatozoa on oxygen concentration[173]. Both motility and respiratory rate were constant in the range of oxygen concentration from that in air to that at a pO_2 of 4-5 mm Hg, below which there was a marked reduction in respiratory rate and inactivation of most of the spermatozoa. Motility and oxygen consumption ceased below an oxygen partial pressure of 1 mm Hg. The critical O_2 values were similar for all 3 species[173]. They are considerably below the pO_2 of 60 mm Hg in the fallopian tube of the unanesthesized rabbit before, and for 3 days after, the induction of ovulation with HCG[149]. If the minimum O_2 partial pressure requirement is similar in other species, a significant safety factor is present.

c. <u>Factors Affecting Spermatozoa Production and Quality</u>. The volume and number of spermatozoa in an ejaculate depend upon the age and breed

of the rabbit and upon characteristics of individual males. For mature rabbits volumes range from 0.4-0.7 ml and concentrations range from 1×10^8-2×10^9 spermatozoa/ml[1]. The variation in the fertility of semen samples is attributed to (1) the inherent quality of the spermatozoa, (2) the composition of the seminal fluid and (3) the interaction of seminal fluid and spermatozoa[169].

Amann and Lambiase studied the changes in semen characteristics and sperm output between puberty and one year of age in New Zealand White bucks[9]. Daily sperm output doubled between 20 and 32 weeks of age, reaching an average of $148 \pm 11 \times 10^6$ spermatozoa per day and did not increase after that time, suggesting that these rabbits reach sexual maturity at about 32 weeks of age. The mean ejaculate volume was 0.56 ml with a spermatozoa concentration of 301×10^6/ml[9].

The effect of the frequency of ejaculation upon the viability of spermatozoa was evaluated by Gregoire, Bratton and Foote who compared the spermatozoa from 2 groups of 4 Dutch Belt rabbits each, ejaculated at daily or weekly intervals[94]. The average number of motile spermatozoa decreased from 243×10^6 to 55×10^6 per ejaculate for the bucks ejaculated daily, and from 138×10^6 to 97×10^6 for the bucks ejaculated at weekly intervals. The weekly output of motile spermatozoa was 385×10^6 compared to 97×10^6 for the bucks ejaculated at daily and weekly intervals, respectively. There was no significant difference in the size of the litters or in the percentage of does kindling following artificial insemination with semen from the 2 groups of bucks after they had been ejaculated for a period of 37 to 40 weeks[94].

Amann evaluated the effect of ejaculation frequency and breed on semen characteristics and sperm output of Dutch and New Zealand White

rabbits using ejaculation frequencies of 1 x 12 hours, 1 x 24 hours,
and 2 x 48 hours with the 2 collections separated by about 15 minutes[8].
Each ejaculation frequency was imposed for 36 days, the last 30 of which
constituted the experimental period. No significant differences were
observed among the daily sperm outputs at the 3 different collection fre-
quencies. The averages were 88 x 10^6 and 123 x 10^6 spermatozoa/day for
the Dutch and New Zealand White rabbits, respectively[8]. Amann did not dis-
cuss the quality of spermatozoa from the different rabbits at the dif-
ferent collection frequencies.

Another study of the sperm output of rabbits at various ejaculation
frequencies was conducted by Desjardins, Kirton and Hafs using 9 Dutch
Belt and 3 New Zealand White rabbits, approximately 12 months of age[64].
Each of the rabbits was subjected to 4 ejaculation frequencies imposed
for 5 week periods, consisting of frequencies of once a week (1 x F),
4 times on Friday (4 x F), twice on Monday, Wednesday and Friday (2 x MWF)
and once daily, except Sunday (1 x M-S). They observed no significant
differences among the first ejaculates at all 4 frequencies. Despite
a decrease in volume in the second ejaculate of the 4 x F and 2 x MWF
groups, an increase in the spermatozoa concentration gave a higher total
sperm output in those ejaculates. In the third and fourth ejaculates
of the 4 x F group, both the volumes and concentrations decreased. There
were no significant differences in the total number of spermatozoa per
week from the 4 x F, 2 x MWF and 1 x M-S groups[64]. No observations on
the quality of the spermatozoa in the different ejaculates were reported.
An analysis of the protein, fructose and citric acid content of the eja-
culates showed both an absolute decrease in successive ejaculates in the
4 x F and 2 x MWF groups, and a decrease in concentration of those sub-

strates, which was most prominent in the third and fourth ejaculates in the 4 x F group[64].

Orgebin-Crist observed that, when rabbits were ejaculated only twice weekly, the daily sperm output was approximately 50% of the estimated testicular sperm production, indicating that a high percentage of the spermatozoa produced were absorbed[181].

Doggett showed that the total sperm count of rabbits ejaculated daily for prolonged periods rose to a definite peak every 2-7 days with an average of 3.16 days[68]. The low values usually were about 50% of the preceding peaks. In mature animals, the peaks were fairly consistent and similar among individual animals. Sperm motility and the volume of liquid semen rose and fell with the total daily sperm production[68].

Degerman and Kihlström have shown a cyclic variation in the body temperature of the male rabbit which correlates statistically with the volume of semen produced daily. Cycles vary in length from 3-9 days with a mean value of 7 days[63]. The authors did not report the quality of the spermatozoa during the cycles.

El Jack and Lake evaluated the effect of resting roosters from ejaculation on the quality of spermatozoa by subdividing the roosters into 5 groups[77]. One group was kept as a control, while the other 4 groups were rested for 2, 4, 6 and 8 weeks, respectively, between ejaculations. Semen was collected from the control group throughout the 10 week experimental period at intervals of 2, 3 or 7 days. Even though there was a tendency for the number of degenerating spermatozoa in an ejaculate to rise after roosters had been rested for more than 2 weeks, there were individual variations in the degree of deterioration of the ejaculates. Some roosters did not appear to be affected appreciably by the change in

collection schedule. When the experimental males were milked frequently after a rest period, the percentage of normal spermatozoa in the semen returned towards the pre-rest value. It was suggested that the rooster should be ejaculated regularly to insure the availability of uniformly good samples[77].

Eliasson showed that frequent ejaculations reduced the volume of human seminal plasma, but did not significantly change the relative amounts of constituents from the seminal vesicles and prostate gland. Ejaculates were obtained from individuals at 24, 12 and 8 hour intervals[76].

Freund and Wiederman evaluated the influence of spermatozoa quality on storage and fertility[88]. They found that compared to high quality spermatozoa, the motility of spermatozoa of poor quality ejaculates was good immediately after cooling to 5°C and after 1 day of storage, but showed a greater loss of motility with longer storage than did ejaculates with high quality spermatozoa[88].

d. <u>Seasonal Variations in Spermatozoa Quality</u>. The seasonal variation in the reproductive traits of New Zealand White rabbits from 1943-1960 was evaluated by Sittman and co-workers at the United States Rabbit Experiment Station, Fontana, California[216]. The maximum average daily temperature increased from 63 to 75°F from January through May, increased to above 80°F in June, was approximately 90°F from July through September, decreased to about 80°F in October, and was about 75°F and 70°F in November and December, respectively. The animals were housed in sheds cooled by sprinklers during the hot, dry summer months. The monthly conception rate was 77.4% from January through May, 62% from June through November, with a low of 52% in September, and was 72% in December. In addition to the decrease in the conception rate during the hot months, the following

also were observed at that time: (1) a decrease in the number of litters born per month, (2) a decrease in the average number of offspring born alive per litter per month, (3) a decrease in the average total litter size per month as a percent of the overall average, and (4) a decrease in the number of rabbits born per month as percentages of the overall total. This variation in reproductive capacity appeared to be more dependent upon changes in temperature than upon changes in light conditions[216].

Orgebin-Crist evaluated the effect of environmental light on the gonadal and epididymal sperm reserve and the daily sperm production in rabbits. Rabbits exposed to 14 hours of light daily for 26.3 ± 1.7 weeks had an average sperm production of 115.8 x 10^6/day and a testes yield of 21.4 x 10^6 sperm/day/gm compared to an average sperm output of 147.4 x 10^6/day and a testes yield of 26.5 x 10^6 sperm/day/gm for bucks exposed to 14 hours of light for only 6.9 ± 8 weeks. This suggests that a constant photo-period is deleterious for sperm production[181].

2. Intrinsic Motility.

This section is concerned primarily with factors influencing the preservation of motility (cell concentration; and the pH, ionic strength and osmolarity of the medium) and factors modifying the degree of motility (temperature and stage of maturation of the spermatozoa). In addition, observations on the fluctuations in swimming rates of individual bull spermatozoa are presented.

a. Spermatozoa Concentration. Emmens and Swyer observed that rabbit spermatozoa from most ejaculates, when suspended in Baker's solution at a concentration of 0.4 x 10^6/ml, became completely immotile within 2 or 3 hours[80]. The same phenomenon occurred with chloride-free diluents and is, therefore, not due to a toxic effect of chloride. They reported that

the rapid immobilization may be prevented by suspension in cell-free
supernatants from other more concentrated suspensions of rabbit semen,
by accessory secretions from a vasectomized buck, and by the addition
of agents such as gum arabic, starch, glycogen, and serum proteins to
Baker's solution. In no case was the action of the additives as effect-
ive in preserving motility as more concentrated suspensions of spermatozoa
in Baker's solution. Washing with Baker's solution did not cause im-
mobilization of spermatozoa at concentrations of 20 x 10^6/ml at a stage
when the concentration of seminal plasma had been reduced to the equiva-
lent of dilution to 0.4 x 10^6/ml. However, 6 washings did immobilize
the spermatozoa and was interpreted by the authors as evidence that the
dilution phenomenon is caused by the loss of intracellular or paracellu-
lar material[80].

Swyer (as noted in the discussion following Kok's report) observed
a significant decrease in the motility of rabbit spermatozoa when the
concentration fell below approximately 4 x 10^6 spermatozoa/ml[125]. Emmens
and Swyer[80] observed that a concentration of 20 x 10^6 spermatozoa/ml main-
tained as good, or nearly as good, a degree of motility as higher concen-
trations.

Freund and Wiederman, using human spermatozoa, observed that with
dilutions of 1:1, 1:5, 1:25 and 1:125 in Norman-Johnson Solution, seminal
plasma and White's phosphate buffer, there was an initial loss of motility
at the greater dilutions[88]. Over a period of 5 hours at 25°C the decrease
in the percentage of motile sperm at all dilutions appeared to be approxi-
mately the same in the Norman-Johnson-1 (NJ-1)Solution. The decrease in the
percentage of motile sperm in raw semen appeared to be greater than in
semen diluted 1:1 and 1:5 in the NJ-1 Solution[88]. The actual spermatozoa

concentration at the different dilutions was not given.

Quinn, White and Wirrick studied the effect of dilution on the concentration of sodium, potassium, calcium and magnesium in ram and bull spermatozoa using an atomic absorption spectrophotometer[193]. They observed an intracellular accumulation of sodium and a loss of potassium, calcium and magnesium upon dilutions as low as 1:8. Equilibrium was reached soon after dilution and little change in the concentration of those cations was observed over a 3 hour period. At dilutions of 1:100 and after washing 4 times, they observed a similar but more severe influx of sodium. This was accompanied by a marked intracellular depletion of potassium which tended to worsen over the 3 hour period. This exchange of sodium and potassium increased with increasing pH[193].

b. pH. Emmens studied the effect of pH on rabbit spermatozoa using a diluent containing 3% dextrose, 0.2-0.4% sodium chloride and sufficient buffer to bring the solutions to a tonicity equivalent to 0.9-1.0% sodium chloride[79]. Suspensions of $10-60 \times 10^6$ spermatozoa/ml were prepared only from active ejaculates with high counts. All observations were made at room temperature. Baker's solution at approximately pH 7.3 was used as a control. Observations included the following: (1) Rabbit semen had a pH between 7.0 and 8.2 with a mean of 24 samples being pH 7.6. (2) Spermatozoa were much more sensitive to acidity than to alkalinity. (3) A pH range of 7.2-7.9 was optimum for maintaining motility IN VITRO. (4) At pH's slightly below 5.8, spermatozoa were immotile. After exposure for 1.25 hours at this pH, the addition of Baker's solution restored the motility to a reasonable degree. With exposure to the low pH for less than 5 hours, a slight degree of motility was restored upon addition of Baker's solution but after 5 hours, the loss of motility was irreversible.

(5) For very short exposures to pH's between 4.4 and 5.4, motility could be restored by the addition of Baker's solution to the medium. In these cases, the duration of exposure was limited to 1 hour or less. (6) At pH 9.5-10.0, spermatozoa remained motile and survived for several hours, but with a depressed degree of motility. (7) At all pH's as death occurred, the sperm tails tended to stain before the heads[79].

Spermatozoa tolerate well a pH range of 6.5-8.0 in most media IN VITRO. The reversibility of the effects of extreme pH's on viability and intrinsic motility is time dependent and therefore of great significance in designing experiments using spermatozoa.

The relationship of these IN VITRO observations to the natural environment of spermatozoa in the female genital tract is illustrated by the following observations. Moghissi observed that human spermatozoa were immobilized in acid mucus while alkaline mucus enhanced motility[161]. According to Stevens, Hafs and Kirton, the uterine fluid of rabbits is pH 7.64[219]. They stress the importance of CO_2 in buffering uterine fluids for they observed a rapid rise in pH upon removal of the fluids from the uterus[219].

In addition to its influence on the preservation of motility, pH also has a significant effect on the electrophoretic mobility of spermatozoa as noted previously.

c. Ionic Strength and Osmolarity of the Medium. Items related to ionic strength which have been mentioned previously include the net surface charge, the relative surface charge on the heads and tails of the spermatozoa, and the effects of ionic strength on the type of agglutination of spermatozoa populations.

Observations by Emmens on the effects of osmotic pressure and electro-

lyte concentration on the motility of rabbit spermatozoa at different

hydrogen ion concentrations included the following: (1) At pH 5.8-

6.6, spermatozoa were less motile than at pH 7-8, were very sensitive to

pH changes, and were more sensitive to hypotonicity than to hypertonicity.

Motility was affected little or not at all by substitution of dextrose

for part or all of a sodium chloride solution. (2) At pH 7-8.7, sperm-

atozoa were relatively insensitive to changes in pH, but hypo- and hyper-

tonicity, whether caused by alterations in sodium chloride or in dextrose

concentration, were about equally deleterious to motility. The replace-

ment of sodium chloride by dextrose in hypertonic diluents tended to lower

initial motility but this level of motility was maintained with little

fall over the next 6 hours. (3) At pH 8.6-9.8, spermatozoa were much

less motile than at pH 7-8, but still were not very sensitive to minor

changes in pH. Hypertonicity was more deleterious than hypotonicity.

The latter may even have been favorable to motility under certain conditions.

(4) The replacement of sodium chloride by dextrose enhanced preservation

of motility in isotonic media, but made little difference in hypo- or

hypertonic solutions. The favorable effect of hypotonicity was expressed

if it involved a reduction in the sodium chloride content of the diluent,

but not if it involved a reduction in the dextrose content. The character-

istic action of dextrose was a lowering of the rate of decline of motility,

not the maintenance of a high initial motility. (5) In alkaline suspen

sions, rabbit spermatozoa were affected adversely by a sodium chloride

concentration greater than approximately 0.2%. Motility was better in

a hypotonic medium of less than 0.2% sodium chloride content than in an

isotonic medium containing more than 0.2% sodium chloride[78].

    d. <u>Temperature</u>. As noted earlier, the use of low temperatures is a

convenient way to reversibly immobilize spermatozoa. Bhattacharya observed that the degree of motility of rabbit spermatozoa at low temperatures depended upon the medium in which the sperm were suspended[35].

The deleterious effect of sudden exposure of mammalian spermatozoa to low temperatures (cold shock) has been known for some time. Epididymal spermatozoa from ram, boar and bull apparently are more resistant to cold shock than are spermatozoa from the ejaculatory ducts[43]. Quinn and White recently have shown that cold shocking and deep freezing bull and ram semen caused an influx of sodium and an efflux of potassium and magnesium from the spermatozoa. They did not observe any significant changes in the cation content of human, dog, rabbit or fowl spermatozoa after cold shocking[192].

At the other extreme, a temperature of 48°C for 30 seconds caused a loss of motility of rabbit spermatozoa IN VITRO[265].

e. Stage of Maturation. Blandau and Rumery have also shown that rat spermatozoa suspensions prepared from the cauda epididymis and inseminated into the left uterine horn gave a 93% fertilization rate, whereas only 8% of ova were fertilized by suspensions of spermatozoa from the caput epididymis when injected into the right uterine horn of the same animals[43]. Most spermatozoa from the caput epididymis were actively motile but swam in circles because of retroflexion of the head and stiffness in the neck piece of the flagellum. They attributed the lower fertilization rate by these spermatozoa to the circular swimming pattern and suggested that it interfered with their ability to ascend to the ampulla of the oviduct[43].

Barack has shown that the amount of fluid produced within the testis of the mouse is sufficient to flush the tubal system at least once a day and that this fluid production is the major factor in the transport

of spermatozoa from the seminiferous tubules to the ductuli efferentes[19].

From their observations, Blandau and Rumery concluded that rat spermatozoa

pass through the epididymis passively because of peristaltic waves that

sweep over the tubules at a rate of approximately 8 per minute[43].

Other changes during maturation are considered in Section 4, Anti-

genicity.

f. Fluctuations in the Swimming Rates of Individual Spermatozoa. In

a study of fluctuations in the swimming rates of individual bull sperma-

tozoa at 37°C, Tampion reported that spermatozoa from some bulls swam at

a rate greater than 150 μ/sec in sperm extract[226]. The mean swimming

rate of straight and circling spermatozoa in 4 ejaculates were estimated

to be 133.4 μ/sec and 134.0 μ/sec, respectively. In a series of 4

experiments, 56-90% of the spermatozoa swam straight over a distance of

more than 310 microns, while 4-10% of the spermatozoa swerved to the

right or left. The remainder of the spermatozoa swam in polygonal pat-

terns. Spermatozoa diluted to a concentration of 5,000/μl with saline

had swimming rates ranging from 57.4 to 103.3 μ/sec. Variations within

individual groups ranged from 15-40 μ/sec. In addition to differences

in swimming rates among spermatozoa, individual spermatozoa changed ve-

locity considerably from time to time[226].

Tampion studied the effects of drugs and hormones on the swimming

performance of bull spermatozoa and showed that the most satisfactory

medium for achieving and maintaining a high rate of motility was 135 mM

NaCl[227]. The addition of fructose in concentrations of 5.0, 0.5 and 0.05

mg/ml to 135 mM NaCl had no significant effect on the swimming rate or on

its maintenance for 1 hour. In 135 mM NaCl, the mean swimming rates

ranged from 130.8-158.0 μ/sec. Despite the greater viscosity of both

fresh and frozen seminal plasma, the swimming rates, after 5 minutes of incubation in these media were still 110.2 ± 4.6 and 99.0 ± 12.6 μ/sec, respectively, compared to 139.2 ± 4.7 and 136.2 ± 7.2 μ/sec in 115 mM NaCl controls. Thus, there does not appear to be a significant inhibitory effect of fresh seminal plasma on motility.

3. Fertilizing Capacity

The fertilizing capacity of spermatozoa is acquired during passage through the epididymis. Orgebin-Crist found that spermatozoa from the caput and the proximal corpus epididymis were infertile when inseminated surgically into the uterus of does[180]. Insemination of does in a similar manner with spermatozoa from the distal corpus epididymis, and from the proximal and the distal cauda epididymis, gave fertilization rates of 57%, 92.7% and 95%, respectively. Compared to control ova fertilized with surgically inseminated ejaculated spermatozoa, the fertilization of ova was delayed significantly when distal corpus spermatozoa were inseminated. Another interesting observation was that spermatozoa isolated surgically in the proximal caput epididymis for 3-8 days developed their capacity for motility but did not develop fertilizing ability[180].

In another paper, Orgebin-Crist reported that, in does inseminated with spermatozoa from the lower corpus epididymis, only 52% of the ova that ultimately would be fertilized had been penetrated 16 hours after the injection of HCG[182]. In about half of these ova, the fertilizing spermatozoon was just penetrating the ovum at that time. Approximately 10% of ova fertilized with epididymal spermatozoa were polyploid. This was attributed to the delayed fertilization because the fertilization of aged rabbit ova is known to lead to polyploidy[182,212].

After separation of epididymis and testis, the fertilizing capacity

of rabbit spermatozoa lasted for 8-10 days, while motility lasted for 14-38 days, indicating that mature sperm can remain in the epididymis for 10 days without loss of fertilizing capacity[54]. Tesh and Glover observed that the fertilizing capacity of rabbit spermatozoa, which had been isolated in the epididymis for periods of up to 2 weeks, was apparently normal[228]. However, it was reduced greatly after 4 weeks and was completely lost after 7 weeks. Increased embryo mortality was observed before a significant failure in fertilization was detected[228].

Bedford compared the interaction between ova and spermatozoa taken from the vas deferens and the cauda epididymis by inseminating spermatozoa of each group into opposite Fallopian tubes in the same rabbits[28]. He found a significantly greater number of spermatozoa from the vas deferens than from the epididymis in association with ova. He suggested that, in the rabbit, capacitation invests spermatozoa with the competence to establish contact with the surface of the zona pellucida and to penetrate ova during the fertile life of the ovum[28].

Burfening and Ulberg incubated portions of split ejaculates at 38°C or 40°C for 3 hours and found no difference in the fertilizing capacity of rabbit spermatozoa incubated at the 2 temperatures[47]. However, the embryonic survival rate was higher in the uterine horns inseminated with semen incubated at 38°C than in the opposite horns inseminated with semen incubated at 40°C[47]. Asdell and Salisbury reported that the morphology of spermatozoa from rabbits in whom the testes had been placed in the abdomen surgically for varying periods of time showed increasing deterioration with time due to the higher temperature in the abdomen[12]. Thus, the short incubation period used by Burfening and Ulberg, although not changing the morphology or fertilizing capacity of the spermatozoa, had caused a more subtle change which showed up in the embryonic survival

rate.

Spermatozoa of normal rabbits retained their full fertilizing capacity in the female genital tract for only about 25 hours before ovulation and had lost fertilizing capacity after 32 hours[54]. McGaughey, Marston and Chang found the fertilizing life of mouse spermatozoa in the female genital tract to be approximately 12 hours[154]. According to the authors, a review of literature gives survival times of spermatozoa of other species in the respective females as follows: 14 hours in the rat, 22 hours in the guinea pig, 30 hours in the rabbit, 126 hours in the ferret, and 144 hours in the horse[154]. Fowl spermatozoa remain fertile in the genital tract of the female for several weeks and turkey spermatozoa retain their fertilizing capacity for at least 30 days[140,220].

The number of spermatozoa required for fertilization varies from one species to another. Even though it is estimated that only $1,000^{14}$ to $2,000^{3}$ spermatozoa reach the site of fertilization after normal mating in rabbits, it appears that with vaginal insemination, $0.5\text{-}1 \times 10^6$ spermatozoa are needed to achieve optimum fertilization results. In poultry husbandry, the usual minimum number of spermatozoa inseminated is 1×10^8 in a volume of approximately 0.1 ml[140]. With both species, high percentages of fertilization have been achieved with lower numbers of spermatozoa[56,140,262]. Additional considerations concerning artificial insemination in the rabbit are given in Appendix B.

4. Antigenicity.

The antigenicity of spermatozoa has been studied with regard to the significance of auto-antibodies against spermatozoa in the blood of men with obstructed vas deferens[186]; the presence of sperm agglutinins in human semen and blood[244]; a comparison of the specificity of antigens

in semen on spermatozoa and in blood of humans[160] and bulls[117]; the failure of the control of fertility in the rabbit by isoimmunization with seminal plasma[240], and success in the mouse[155,250]; and the induction of antibodies against spermatozoa in female rabbits by intravaginal or intramuscular injections of rabbit semen and bull semen[73]. Edwards, Ferguson and Coombs found human A and B blood group isoantigens on the spermatozoa of secretors but not on the spermatozoa of non-secretors[75]. The presence of the isoantigens M, N and Tja was established. However, the sex-linked antigen Xga could not be shown on the spermatozoa[75]. Boettcher found ABO antigens on seminal spermatozoa from secretors but not on spermatozoa from non-secretors, and concluded that the ABO antigens are adsorbed from the seminal plasma[44].

Although studies such as those mentioned in the above paragraph are of great interest and may eventually be incorporated into the experimental program in progress in this laboratory, the problem of antigenicity is of importance in current work only with regard to agglutination in experimental procedures. Because clumps of spermatozoa behave quite differently from individual spermatozoa during sedimentation and electrophoresis, it is desirable to avoid agglutination[158].

In a study of the agglutination of mammalian spermatozoa, Bedford found a tendency for washed ejaculate or epididymal spermatozoa of several mammalian species to agglutinate by the head region in physiological media such as Ringer's and Tyrode's solutions, 0.9% sodium chloride, and especially in either normal heated isologous, homologous, or heterologous sera[27]. In these aggregates, the sperm tails remained quite free and showed persistent, vigorous activity. He stated that tail-tail agglutination was never observed between mature rabbit spermatozoa in electrolyte solutions or in normal homologous or non-specific hetero-

logous sera. Tail-tail agglutination did occur readily when rabbit
or other spermatozoa were suspended in specific heterologous antisera.
He attributed this to antibodies against tail and head specific antigens
giving possible combinations of head-head, head-tail and tail-tail agglu-
tination. Thus, tail-tail agglutination in homologous and, especially,
isologous sera was considered to be due to specific antibodies against
tail antigens[27].

When mature rabbit spermatozoa were suspended in isotonic solutions
of sucrose, glucose or fructose at 37°C, the aggregation of motile sperm-
atozoa by the tail region similar to tail-tail agglutination seen in
specific antisera occurred rapidly[27]. In isotonic media of low electro-
lyte concentration, the surfaces of the sperm tails became attracted
not only to other tail surfaces, but also to particulate matter in the
medium. Materials that adhered to the surface of the tail never did so
to the surface of the sperm head. Addition of increasing amounts of
Ringer's solution to a suspension of motile spermatozoa in an isotonic
solution of sucrose or glucose caused a transition from the tail-tail
agglutination to head-head agglutination[27].

The agglutination properties of rabbit spermatozoa changed during
passage through the epididymis[27]. There was little tendency for head-
head agglutination of spermatozoa from the caput epididymis, but there
was for tail-tail agglutination in 0.145 M NaCl. Spermatozoa from the
corpus epididymis showed some tendency for head-head agglutination, but
much less so than spermatozoa from the cauda epididymis. Spermatozoa
from the cauda epididymis, vas deferens and mature, washed spermatozoa
from the ejaculate tended to agglutinate by the head region in homolo-
gous heated serum or in 0.145 M NaCl but showed no tail-tail agglutination[27].

Bedford concluded that the orientation of many caput spermatozoa in an electric field with heads pointing towards the anode indicated the existence of a relatively high net surface charge over the head region[24]. The change to tail anode orientation and the relatively higher electrophoretic mobility and speed of re-orientation of cauda spermatozoa under the same conditions reflected an absolute increase in the net negative surface charge over the tails during maturation of the spermatozoa. He raised the question of whether or not an absolute reduction in the negative surface charge density of the head region occurred during epididymal passage of the sperm. Spermatozoa failed to exhibit head-head agglutination in media giving head anode orientation in a microelectrophoresis cell using media of low ionic strength. This suggested that the increased net surface charge over the head region in all epididymal spermatozoa might exert a repulsive force great enough to negate any inherent attractive forces on the sperm surface[24]. However, the failure of caput spermatozoa to agglutinate and their weak head anode orientation response in high ionic strength media suggested that the failure to agglutinate also may be due equally to the absence of attractive forces over the immature sperm surface[24]. Bangham showed that with isotonic reduction in ionic strength of the surrounding medium, the increase in sperm surface potential was distinctly non-uniform in character and that the orientation of spermatozoa in the electric field changed from predominantly tail-anode to head-anode[18]. Bedford suggested that this change in surface property observed by Bangham reflected the interaction of free radicals, such as the amino or imino groups at the sperm surface, with solute molecules in the medium[27]. This occurred to a greater extent over the head region emphasizing the different characteristics of the sperm surface over the two regions[27].

5. Genetic Composition.

Somatic cells of the rabbit, bull and domestic cock normally contain 44, 60 and 78 chromosomes, respectively. Chiarelli, De Carli and Nuzzo present excellent pictures of the chromosomes of the rabbit in which it appears that the Y-chromosome is approximately one-quarter to one-third of the length of the X-chromosome[57]. Gustavsson presents photographs of chromosomes from cattle showing the normal patterns for both male and female with 60 chromosomes[96]. In addition, abnormal patterns with 59 and 58 chromosomes, show the replacement of 2 telocentric chromosomes by a long, sub-telocentric chromosome, and the replacement of 2 telocentric chromosome pairs by a sub-telocentric chromosome pair, respectively[96]. Thus, even though the organization of the chromosomes has changed, the total genetic composition is unchanged.

In regard to this paper, the interesting question is whether or not differences in the genetic composition of spermatozoa are reflected in differences in physical properties which could be detected by experimental techniques. The most obvious and most interesting difference in genetic composition is that between the X- and Y-chromosome bearing spermatozoa of mammals. Many attempts to segregate these 2 types of spermatozoa have been mentioned in Section B. As noted above, the sex-linked antigen Xga has not been shown to be present on spermatozoa.

Because all spermatozoa in the domestic fowl normally bear X-chromosomes, there is no opportunity to effect a fractionation of fowl spermatozoa based on sex chromosomes. Work with fowl spermatozoa was motivated by the availability of a line of cocks with spermatozoa, some of which have a translocation which was produced by X-irradiation with 4,000 r at the Department of Poultry Husbandry in Berkeley, California. The trans-

location, TN IV, has been demonstrated genetically and cytologically[120].
The semi-sterility of the translocation heterzygotes furnishes a simple
and practical means of detecting the translocation in spermatozoa. The
spermatozoa carrying the translocation apparently are motile and capable
of fertilizing ova, which, however, will give non-viable zygotes.

D. Summary

The interrelationships among the physical and biological properties
of spermatozoa and the important experimental variables, listed in Table
I, are too numerous to recapitulate in detail. It is sufficient to say
that the characteristics of the dilution medium affect the electrophor-
etic mobility, sedimentation rate and specific gravity as well as the
viability, intrinsic motility, fertilizing capacity and agglutination
of spermatozoa. In certain experimental systems, the spermatozoa con-
centration can jeopardize electrophoretic mobility and sedimentation
rate measurements and is also a critical factor in preserving the viabi-
lity, intrinsic motility and fertilizing capacity of spermatozoa. The
ionic strength, viscosity, and, to a lesser extent, the specific gravity
of the medium are affected by temperature and consequently influence
the results of electrophoresis and sedimentation experiments. Temper-
ature also influences the preservation of the vital functions of sperm-
atozoa IN VIVO and IN VITRO.

The time-dependence of the preservation of viability, intrinsic
motility and fertilizing capacity of spermatozoa is a primary considera-
tion in the design of experiments. If one is trying to exploit small
differences in sedimentation rate, electrophoretic mobility or specific
gravity among spermatozoa, relatively more time for experiments might be
necessary to achieve satisfactory resolution of the components. Thus,

the selection of media, spermatozoa concentration, temperature and experimental operating conditions are all related by time.

Finally, the external forces are determined primarily by the physical properties of the spermatozoa, and, in turn, influence the preservation of the vital functions of spermatozoa generally in a deleterious manner. Thus, as will become evident in the subsequent chapters, these interrelationships remind the author of one's attempting to put one too many marbles into a dish: "for every marble put in, one pops out".

In the review of the physical and biological properties in Sections B and C above, many possible methods were presented by which spermatozoa populations might be fractionated into groups with interesting biological differences. Gordon reported a partial dimorphism in the rabbit spermatozoa populations he studied, but found no correlation with the sex ratio of offspring from the insemination of electrophoretically fractionated samples[92]. Shettles reported differences in the size of human spermatozoa and speculated that the larger contained the X-chromosome[214]. Laird reported a correlation between the morphological features of bull spermatozoa with two-way electrophoretic migration in an apparatus similar to that used by Gordon[129]. However, only Bhattacharya has reported a correlation of the size of spermatozoa with the sex of offspring therefrom (large sperm give female offspring)[35].

The electrophoretic separation of X- and Y-chromosome bearing rabbit spermatozoa reported by Schröder[206,207] and by Gordon[91,92] has not been confirmed by other investigators. In fact, Bangham and others have shown that above pH 4, all spermatozoa have a net negative charge[18,174,233]. Bangham has shown slight differences in the electrophoretic mobility of head anode and tail anode ram and rabbit spermatozoa under extreme conditions

of pH and ionic strength[18]. The latter differences have not been studied
with regard to sex ratio of offspring. There are changes in the electro-
phoretic properties of rabbit spermatozoa during maturation[24], but these,
again, do not appear related to the properties of spermatozoa in the
ejaculate.

Bhattacharya showed that the faster sedimenting rabbit spermatozoa
gave predominantly female offspring after insemination[35]. Schilling,
working with bull spermatozoa, obtained slightly more female than male
offspring from the insemination of the faster sedimenting group of sperm-
atozoa[205], lending support to Bhattacharya's hypothesis. However, Bhatta-
charya and co-workers, in a study similar to that by Schilling, found
no difference in the sex ratio from the insemination of fast and slow
sedimenting groups of bull spermatozoa[37]. Recently, Bedford and Bibeau
found no correlation between the sedimentation rate of rabbit spermatozoa
and the sex ratio of offspring[29]. Thus, Bhattacharya's results with
rabbit spermatozoa and Schilling's work with bull spermatozoa have yet
to be duplicated.

Lindahl and Thunqvist have shown a difference between the specific
gravities of epididymal and ejaculate spermatozoa that does not have
much relationship to the fractionation of mature spermatozoa in the
ejaculate[139]. Benedict and co-workers have found light and heavy groups
of mature rabbit spermatozoa using a hypertonic equilibrium density gradient[32].
There is a suggestion that dead spermatozoa have a higher apparent buoyant
density in this hypertonic medium than do live spermatozoa[32]. Thus, the
heavy group may be an artifact due to uptake of the density gradient
material. The use of small, heavy molecules to prepare high specific
gravity density gradients may account for the great discrepancies in
the apparent buoyant densities of rabbit and bull spermatozoa mentioned above.

III. THE STAFLO METHOD AND FRACTIONATION PRINCIPLES

Contents

A. Introduction

In the previous chapter, electrophoretic mobility, sedimentation rate
and intrinsic motility were considered among the physical and biological
properties by which semen spermatozoa might be fractionated. One purpose
of this section is to describe the stable-flow free-boundary (Staflo) ap-
paratus and ancillary equipment used in this study. In so doing, the
flexibility of use of the Staflo apparatus, which makes it possible to
investigate the properties of spermatozoa mentioned above, either indivi-
dually or in various combinations, will be illustrated. The other objec-
tive of this section is to present a new approach to quantitate the design
and interpretation of Staflo experiments, including equations for deter-
mining migration distances and rates for particles in each collection
fraction in Staflo-sedimentation and -sedimentation-electrophoresis experi-
ments.

Except for the interesting ramifications of migration patterns caused
by the intrinsic motility of spermatozoa, and conceivably by other cells
or organisms with significant motility, the discussion of the Staflo method
and fractionation principles applies to all migrating species. In addition,
the fractionation principles, with slight modification, may serve as a
model for the analysis of component parts of migration patterns in other
fractionation devices.

B. The Staflo Method

The theory of the Staflo method has been described by Mel[156-158] and amplified recently with regard to the three dimensional aspects of flow by Tippetts[263] and by Tippetts, Mel and Nichols[229]. This section describes the different models of the Staflo apparatus and ancillary equipment used in the studies reported in subsequent chapters. In addition, a partial listing of the types of experiments which can be conducted with the Staflo apparatus is presented to demonstrate the versatility of the apparatus.

1. The Staflo Apparatus.

Figure 3 is a photograph of the 3 x 16 Staflo apparatus in a refrigerated room. The letters on Figure 3 correspond to those in Figure 4 which is a schematic drawing of the Staflo apparatus. The essential features of the Staflo apparatus are as follows: (A) A multi-syringe pumping system for introducing solutions into the flow-cell, (B) the flow-cell proper and (C) a multiple-tube collection system for solutions exiting the flow-cell. Ancillary equipment includes the electrical system consisting of electrode compartments, an electrode wash flow system (D), an electric power supply unit (E) and the Staflo optical system, (F) shown only in Figure 3. Each of these features and its modifications during the course of the study is discussed below.

a. Flow-Cells.

The key feature of the Staflo apparatus is the flow-cell proper, 3 types of which were used in the experiments discussed in subsequent chapters. A flow-cell consists of a flow (migration)-chamber and upper and lower electrode chambers separated by cellophane membranes (dialysis tubing). They are distinguished by the design of the inlet and outlet flow dividers. Greater importance is attached to the latter because it determines the extent of fractionation of the migration profile. The
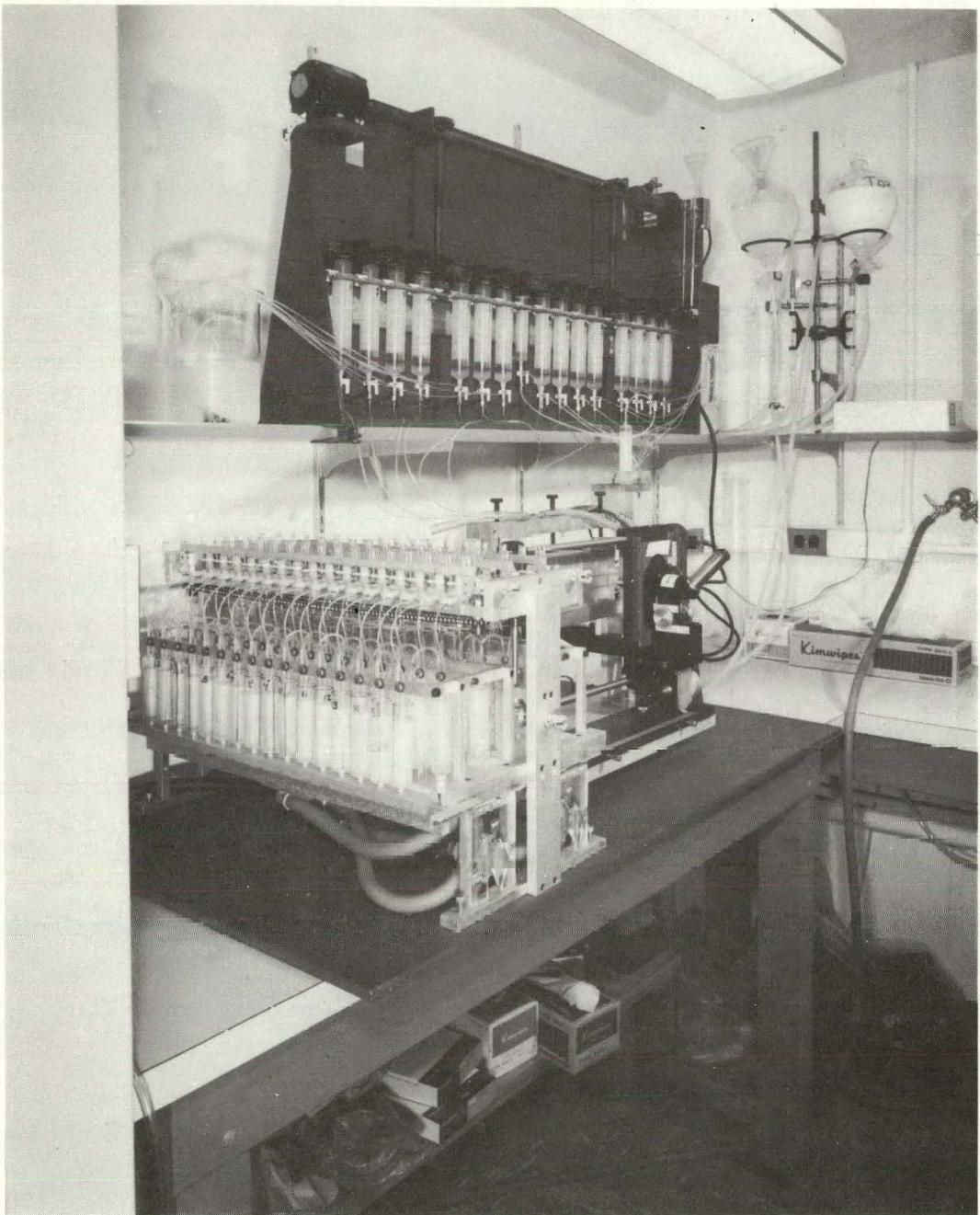
XBB 689-5751

Fig. 3. Photograph of 3 x 16 Staflo apparatus. Letters correspond to components similarly designated in Figure 4.
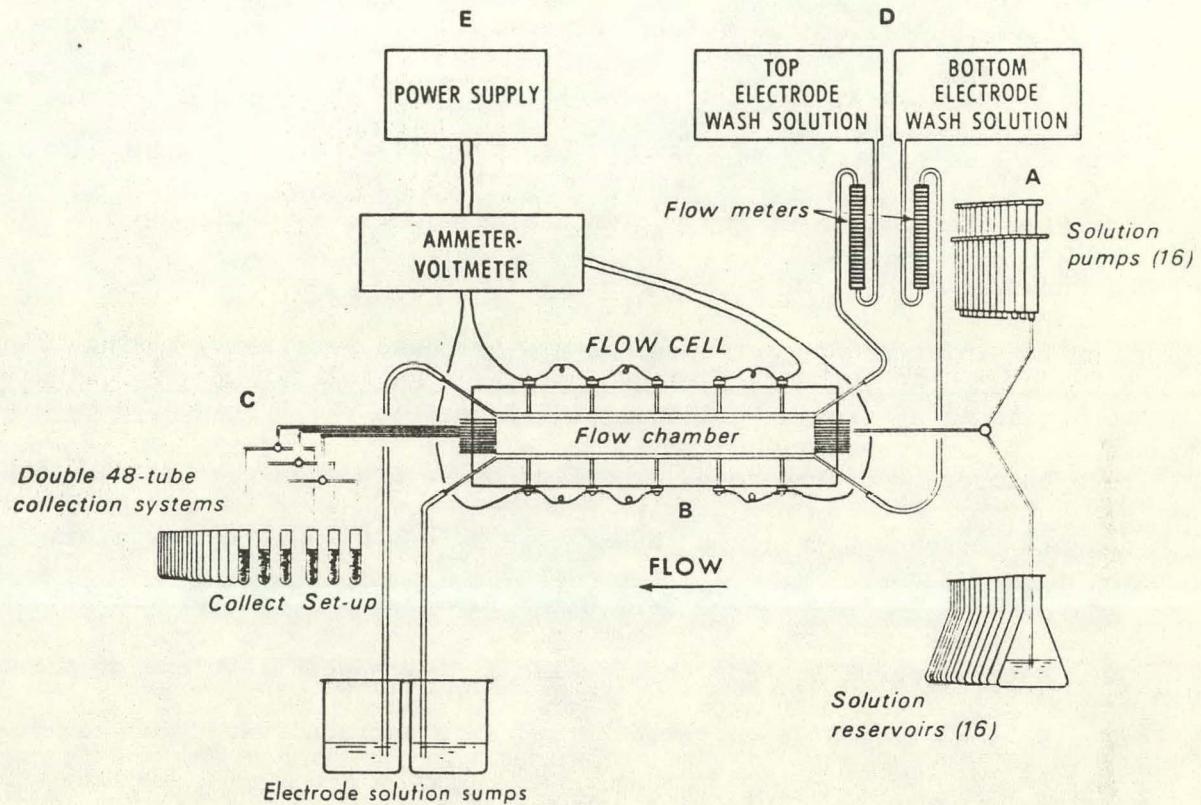
Fig. 4. Schematic diagram of Staflo apparatus showing main components.

characteristics of the 3 flow-cells used in this study (designated numbers VII C, XI and XIII), are presented in Table II. The flow-cells, including the flow dividers, were machined from lucite. On the newer flow-cells, a wall thickness of 0.8 mm was used to provide better observation with the microscope described below. Figures 5 and 6 show flow-cell number XI in place with and without the Staflo microscope, respectively. Figure 7 is a close-up photograph of the 3 x 16 flow-cell on its mount showing the flow-chamber, inlet and outlet tubing and wires to the top and bottom electrodes.

The inlet and outlet openings are arranged vertically giving 12 or 16 horizontally flowing 0.125 cm high layers in the 1.5 and 2.0 cm high flow-chambers, respectively. The flowing layers are stabilized by hydro-dynamic feedback principles rather than by use of a solid supporting medium[156,157]. The average horizontal flow rate is the same in all layers. If a heterogeneous suspension of cells is introduced into one of the upper layers, the gravitational force acting in a vertical direction can be expected to cause a differential downward migration of the cells[158]. This is illustrated in Figure 8 where a cell suspension introduced into layer 8 of the 3 x 16 Staflo apparatus gives a downward migration pattern over 3 layers.

The 1 x 12 flow-cell (No. VII C) was used in all of the early sedimentation experiments (Chapter IV) and some of the early electrophoresis experiments (Chapter V). The 3 x 12 flow-cell (No. XIII) was used only in 2 sedimentation experiments discussed in Chapter VIII. In all other Staflo experiments, the 3 x 16 flow-cell (No. XI) was used.

b. Pumping System and Sample Introduction. Different pumping systems were used with the 3 types of flow-cells described above. Although differing in design, the principle of operation is the same for all pumping

Table II. Characteristics of Staflo Flow-Cells

Design Features	Flow-Cell Type (Number)		
	1 x 12 (VII C)	3 x 12 (XIII)	3 x 16 (XI)
Flow-Chamber:			
Length (cm)	30	30	30
Width (cm)	0.7	0.7	0.8
Height (cm)	1.5	1.5	2.0
Inlet array:			
Horizontal (no.)	1	3	3[a]
Vertical layers (no.)	12	12	16
Layer height (cm)	0.125	0.125	0.125
Outlet array:			
Horizontal (no.)	1	3	3
Vertical layers (no.)	12	12	16
Wall thickness (cm)	1.33	0.8	0.8
Electrode chamber:			
Height (cm)	0.25	0.25	0.25
Number of electrodes	1	6	6
Electrode length (cm)	29.5	4.95	4.95
Electrode spacing (cm)	0	0.05	0.05

a. In practice, solutions were introduced only in the center inlet in each of the 16 layers.
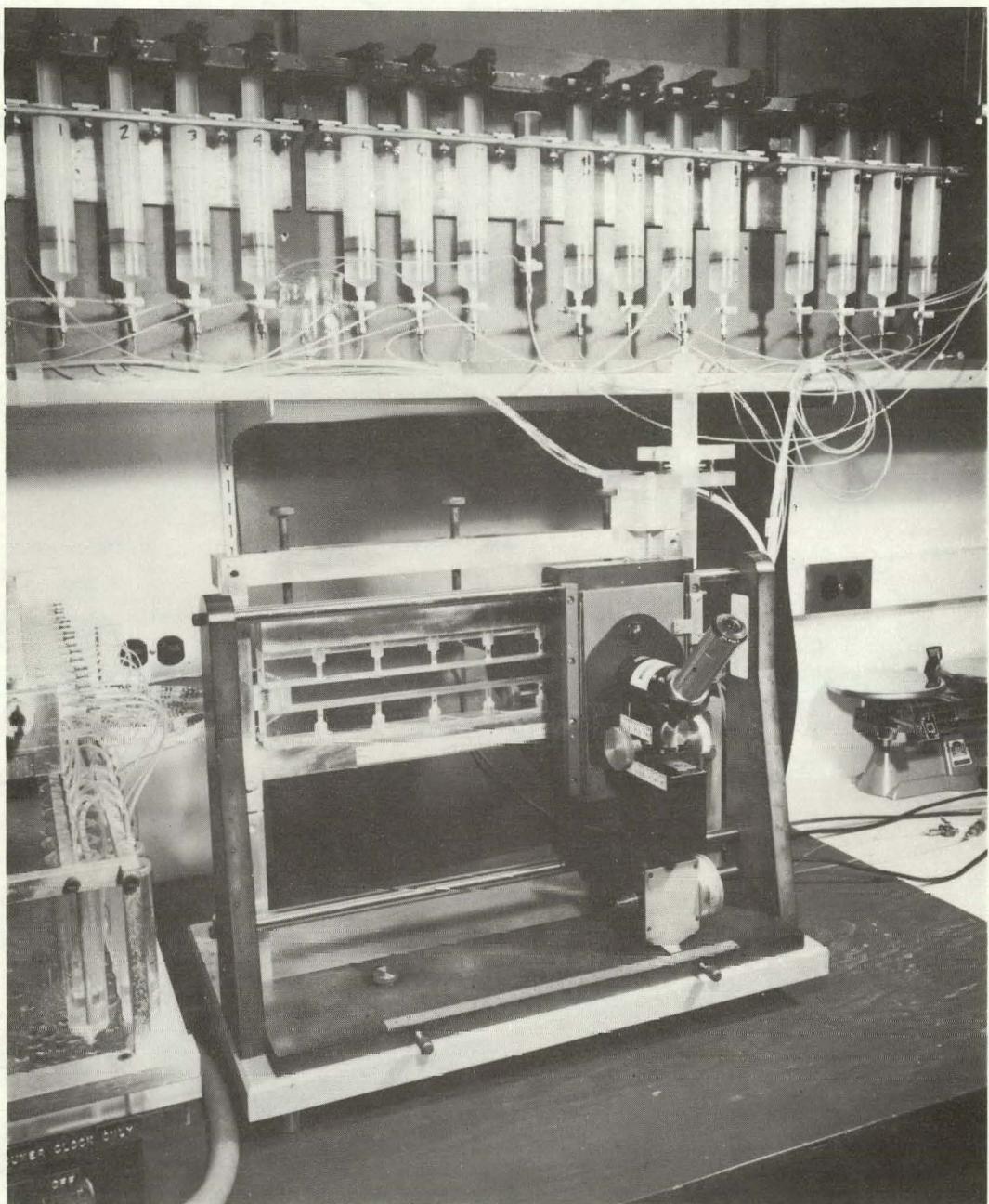
CBB 6812-7720

Fig. 5. Photograph of Staflo flow-cell and mount with microscope in place.
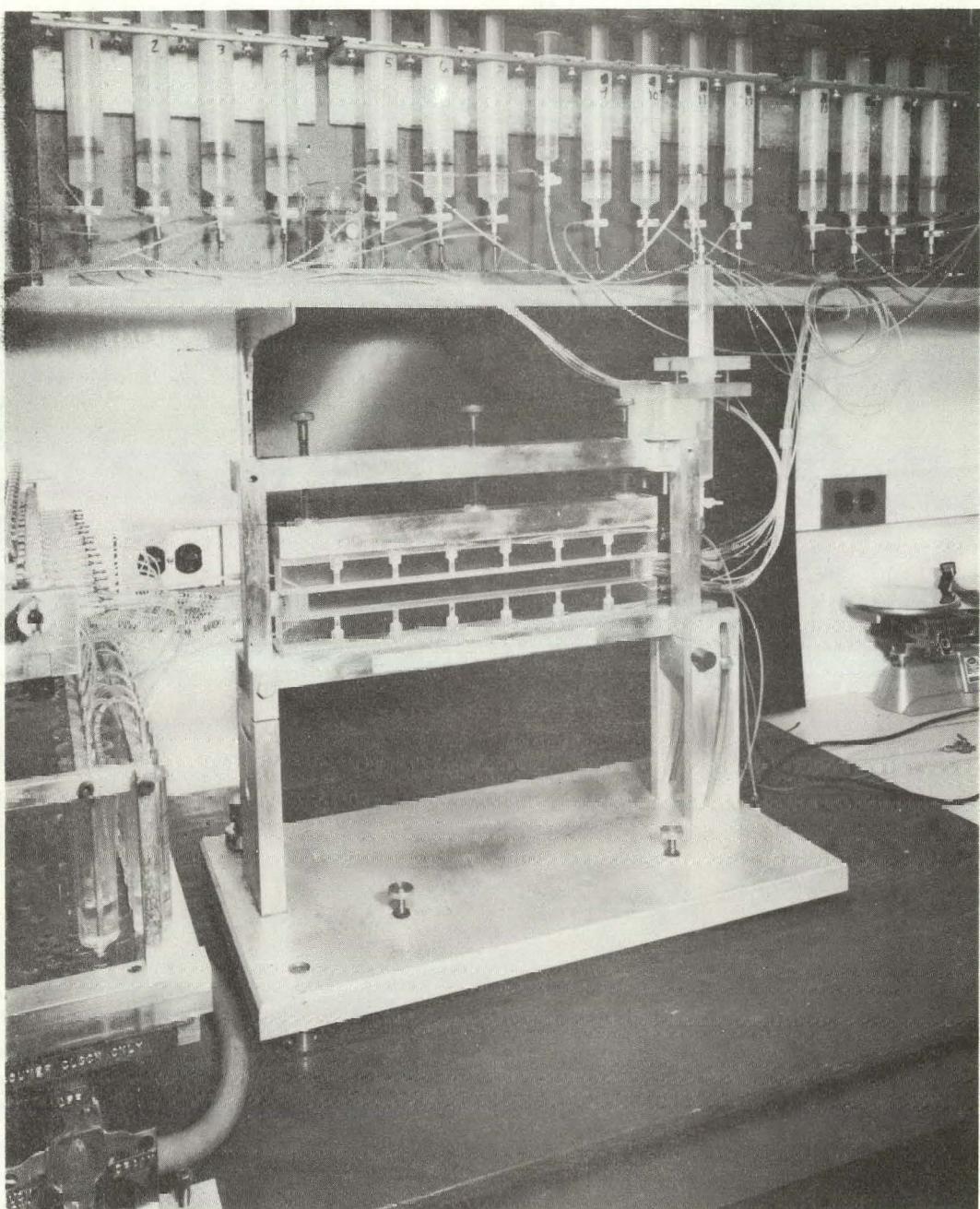
CBB 6812-7724

Fig. 6. Photograph of flow-cell and mount with microscope removed.
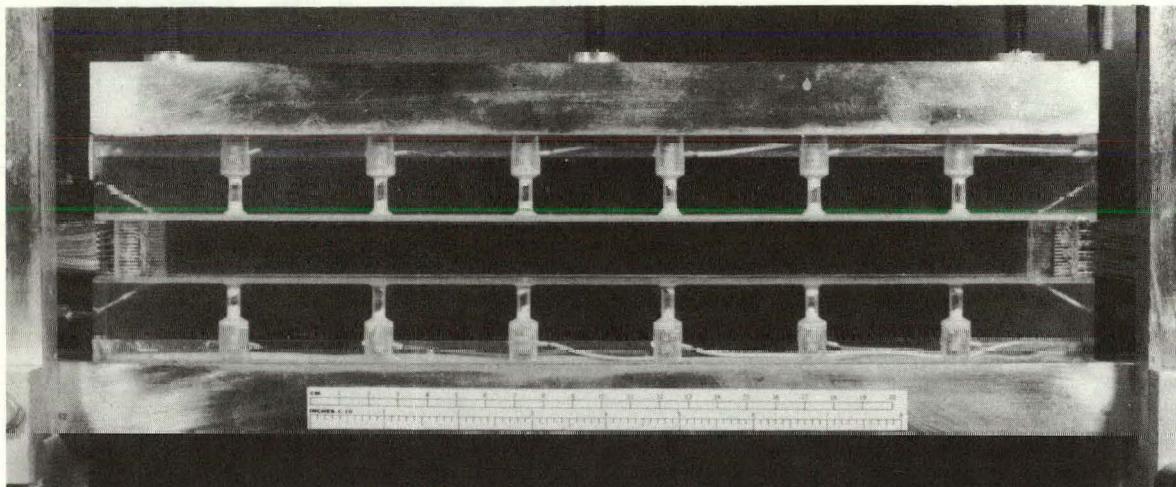
CBB 6812-7726

Fig. 7. Closeup of flow-cell showing flow-chamber and part of wiring to electrodes.

MEMBRANE　TOP ELECTRODE

OUTLET　INLET

FLOW

1
2
3
4
5
6
7
8
9
10
11
12

MAIN CHAMBER　BOTTOM ELECTRODE　MEMBRANE
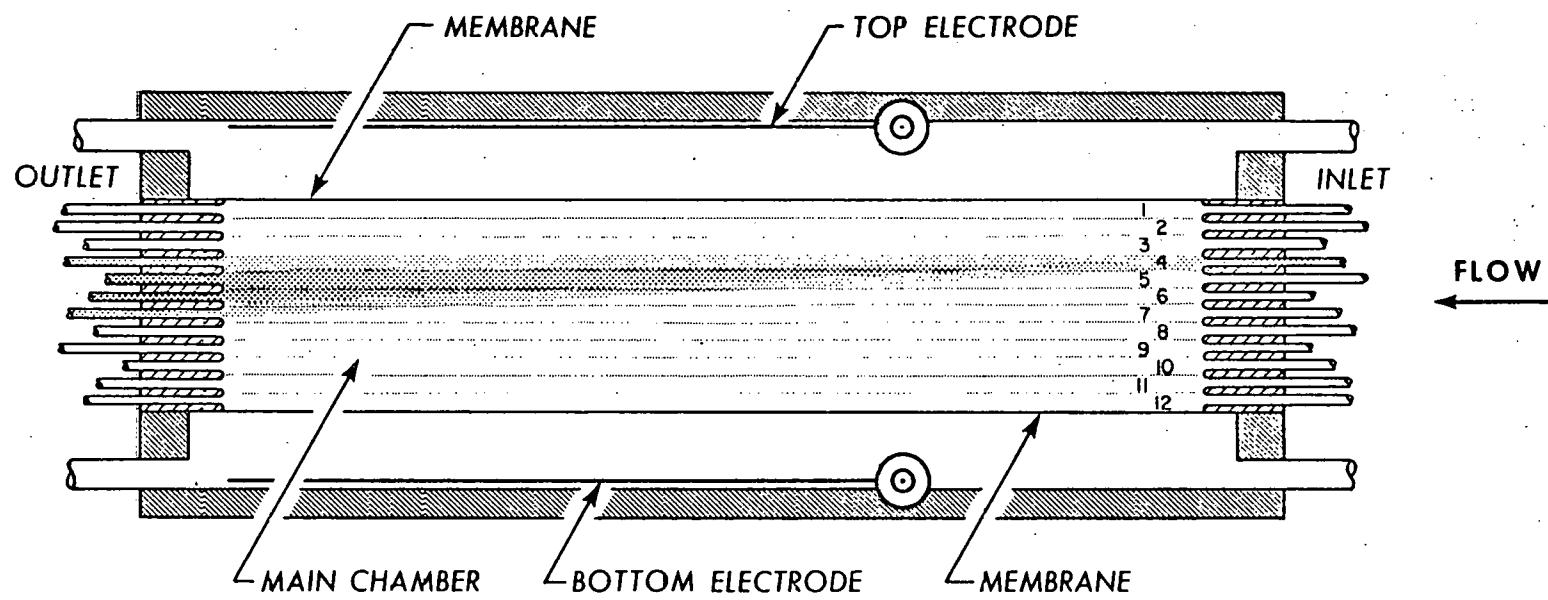
MU-32018-B

Fig. 8.　Schematic diagram of flow-cell showing spermatozoa migration pattern.

systems. Each system consists of a rack of plastic syringes containing solutions for the respective inlets on the flow-cell (see Figure 3). The rack is driven by a variable speed motor which allows a wide range of accurately controlled solution flow rates. The pumping system used with the 1 x 12 flow-cell consisted of a linear rack containing twelve 20 ml plastic syringes (TOMAC Disposable Syringe, American Hospital Supply Corp., Evanston, Ill.). It is similar in design to that used with the 3 x 16 flow-cell which is described in more detail below. The pumping system used with the 3 x 12 flow-cell holds thirty-six 20 ml plastic syringes.

The pump rack for the 3 x 16 flow-cell is shown in Figure 3 (A) and has holders for a linear array of sixteen 50 ml plastic syringes (PLASTIPAK, Discardit, 50 ml, Becton, Dickinson and Co., Rutherford, N.J.). The syringes are connected by three-way valves to the flow-cell inlets and to solution reservoirs. The pump rack is driven by a 1/70 horsepower, 0 to 1725-rpm shunt-wound electric motor (Bodine Electric Co., Chicago, Ill.), with a variable-speed power supply (Minarik Electric Co., Los Angeles, Calif., Model Sh-14). The low speed drive rate is controlled by a variable speed Zero-Max Torque Converter (Revco Incorporated, Minneapolis, Minn., Model 14 Standard) giving a solution flow rate range of 0.075-2.62 ml/minute/ inlet with 50 ml syringes. The pump rack can be driven in reverse to fill the syringes from solution reservoirs.

The sample suspension can be introduced into the flow-cell with one of the syringes on the pump rack giving a sample stream 0.125 cm. high. In order to preserve the viability of many biological species, it is desirable to reduce the length of inlet tubing by mounting the sample syringe near the inlet and controlling its flow rate from the pump rack. For greater resolution of the migrating particles, often it is desirable to reduce

the height of the sample stream by using a syringe size smaller than that used for the other 15 inlets[263]. For instance, 10 and 20 ml plastic syringes give solution flow rates approximately 37% and 52% that of the 50 ml plastic syringe, respectively. The corresponding sample streams would be 0.046 and 0.065 cm high compared to 0.125 cm in the other layers. This arrangement is shown in Figure 7 where syringe #8 on the rack has been replaced by a 20 ml plastic syringe which drives the 20 ml sample syringe mounted on the right side of the flow-cell near the inlet. Generally, magnetic stirring is used to maintain a uniform suspension in the sample syringe throughout the experiment.

c. Collection Systems. An adequate collection system for the outlet solutions from the flow-chamber is a level platform holding one plastic syringe barrel (with plugged tip) for each outlet tubing. This system has the shortest possible outlet tubing and was used effectively with the 3 x 12 and 3 x 16 flow-cells with 10 ml and 20 ml plastic syringe barrels, respectively.

If use of longer tubing is compatible with the type of particle being studied, operation of the Staflo apparatus is simplified by a valved collection system such as that shown for the 3 x 16 flow-cell in Figure 3. In this dual collection system, the outlet flow can be directed to either of the two sets of forty-eight 20 ml collection containers (plastic syringe barrels). One set is used for setting up ("SET UP" side) and the other for collection ("COLLECTION" side) of the fractionated sample. Prior to introducing the sample suspension, density gradient solutions are run to both the SET UP and COLLECTION sides to establish steady state conditions in the flow-chamber. This condition is ascertained by observing the level of entrance and exit of fluid in one layer. This is facilitated by introducing a blue dextran solution in the sample layer. Only enough solution

is run to the COLLECTION side to insure steady state conditions in order to minimize the volume of solution in the collection containers. Flow is switched to the SET UP side for the introduction of the sample suspension. After the sample stream has attained the desired migration pattern across the flow-cell and has been maintained for 1/6 of the average transit time across the flow-cell, the outlet flow is switched to the COLLECTION side. Thus, any initial instability can be corrected without contaminating the final collection fractions. Generally, only the center fraction of each layer is saved for evaluation, e.g., that coming from the middle outlet channel of the three horizontal ones in each layer.

Both sides of the collection system can be emptied rapidly by a vacuum system to facilitate cleaning in place. The central row of containers on the COLLECTION side can be removed for more thorough cleaning and for working with the fractionated samples.

d. Electrode/Electrical System. The electrical system for electrophoresis experiments consists of electrodes in the electrode chambers, a power supply unit and an electrode wash system. As noted above, the electrode chambers are separated from the flow-chamber by cellophane membranes (dialysis tubing). The electrode chambers are 0.25 cm deep and have the same length and width as the flow-chamber. On the 3 x 16 flow-cell, there are 6 platinum electrodes 0.8 cm wide and 4.95 cm long, leaving spaces of approximately 0.05 cm between adjacent electrodes. The 6 sections of electrode area can be controlled individually, giving the flexibility of a programmed electric field[263,229]. As discussed by Tippetts, a bubble forms in each of the 0.05 cm gaps due to extreme polarization at those sites and effectively insulates the shorted-out electrodes from the active electrophoresis electrodes[263]. Thus, one can get a time average uniform electric field with

high currents and without lethal polarization. Power is supplied to the electrodes by a regulated DC power supply (Power Designs, Inc., Model 5015S) monitored by a General Electric voltmeter and ammeter.

The electrode wash flow system consists of two 2,000 ml separatory funnels, acting as reservoirs, connected to the electrode chambers by plastic tubing. Solutions flowing in the top and bottom electrode chambers generally are the same as the solutions in layers 1 and 16, respectively, of the flow-chamber. The flow rates can be regulated over the range 0-34 ml/min with floating-ball flow meters (Fischer and Porter Co., Holbord, Pa.). Changes in pH and temperature in the flow-chamber due to the electric field can be controlled by increasing the electrode wash flow rate (EWFR) without excess hydrodynamic shock.

Temperature measurements were made in the 3 x 16 flow-chamber with a Varian Temperature Recorder connected to a probe at the level of layer 5 approximately 3 cm from the outlet. The results showed that the temperature rise in the flow-chamber depends upon the electric field strength, the solution flow rate and especially the EWFR.

With room and solution temperatures of 4-6°C, an average transit time of 21 minutes, an electric field over the last 25 cm of the flow-chamber giving a heat production of 1,200 watt-sec, and an EWFR of 20 ml/min, the maximum and average changes in temperature in the flow-chamber would be + 1.0°C and + 0.5°C, respectively. With rare exceptions, the operating conditions used in this study gave temperature rises of this or lesser magnitude.

e. Optical Equipment. The slit lamp technique was used for observations and measurements of the gross migration pattern and profile[229,263].

The acquisition of several long working distance objectives and a

reduction of flow-cell wall thickness made microscopic observations of cells in the flow-chamber possible. Initially, the microscopic system consisted of a horizontally mounted microscope and an independent light source. Because difficulties in alignment resulted in poor observation and were time consuming, the microscope shown in Figures 9 and 10 was constructed specifically for use with the Staflo apparatus. An adjustable light source, a condenser and the microscope were aligned on a carriage which can move laterally the length of the flow-chamber. Complete vertical movement and depth focusing makes it possible to observe and locate cells anywhere in the flow-chamber within the accuracy of the millimeter scales on all 3 axes of movement. Although excellent viewing was possible at magnifications up to 640 X, a magnification of 320 X with dark field illumination was generally used for observing spermatozoa.

The stand to which the microscope carriage is attached can be fastened by set screws to the Staflo mount to prevent inadvertent movement and is interchangeable with all current Staflo apparatuses.

2. Modes of Operation

The versatility of use of the Staflo apparatus in electrophoresis, sedimentation and sedimentation-electrophoresis experiments has been described by Mel[158]. With only a change in the medium used in the flow-chamber, a variety of studies can be conducted for either analytical or preparative purposes. The experimental design considerations presented in Chapter II with regard to studies on spermatozoa are applicable to, and place many restrictions upon, Staflo experiments with those biological cells. This section presents general experimental design considerations pertaining to different modes of operation of the Staflo apparatus without regard to the type of cell being studied.
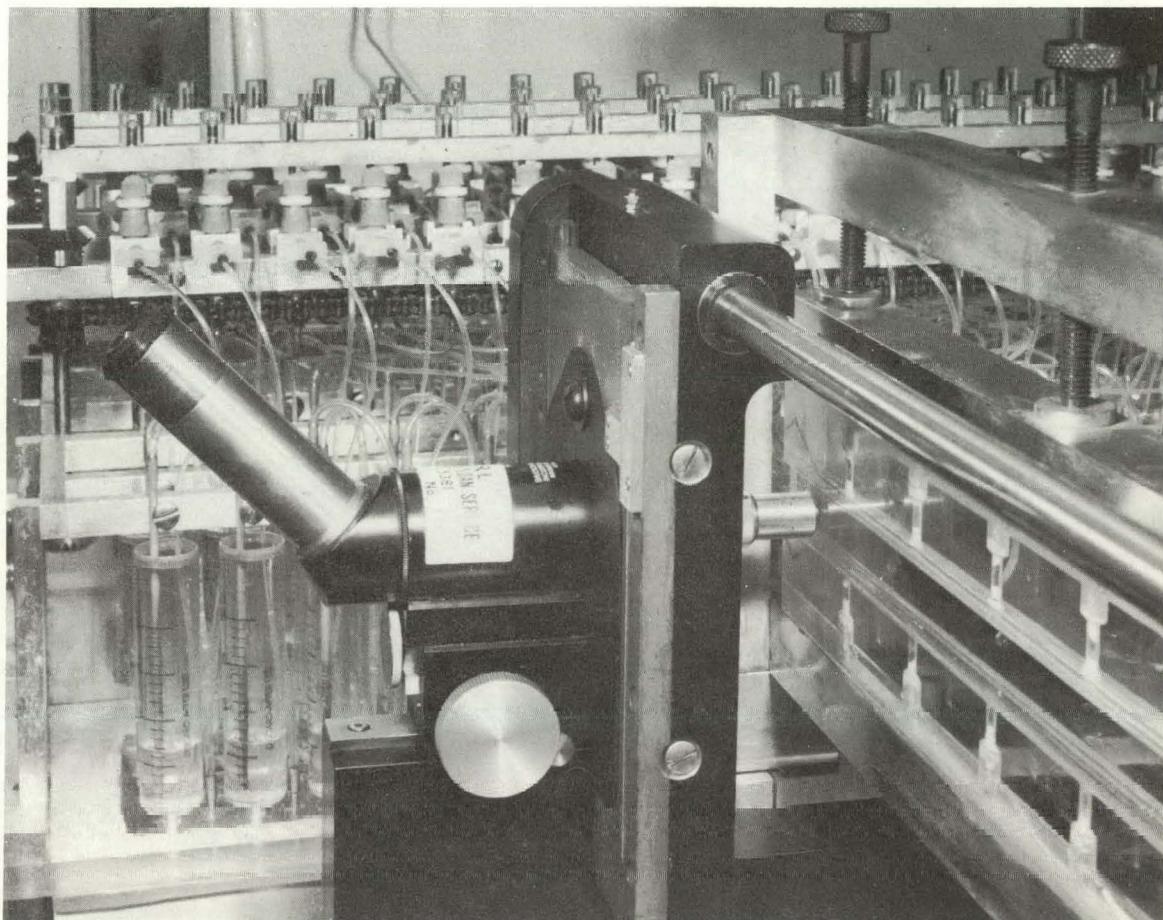
CBB 6812-7722

Fig. 9. Closeup of Staflo microscope mounted on Staflo apparatus.
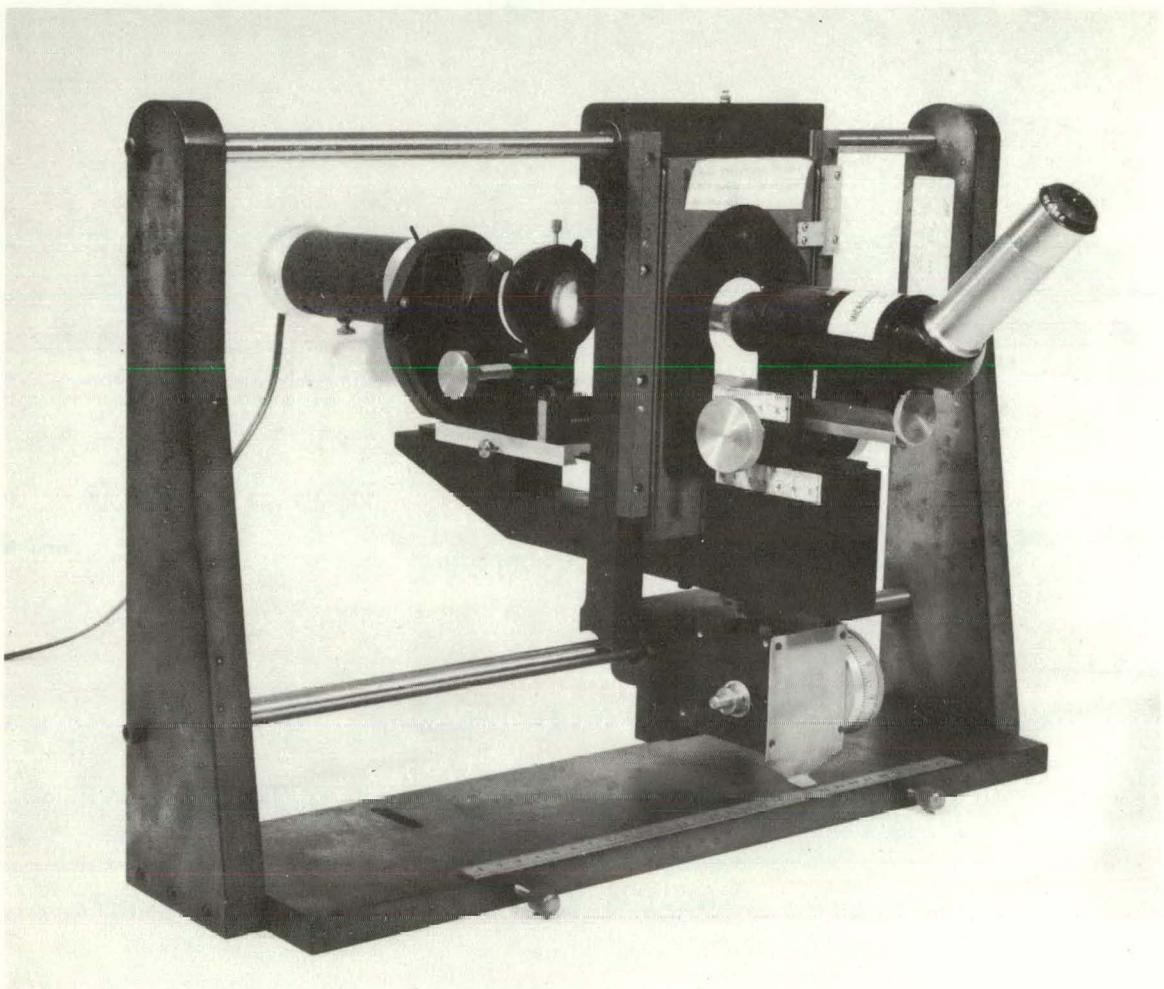
XBB 689-5752

Fig. 10. Photograph of Staflo microscope showing component parts.

a. <u>Types of Experiments</u>. The types of Staflo experiments which can be conducted with populations of cells or particles include:

(1) 1g sedimentation

(2) Electrophoresis with upward and/or downward migration

    (a) Single pH

    (b) Two or three pH regions within the Staflo chamber

    (c) pH gradient

    (d) Conductivity gradient

(3) Sedimentation-upward-electrophoresis (SUE)

(4) Sedimentation-downward-electrophoresis (SDE)

(5) Exploitation of intrinsic motility, alone or in combination with sedimentation, electrophoresis, or both.

b. <u>Experimental Variables</u>. Experimental variables and the ways in which they influence each of the above groups of Staflo experiments are as follows:

(1) <u>Transit time</u>: Under a fixed set of conditions, the migration distance will be proportional to the transit time in all types of experiments. The appropriate transit time for any one experiment will depend upon the specific migration properties and the requirements for the preservation of vital functions of the species being studied.

(2) <u>Staflo medium</u>: The specific gravity of the medium will determine whether one gets sedimentation and/or flotation of particles and the degree thereof depending upon the difference between the specific gravities of the medium and the particles. Because of the limited height of the flow-chamber, large differences between the specific gravities of the particles and the medium would give high migration rates for all particles, and consequently, poor resolution in fractionation. Ideally, an intermediate specific gravity would give flotation of the light particles and sedimentation of the heavy particles in the population. The osmolarity

of the medium may influence the specific gravity of cells through changes in hydration.

Increases in viscosity of the medium will decrease all migration rates, thereby requiring longer transit times, especially in sedimentation experiments. Results from experiments with media of different viscosities can be normalized for comparison.

In aqueous solutions, the effects of changes in temperature are primarily reflected in changes of viscosity and conductivity; relatively smaller changes occur in specific gravity. The changes in conductivity are very important in electrophoresis experiments so the temperature and the ionic strength of the medium must be known.

For microscopic observations in the flow-chamber, a clear medium must be used.

The pH of the medium primarily affects the electrophoretic mobility of the migrating species and the conductivity of the medium and thus, is important in electrophoresis and sedimentation-electrophoresis experiments. Its effect on the vital functions of cells should be considered, also.

(3) Density gradient: Materials added to the medium in low concentrations to give a density gradient usually do not cause significant changes in the specific gravity. Sucrose, a common density gradient constituent, increases osmolarity with little effect on viscosity, while dextran has the opposite effect. These substances and other common density gradient materials have little effect on conductivity and pH other than through volume changes. Too large an increment between layers can give a density shelf which acts as a barrier to migration of particles. However, diffusion of the density gradient molecules tends to minimize this problem, especially if a low molecular weight substance such as sucrose is used for the density

gradient[157].

In sedimentation-upward-electrophoresis experiments, large density gradient increments may maintain the differential migration achieved against gravity by preventing convection[263].

(4) Temperature: In addition to its effects on the medium as noted above, the temperature has a strong influence on the intrinsic motility of spermatozoa and other motile organisms. As the temperature approaches 0°C, spermatozoan motility ceases. Low temperatures also aid in the dissipation of heat generated by the electric field.

(5) Electric field: The strength of the electric field determines the rate of electrophoretic migration and dictates the transit time. It also affects the temperature in the flow-chamber. A programmed electric field can be used for special purposes[229,263].

c. Interrelationships Among Variables. As noted in Chapter II, the viability, motility and fertilizing capacity of spermatozoa are affected strongly by the characteristics of the medium. If a migrating species has both a significant sedimentation rate and electrophoretic mobility, the relative contributions of each can be controlled most readily by variations in the electric field strength. If it is desired to minimize or eliminate the sedimentation component of migration in electrophoresis experiments, one can increase the specific gravity and viscosity of the medium, increase the density gradient or reduce the transit time. To do the latter, the electric field strength may have to be increased to get sufficient migration of the particles.

In experiments combining electrophoresis and/or sedimentation, and/or intrinsic motility, all of these variables must be considered in terms of their effects on the physical and biological properties of the migrating

species and on the physical stability of the experimental system. These interrelationships are illustrated in Chapters IV, V, VI and VIII.

C. Staflo Migration-Fractionation Principles

As noted above, the Staflo migration profile is fractionated at the outlet flow divider into 0.125 cm high layers which are collected separately. In addition to evaluating the particles in each collection fraction for differences in morphology, size and biological activity, it is often desirable to characterize each fraction by an average and range of sedimentation rates or electrophoretic mobilities. The limits of migration can be measured with the slit lamp technique[229] and intermediate measurements are calculated from the known positions of the outlet flow dividers. The figures presented in this section show the range and distribution of migration distances in the flow-chamber and the interplay of various combinations of sedimentation, electrophoresis and intrinsic motility. Expressions for calculating the minimum and maximum migration distances for particles in each layer in sedimentation and sedimentation-electrophoresis experiments are developed below. In addition, equations for calculating the average migration rates for particles in each layer are presented for populations having a uniform distribution of migration rates. These equations are applicable to the fractionation of all types of particles in the Staflo apparatus within the limitations upon which they are based. Only migration profiles exiting the flow-chamber in 2 or 3 adjacent layers are considered but the equations can be readily extended to 4 or more adjacent layers. The special problem of the effect of the intrinsic motility of spermatozoa on sedimentation and sedimentation-electrophoresis migration patterns is considered separately.

1. Sedimentation Experiments.

Figures 11 and 12 are schematic diagrams of Staflo-sedimentation migration patterns spreading over 2 and 3 adjacent layers, respectively. Figures 11A and 12A depict sedimenting particles exiting from the layer in which they enter and 1 or 2 adjacent layers below, respectively. Figures 11B and 12B show general conditions in which the particles exit the flow-chamber in any 2 or 3 adjacent layers below the inlet layer, respectively. The following discussion develops equations for calculating (1) the range and distribution of sedimentation migration distances, (2) the average migration distances for the appropriate collection fractions and (3) the weighted average sedimentation distance for the migration pattern as a whole for each of the conditions shown in Figures 11 and 12.

a. Migration Distances. Because the average sedimentation migration distance for most collection fractions is not a simple average of the minimum and maximum migration distances, expressions for these distances will be developed for migration patterns spreading over 2 or 3 adjacent layers. Then expressions for the average migration distances will be developed for the same migration patterns.

Assumptions applicable to both 2- and 3-layer migration patterns are as follows: (1) The bottom of the inlet sample stream is at the bottom of the inlet layer under steady state conditions. (2) The maximum migration distance in layer 1 is less than or equal to h, the height of the sample stream at the inlet. (3) Upon entry into the flow-chamber, the sample suspension is homogeneous. (4) The leading (bottom) edge of the migration pattern is determined by the fastest migrating particles, some of which were at the lower border of the sample stream at the inlet. (5) The top edge of the migration pattern is determined by the slowest migrating particles, some of which were at the upper border of the sample

STAFLO "MIGRATION PATTERNS"
Sedimentation and sedimentation-downward-electrophoresis

Collection fraction

A. Two layers; 1 and 2

Migration distance of leading edge (mm)

1
2
3, ...
n−1
n

h
0
1.25
1.25 + h
(n−3) × 1.25
(n−3) × 1.25 + h
(n−2) × 1.25
(n−2) × 1.25 + h

B. Two layers; n−1 and n

1
2
3, ...
n−1
n

h
0
1.25
1.25 + h
(n−3) × 1.25
(n−3) × 1.25 + h
(n−2) × 1.25
(n−2) × 1.25 + h
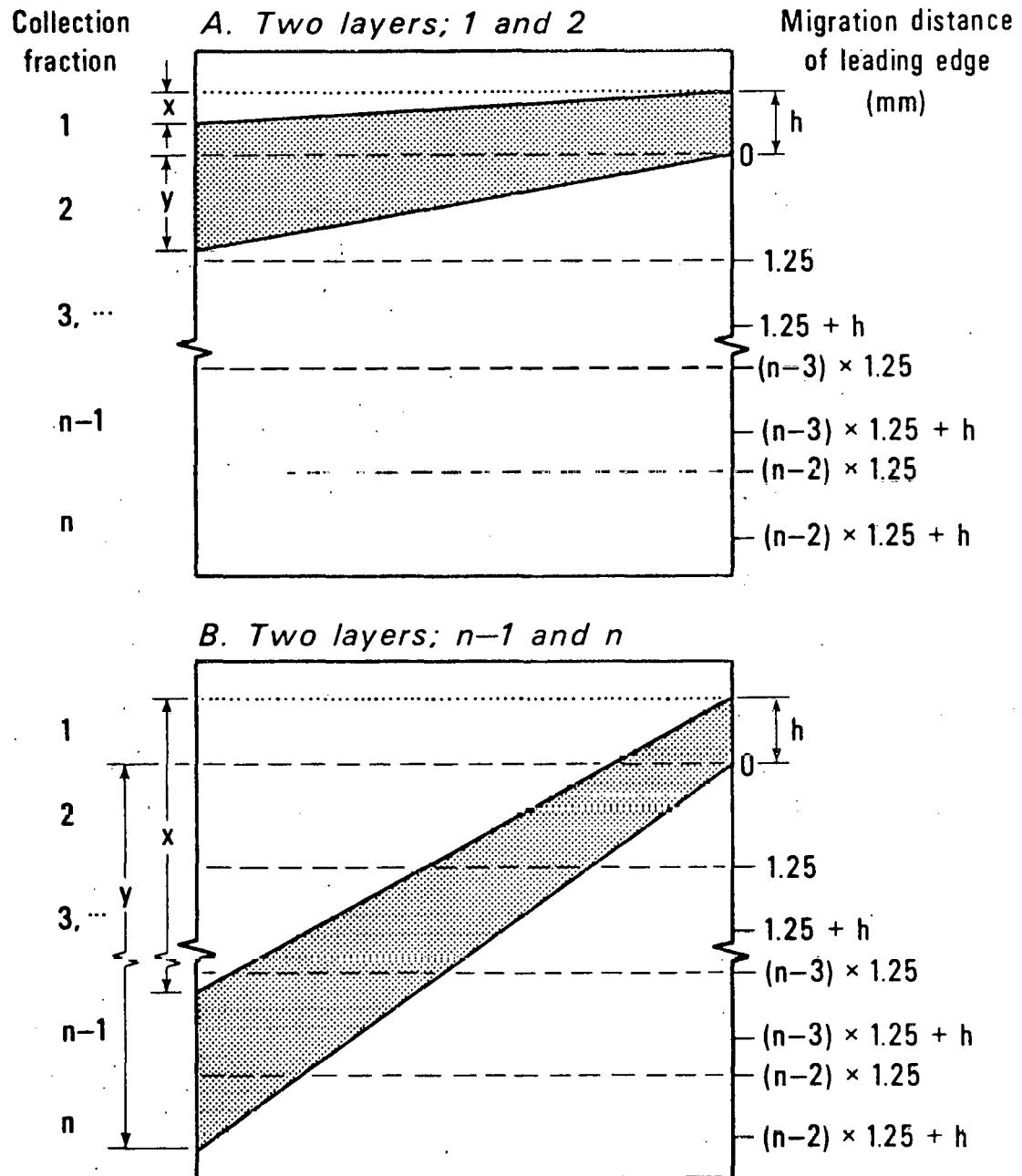
DBL 689-5473

Fig. 11. Schematic diagram of sedimentation or sedimentation-downward-electrophoresis migration pattern; two-layer spread. A. Exit in layers 1 and 2. B. Exit in layers n-1 and n.

STAFLO "MIGRATION PATTERNS"
Sedimentation and sedimentation-downward-electrophoresis

A. Three layers; 1, 2 and 3

Collection fraction

Migration distance of leading edge (mm)

1

2

3, ⋯

n−1

n

x

y

h

0

1.25

1.25 + h

$(n-3) \times 1.25$

$(n-3) \times 1.25 + h$

$(n-2) \times 1.25$

$(n-2) \times 1.25 + h$

B. Three layers; n−2, n−1 and n

1

2, ⋯

n−2

n−1

n

x

y

h

0

1.25

$(n-4) \times 1.25 + h$

$(n-3) \times 1.25$

$(n-3) \times 1.25 + h$

$(n-2) \times 1.25$

$(n-2) \times 1.25 + h$
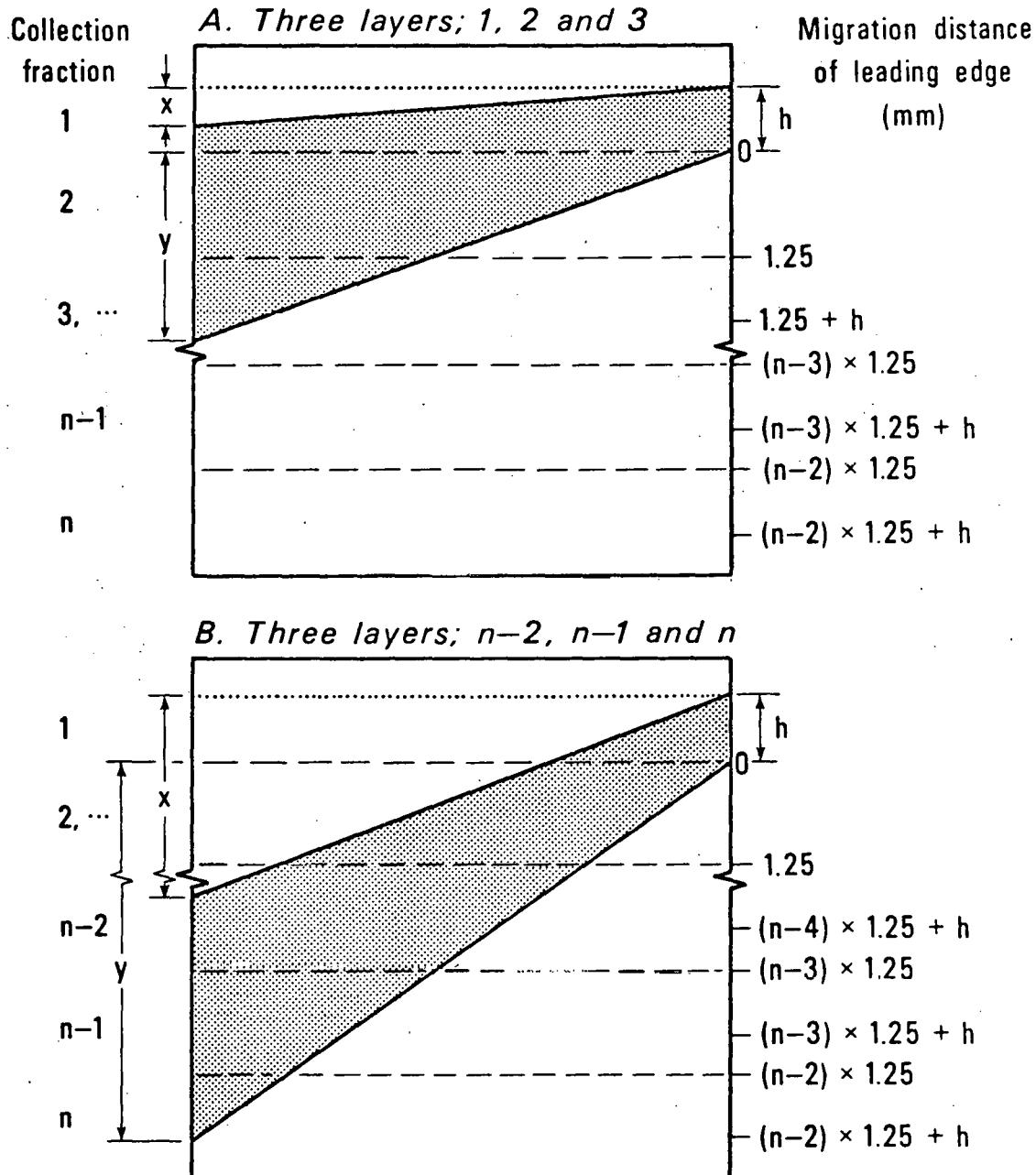
DBL 689-5474

Fig. 12. Schematic diagram of sedimentation or sedimentation-downward-electrophoresis migration pattern; three-layer spread. A. Exit in layers 1, 2 and 3. B. Exit in layers n-2, n-1 and n.

stream at the inlet.

(1) <u>Minimum and Maximum Migration Distances</u>. Referring to Figure 11A, the minimum migration distance for both collection fractions in the 2-layer migration pattern is x. The maximum migration distance in both fractions depends upon the distance the particles migrate into layer 2 since, by reason of assumption (4), this is the maximum sedimentation distance for the population of particles. Thus, if $y < h$, y is the maximum migration distance for both fractions. If $y > h$, the maximum migration distances are h and y for particles in fractions 1 and 2, respectively. These migration distances are summarized in Appendix E1.

The minimum and maximum migration distances for the collection fractions of the 3-layer migration pattern shown in Figure 12A are determined as follows:

Fraction 1: The minimum and maximum migration distances are x and h, respectively.

Fraction 2: The minimum migration distance is x. As in the case of fraction 1 for a 2-layer migration pattern, the maximum migration distance for particles in fraction 2 depends upon the relationship between the distances h and y.

If $(y - 1.25) < h$, $\lambda_{2_{max}}$ = y mm. If $(y - 1.25) > h$, $\lambda_{2_{max}}$ = 1.25 + h mm.

Fraction 3: The minimum and maximum migration distances are 1.25 mm and y mm, respectively.

These migration distances are summarized in Appendix E2.

The same principles apply to the determination of the minimum and maximum migration distances for particles exiting in layers n-1 and n in Figure 11B and n-2, n-1 and n in Figure 12B. These expressions are also summarized in Appendices E1 and E2, respectively.

(2) <u>Range and Distribution of Migration Distances</u>. An understanding of the range and distribution of sedimentation distances characterizing each of the collection fractions is helpful in developing equations for the average migration distances. In Figures 13 and 14, the abscissa represents the total range of migration distances and the ordinate represents the migration distance of the leading edge of the migration pattern starting at the bottom of the sample stream at the inlet (see assumption (1)). Thus, assuming a homogeneous distribution of particles in the sample stream at the inlet, particles characterized by a specific migration distance will be found in a column "h" mm high with the bottom of the column at the intersection of the lines representing that migration distance drawn from both the ordinate and the abscissa. For example, referring to Figure 13A, particles characterized by a sedimentation velocity that gives a maximum sedimentation distance of 1.25 mm will be distributed throughout the bottom h mm of layer 2. It should be noted that in Figures 13 and 14, the migration distances x and y are both drawn from the origin (o) even through they are measured differently in Figures 11 and 12.

Figure 13A shows that the particles characterized by the slowest sedimentation velocity (migration distance x') are distributed between layers 1 and 2 in inverse proportion to the ratio $\frac{x'}{h}$. If x' = 0, all of the particles will be in layer 1; whereas, if x' = h, all of the particles will be in layer 2. For $0 < x' < h$, the percentage of particles in layer 1 characterized by a sedimentation velocity giving a migration distance x' is $(1 - \frac{x'}{h}) \cdot 100\%$ and in layer 2, $\frac{x'}{h} \cdot 100\%$. This relationship is more obvious in Figure 15 which presents the same information as Figures 13 and 14 in a different manner. The area of the distribution band falling within each collection fraction in Figures 13 and 14 is shown in Figure

RANGE AND DISTRIBUTION
OF STAFLO MIGRATION DISTANCES
Sedimentation and
sedimentation-downward-electrophoresis

A. Two layers; 1 and 2

B. Two layers; n−1 and n

Limits of migration profile
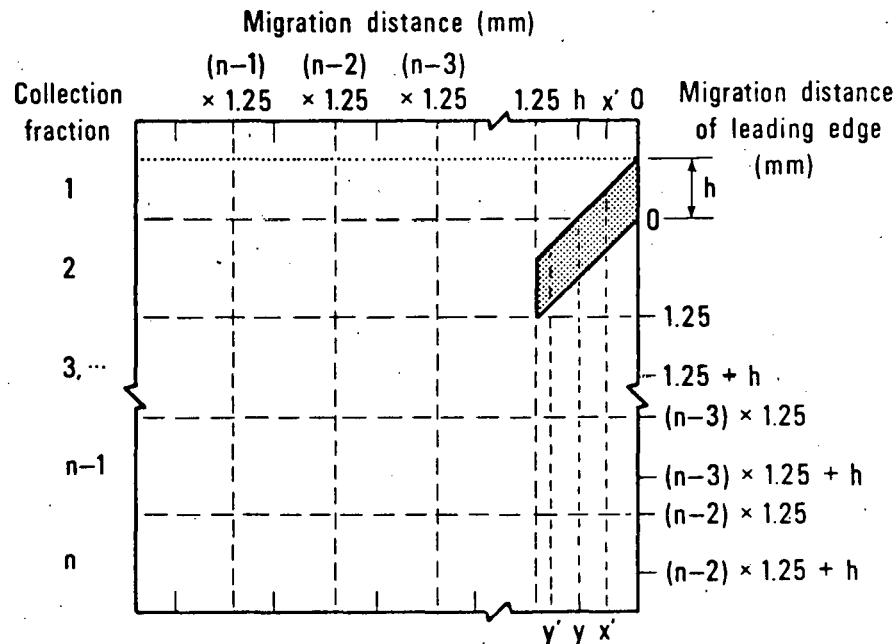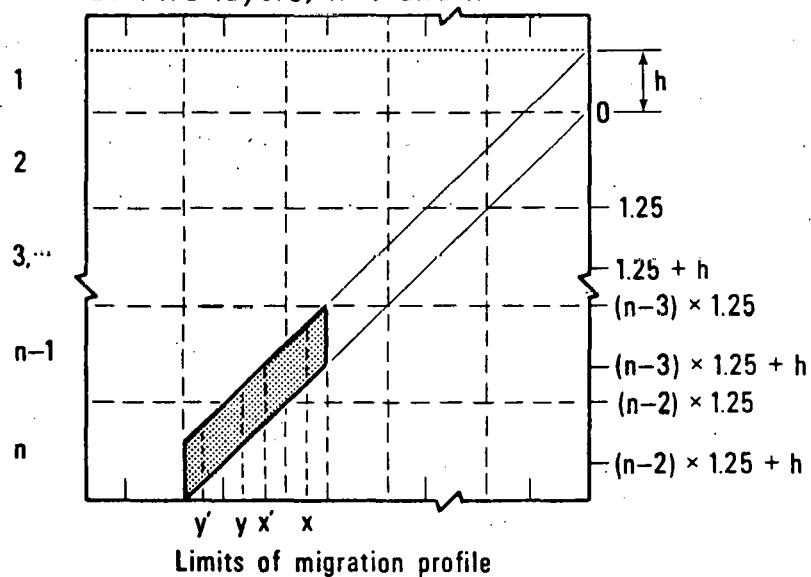
DBL 7011 5968

Fig. 13. Range and distribution diagram for migration distances in
sedimentation and sedimentation-downward-electrophoresis
experiments; two-layer spread. A. Exit in layers 1 and 2.
B. Exit in layers n-1 and n.

RANGE AND DISTRIBUTION
OF STAFLO MIGRATION DISTANCES
Sedimentation and
sedimentation-downward-electrophoresis

A. Three layers; 1, 2 and 3

B Three layers; n-2, n-1 and n

Limits of migration profile
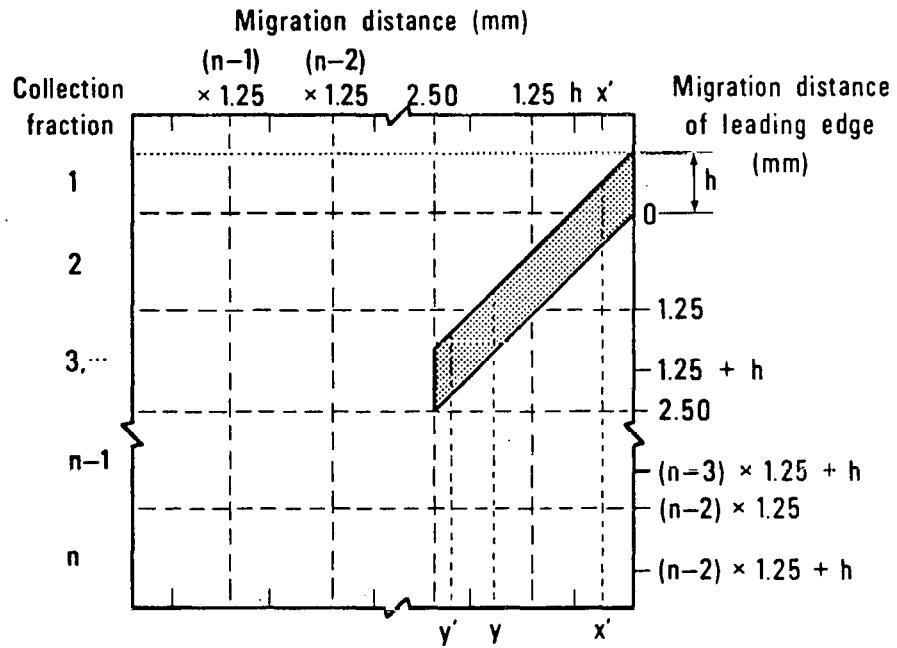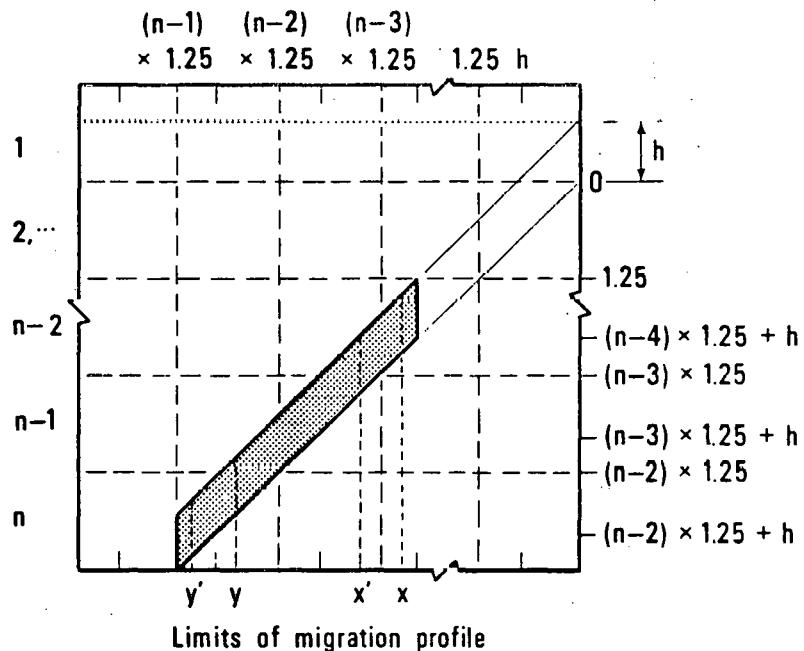
DBL 7011 5969

Fig. 14. Range and distribution diagram for migration distances in sedimentation and sedimentation-downward-electrophoresis experiments; three-layer spread. A. Exit in layers 1, 2 and 3. B. Exit in layers n-2, n-1 and n.

RANGE AND DISTRIBUTION
OF STAFLO MIGRATION DISTANCES
Sedimentation and
sedimentation-downward-electrophoresis

Migration distance (mm)
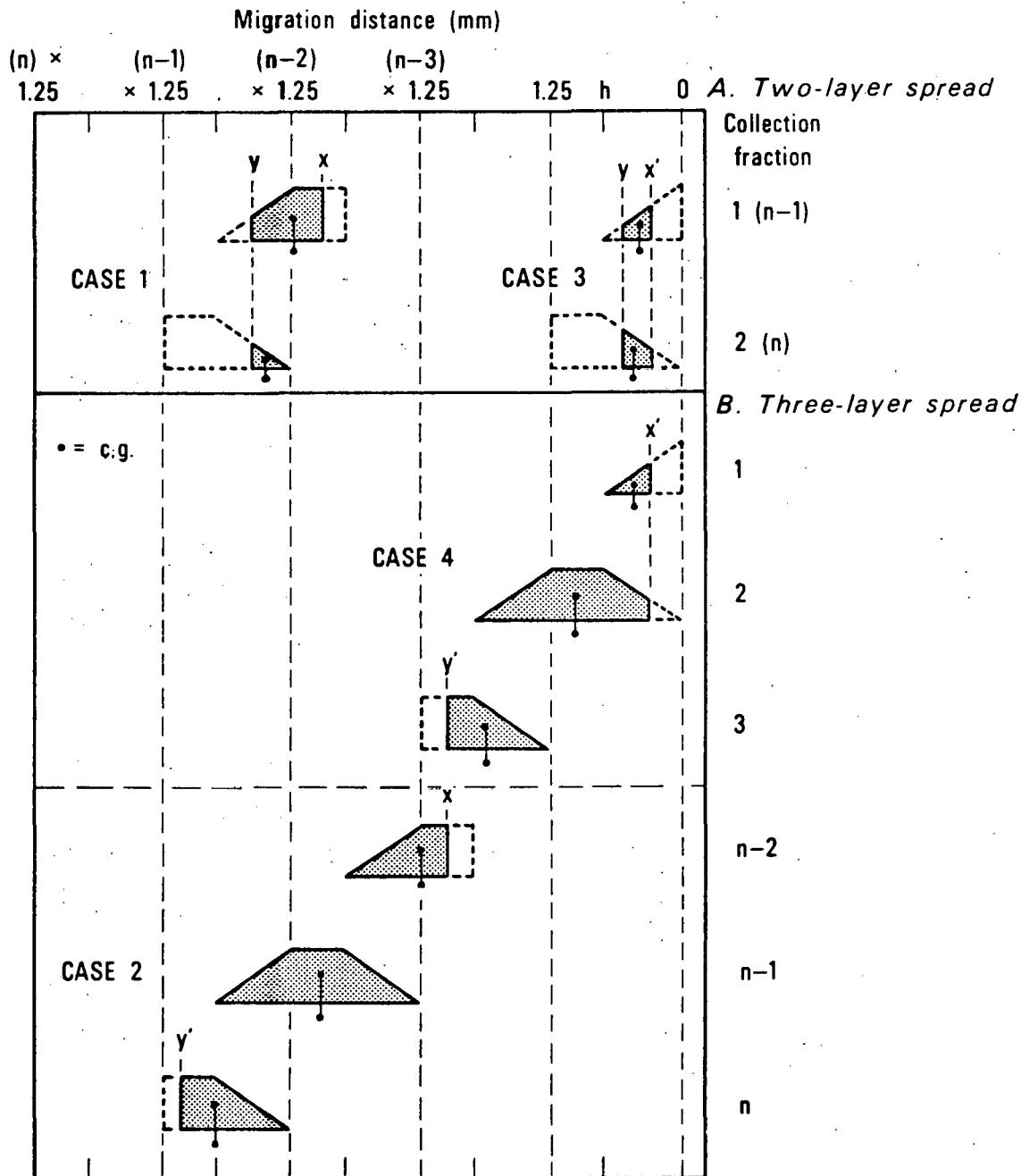
DBL 7011 5970

Fig. 15. Alternative range and distribution diagram for migration
distances in sedimentation and sedimentation-downward-electro-
phoresis experiments; two- and three-layer spread. Dots show
the centers of gravity of the respective areas.

15 in such a way that the relative height of the geometrical figure represents the fraction of particles characterized by a specific migration distance found in that collection fraction.

Figure 15 shows that the extent of the overlap in the distribution of migration distances among the collection fractions for 2- and 3-layer migration patterns depends upon the relationships among the distances h, x and y. Figure 15A shows that, for 2-layer migration patterns, Case 1 (layers n-1 and n) and Case 3 (layers 1 and 2) give similar average migration distances for the examples shown. If the medium, density gradient and operating conditions are selected to give the maximum distribution of migration distances (represented by the entire area shown in Figure 15A), the overlap is relatively less and the difference between the average migration distances is much greater.

Figure 15B shows that the extreme collection fractions of a 3-layer migration pattern have completely different ranges of migration distances while the center fraction overlaps the other 2. Conditions that give the widest spread of the 3-layer migration pattern will give the greatest number of particles in the top and bottom fractions. This would make available for analysis the largest number of particles with distinctly different migration properties.

(3) Average Migration Distances. The basic assumption of the center of gravity method of calculating the average migration distance is that the cells are uniformly distributed throughout the migration band. For this to be true, the range of sedimentation velocities characterizing the particles must be distributed uniformly throughout the population of particles. With this assumption, the fractional area of the migration band in each layer is equal to the fraction of the total number of particles

exiting in that layer. In the absence of specific information about the characteristics of the population of particles, this method is believed to give a good approximation of the average migration distance. This assumption is discussed in Chapter VIII in regard to the results of a specific Staflo sedimentation experiment.

Using Figures 13 and 14 and the center of gravity principle, equations can be derived for the average sedimentation migration distance for particles in each collection fraction. In fractions with 2 or 3 different geometrical areas representing particles with different ranges of sedimentation velocities, the average migration distance for the fraction as a whole is calculated as follows:

$$\lambda_{ave} = \frac{A_1 \lambda_1 + A_2 \lambda_2 + A_3 \lambda_3}{A_1 + A_2 + A_3} \tag{1}$$

where A_1, A_2 and A_3 are the areas for ranges of sedimentation distances such as $0 \rightarrow h$, $h \rightarrow 1.25$ and $1.25 \rightarrow (1.25 + h)$ mm for collection fraction 2 in Figure 14A. λ_1, λ_2 and λ_3 are the migration distances to the centers of gravity of the respective areas.

Equations for 2- and 3-layer migration patterns are given in Appendices E1 and E2, respectively.

(4) <u>Weighted Average Migration Distance</u>. In experiments for which migration profile measurements and the distribution of particles among collection fractions are available, the weighted average sedimentation distance ($\overline{\lambda}_S$) is determined as follows:

$$\overline{\lambda}_S = \Sigma f_i \lambda_{s_i} \tag{2}$$

where f_i is the fraction of the total sample in collection fraction "i"; and λ_{s_i} is the average sedimentation distance for particles in collection

fraction "1".

(5) <u>Correction of Sedimentation Migration Distances for Temperature

and Viscosity</u>. The viscosity of solutions used in the Staflo

apparatus changes significantly with temperature, whereas the change in

specific gravity is relatively small and generally can be ignored. For

the purpose of these calculations, it is believed adequate to correct the

sedimentation migration distances (λ_S) solely for changes in viscosity

as follows:

$$(\lambda_S)_{k°C} = (\lambda_S)_{r°C} \cdot \frac{\eta\,k°C}{\eta\,r°C} \tag{3}$$

where η = viscosity at temperature k°C or r°C, as appropriate.

An additional refinement can be made by correcting for differences

in viscosity among the solutions in different layers in the flow-chamber.

For instance, if the sedimentation rate $(dx/dt)_{k°C}$ is determined from

measurements made in layers 8, 9 and 10, a correction is necessary with

upward electrophoresis where the sedimentation would take place in layers

6, 7 and 8. Depending upon the component of the migration pattern for

which the calculation is being made, it may be appropriate to correct

$(\lambda_{9S})_{k°C}$ to $(\lambda_{7S})_{r°C}$. This is done by using the viscosities of the

medium for the appropriate solutions and temperatures in equation (3).

b. <u>Calculation of Sedimentation Velocity</u>. The sedimentation velocity

(dx/dt) is calculated by dividing the sedimentation distance (λ_S) by the

minimum transit time (τ_{min}) for the experiment:

$$(dx/dt)_i = \frac{\lambda_{S_i}}{\tau_{min}} \tag{4}$$

where i indicates that the equation is general and can be used to calculate

the minimum, maximum or average sedimentation velocities for individual

collection fractions or the entire migration pattern as desired.

2. Sedimentation-Electrophoresis Experiments.

The relative contributions of sedimentation and electrophoresis to the net migration of particles in the flow-chamber depend primarily upon the particle sedimentation rate, the average transit time and the strength and length of the electric field. Additional factors were considered in Section 1 above. A general formula for relating the observed sedimentation-electrophoresis migration distance (λ_{OBS}) to the true electrophoretic (λ_E) and sedimentation (λ_S) migration distances is:

$$\lambda_{OBS} = \lambda_E \pm \lambda_S \qquad (5)$$

where (+) applies to downward electrophoresis ($\lambda_{OBS} = \lambda_{SDE}$); and (-) applies to upward electrophoresis ($\lambda_{OBS} = \lambda_{SUE}$).

General statements with regard to equation (5) include the following: 1) It is applicable only if the migrating species has no intrinsic motility under the conditions of the experiment. 2) As $\lambda_S \to 0$, $\lambda_{OBS} \to \lambda_E$, i.e., true electrophoresis is achieved only when the term λ_S approaches zero. Inasmuch as a finite time is required for transit across the flow-chamber, one must reduce λ_S in order to approach true electrophoresis. This can be done by increasing the specific gravity and/or the viscosity of the medium. 3) λ_S can be calculated from sedimentation experiments. Thus, if one assumes that the electrophoretic mobility and sedimentation rate of individual particles are independent, λ_E can be calculated from equation (5) if λ_S is known.

a. Sedimentation-Downward-Electrophoresis Migration Distances. The migration pattern in sedimentation-downward-electrophoresis (SDE) experiments is similar to that shown in Figures 11 and 12 for 2 and 3-layer patterns, respectively, except the electric field generally starts 1/6 or 1/3 of the way across the flow-chamber. The range and distribution of migration

distances are as shown in Figures 13, 14 and 15. The equations presented in Appendices E1 and E2 apply to SDE experiments, also. The calculation of electrophoretic mobilities is discussed below.

Figure 16 shows the relationships between the sedimentation and electrophoretic components of the migration pattern in a SDE experiment in which there is a significant sedimentation component. The minimum and maximum sedimentation distances, $\lambda_{S_{min}}$ and $\lambda_{S_{max}}$, respectively, which would have been observed in the absence of an electric field are shown in Figure 16. The minimum and maximum SDE migration distances, $\lambda_{SDE_{min}}$ and $\lambda_{SDE_{max}}$, are given by x and y, respectively. The minimum electrophoretic migration distance ($\lambda_{E_{min}}$) is equal to the minimum observed migration ($\lambda_{SDE_{min}}$) less the expected minimum sedimentation distance ($\lambda_{S_{min}}$). The maximum electrophoretic migration distance ($\lambda_{E_{max}}$) is equal to the maximum observed migration distance ($\lambda_{SDE_{max}}$) less the expected maximum sedimentation distance ($\lambda_{S_{max}}$). These relationships are summarized in equations (6) and (7) below.

The lines in Figure 16 labelled s and S represent the expected distribution of the slowest and fastest sedimenting spermatozoa in the absence of an electric field and are equal in height to "h". The lines labelled E·S, e·s, E·s and e·S represent the expected distribution of the fastest electrophoresing-fastest sedimenting, slowest electrophoresing-slowest sedimenting, fastest electrophoresing-slowest sedimenting, slowest electrophoresing-fastest sedimenting populations of spermatozoa, respectively.

b. Sedimentation-Upward-Electrophoresis Migration Distances. In electrophoresis experiments, the electric field generally is applied over the last 20-25 cm of the flow-chamber to allow density gradient stabilizing
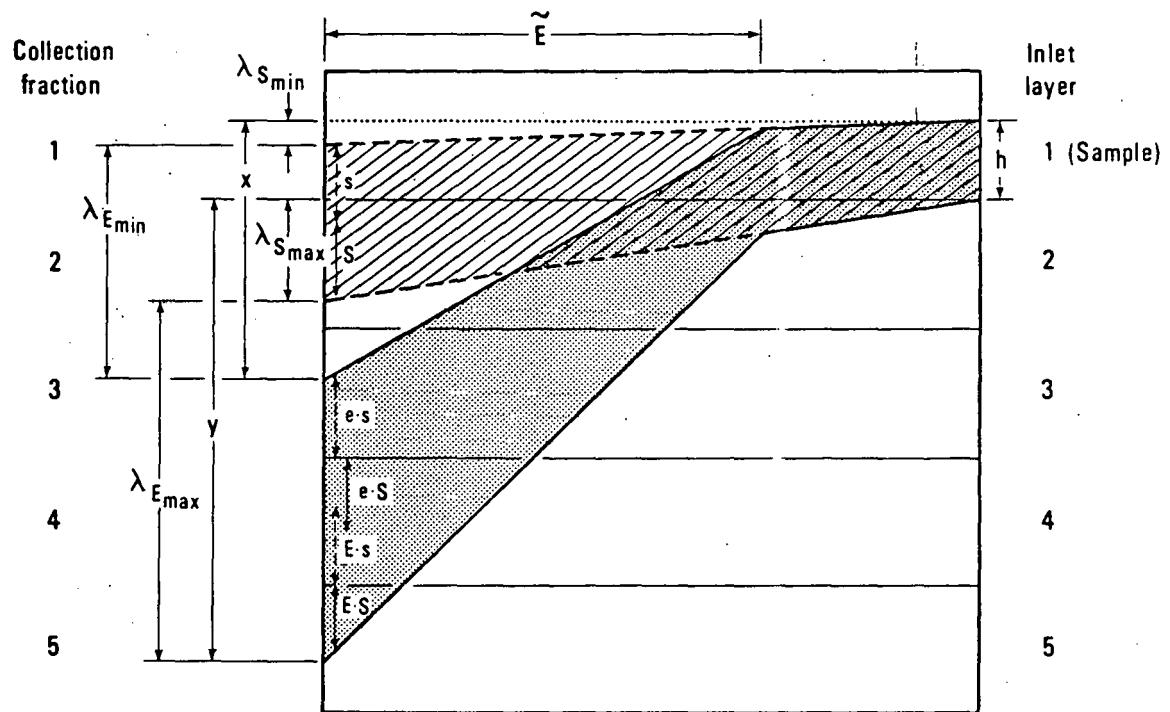
Fig. 16. Schematic diagram of sedimentation-downward-electrophoresis migration pattern showing separate components.

diffusion to take place.[157] The upward migration is illustrated in Figures 17 and 18 which represent a vertical plane in the centerline through the long axis of the flow-chamber in 2- and 3-layer sedimentation-upward-electrophoresis (SUE) migration patterns, respectively. In addition to the assumptions given above for sedimentation experiments, it is assumed that the leading and trailing edges of the migration pattern determine the maximum and minimum SUE migration distances, respectively.

(1) <u>Minimum and Maximum Migration Distances</u>. Figure 19 shows the relationships among the various migration distances in a SUE experiment in which there is a significant sedimentation component. The minimum and maximum sedimentation distances ($\lambda_{S_{min}}$ and $\lambda_{S_{max}}$) which would have been observed in the absence of an electric field are shown in Figure 19. The distances x and y are the minimum and maximum SUE migration distances, $\lambda_{SUE_{min}}$ and $\lambda_{SUE_{max}}$, respectively.

The minimum electrophoretic migration distance is equal to the sum of the maximum sedimentation distance and the minimum SUE distance. The maximum electrophoretic migration distance is equal to the sum of the minimum sedimentation distance and the maximum SUE migration distance. These relationships are summarized in equations (8) and (9) below.

In Figure 19 the lines labelled s, S, E·S, E·s, e·S and e·s have the same meaning as in Figure 16 which was discussed above.

(2) <u>Range and Distribution of Migration Distances</u>. The same general principles, discussed in relation to sedimentation, concerning overlapping ranges of migration distances and the need for special equations depending upon the relationships between the location of the sample stream at the inlet and the limits of the migration pattern at the outlet apply to SUE experiments. Because the sample stream is at the bottom of the inlet

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STAFLO "SUE" MIGRATION PATTERN

A. Two layers; 1 and 2
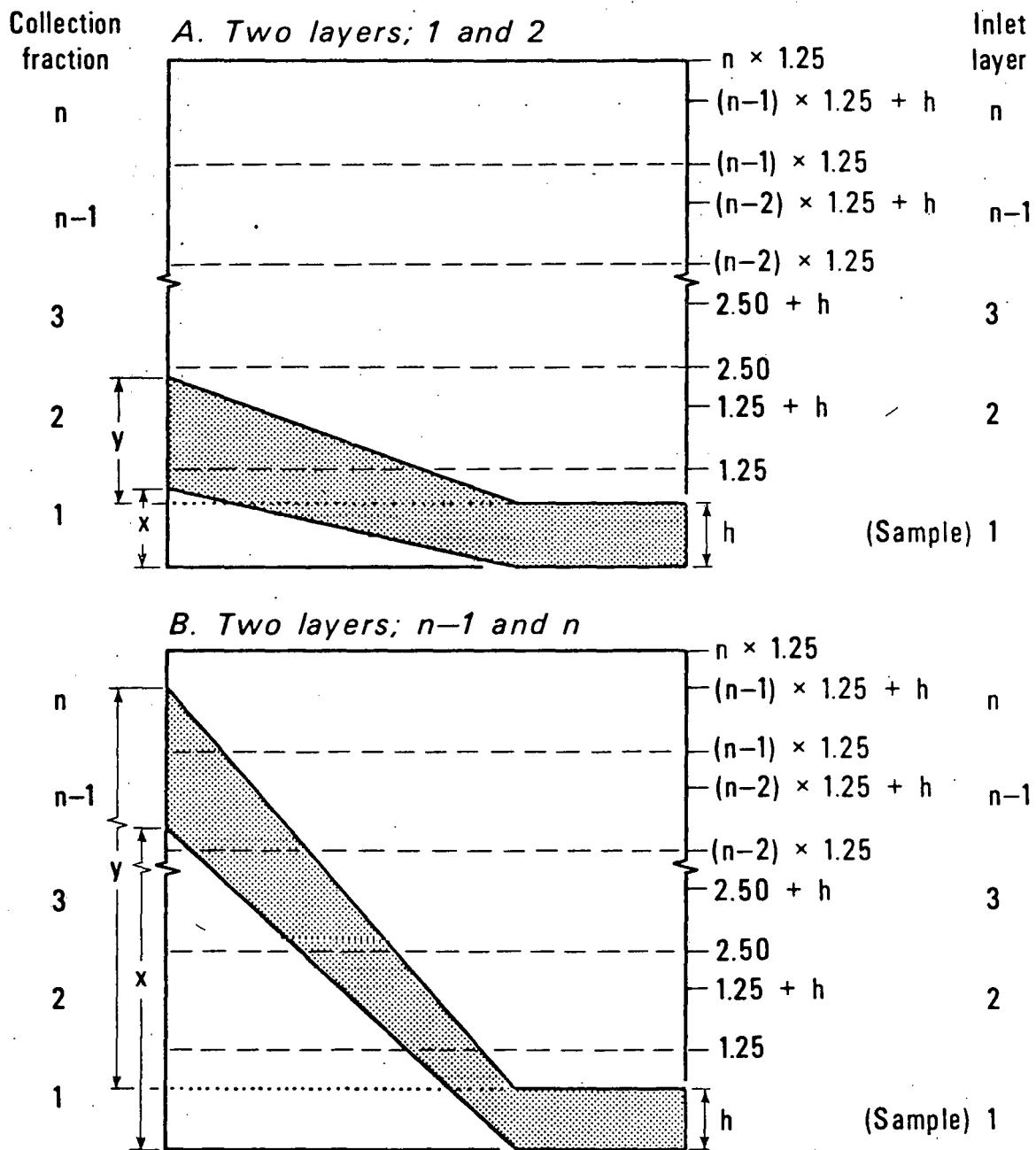
B. Two layers; n—1 and n

DBL 7011 5972

Fig. 17. Schematic diagram of sedimentation-upward-electrophoresis migration pattern; two-layer spread. A. Exit in layers 1 and 2. B. Exit in layers n-1 and n.

STAFLO "SUE" MIGRATION PATTERN

Collection fraction

Inlet layer

A. Three layers; 1, 2 and 3

n — n × 1.25
— (n−1) × 1.25 + h   n
— (n−1) × 1.25
n−1 — (n−2) × 1.25 + h   n−1
— (n−2) × 1.25
3 — 2.50 + h   3
— 2.50
2 — 1.25 + h   2
— 1.25
1 — (Sample) 1

y

x

h

B. Three layers; n−2, n−1 and n

n — n × 1.25
— (n−1) × 1.25 + h   n
— (n−1) × 1.25
n−1 — (n−2) × 1.25 + h   n−1
— (n−2) × 1.25
n−2 — (n−3) × 1.25 + h   n−2
— (n−3) × 1.25
2 — 1.25 + h   2
— 1.25
1 — (Sample) 1

y

x

h
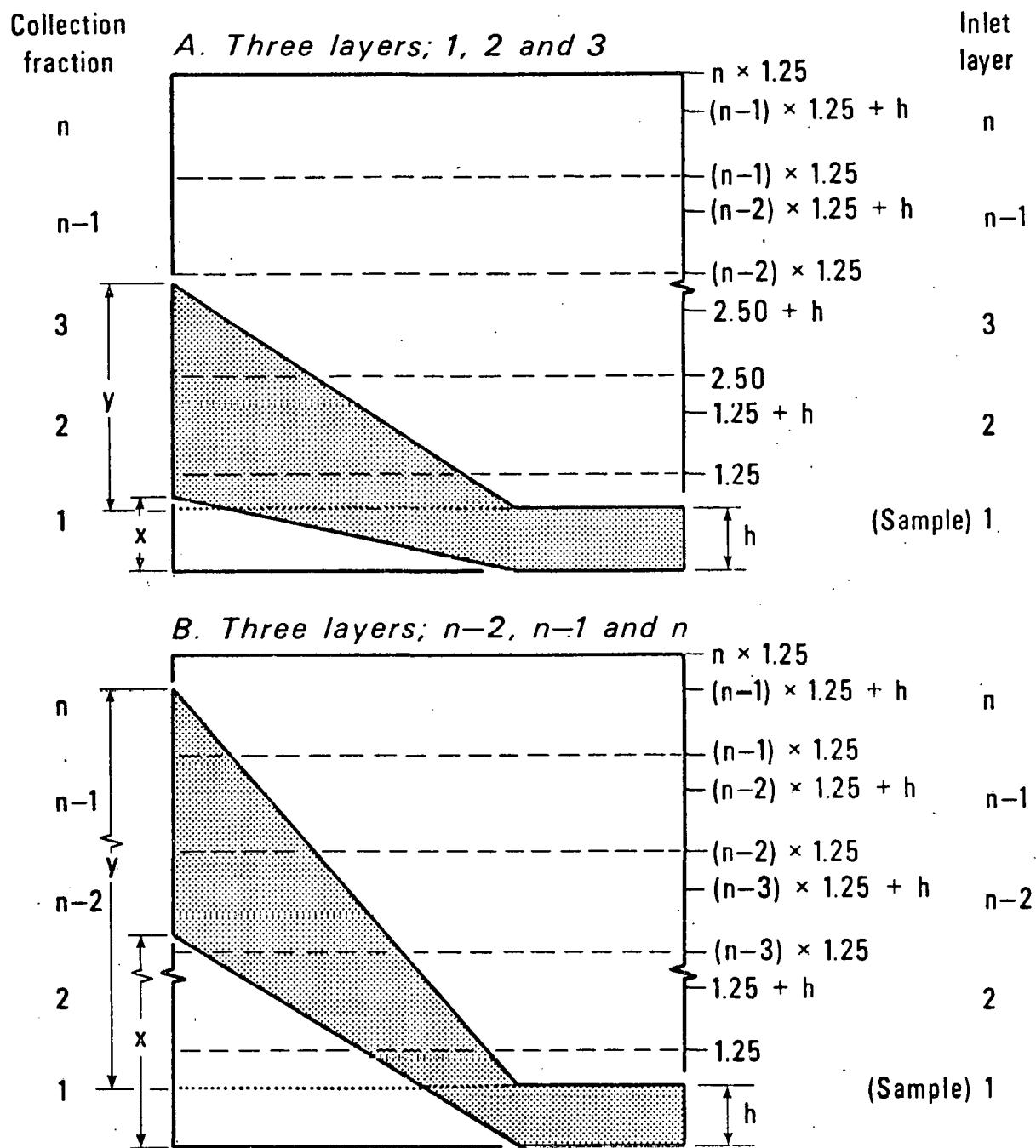
DBL 7011 5973

Fig. 18. Schematic diagram of sedimentation-upward-electrophoresis migration pattern; three-layer spread. A. Exit in layers 1, 2 and 3. B. Exit in layers n-2, n-1 and n.

STAFLO "SUE" MIGRATION PATTERN
Separate components
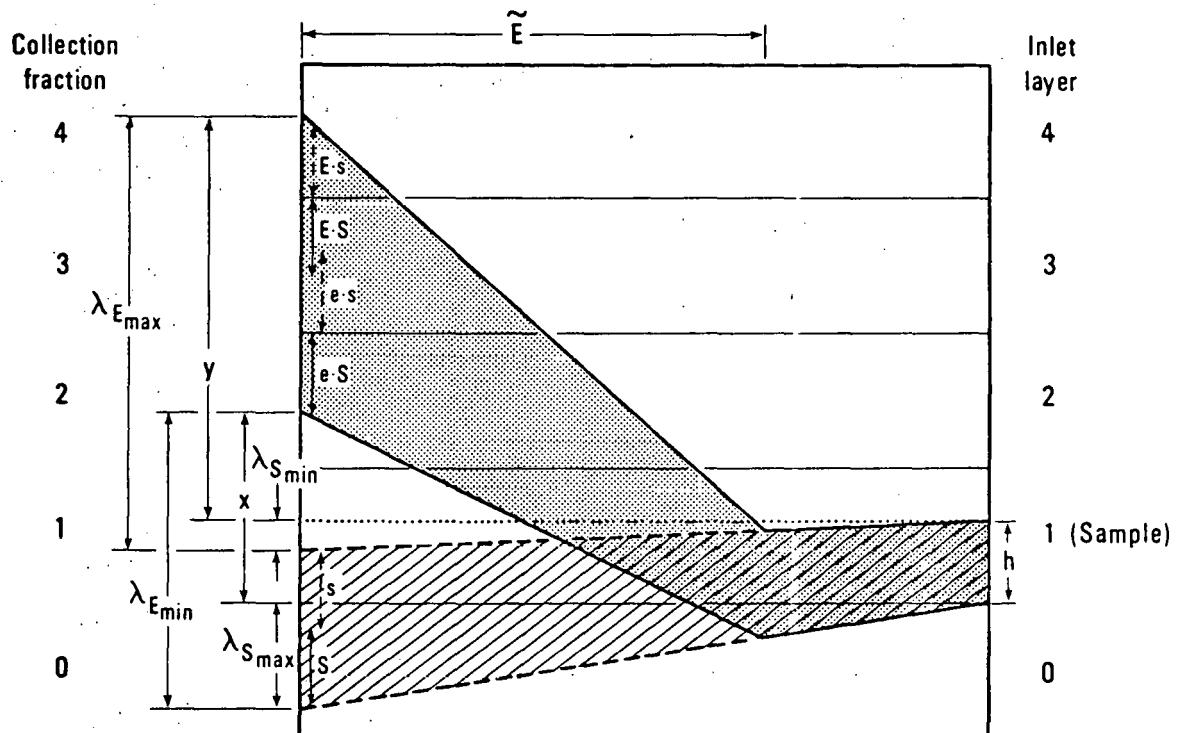
DBL 7011 5974

Fig. 19. Schematic diagram of sedimentation-upward-electrophoresis migration pattern showing separate components.

layer under steady state conditions, the distribution of SUE migration distances can have any 1 of 4 different patterns. These are shown in Figures 20 and 21 which are diagrams of the possible ranges of SUE migration distances. The relationships among the possible distributions for 2- and 3-layer migration patterns are summarized mathematically in Appendices E3 and E4, respectively. Note that x and y in Figures 20 and 21 are both drawn from the origin (o) even though they are measured differently in Figures 18 and 19.

In Figures 20 and 21, the migration distance of the trailing edge of the column of particles is plotted on the ordinate against the same migration distance on the abscissa for migration patterns spreading over the 2 and 3 layers, respectively. For example, when those particles at the bottom of the sample stream at the inlet, which are characterized by an electrophoretic mobility (μ) giving a migration distance of 1.25 mm, reach the bottom of the next layer, the remainder of the particles characterized by that value of μ will be found in the lower part of that layer up to height "h" as shown in Figures 20 and 21.

The geometry of the upward migration pattern makes it possible to write general expressions for the minimum, maximum and average SUE migration distances regardless of the outlet layers. These equations are presented in Appendices E3 and E4 for 2- and 3-layer migration patterns, respectively.

Figures 22 and 23 illustrate all 4 combinations of minimum and maximum migration distances for 2- and 3-layer upward migration patterns, respectively. For each migration distance, the relative heights of the figures represent the fraction of particles in each collection fraction characterized by that migration distance. As shown in Figure 22 for a 2-layer migration pattern, Case 2 gives the least overlap and the great-

RANGE AND DISTRIBUTION OF
STAFLO "SUE" MIGRATION DISTANCES

A. Two layers; 1 and 2

Migration distance (mm)

B. Two layers; n—1 and n

Limits of migration profile
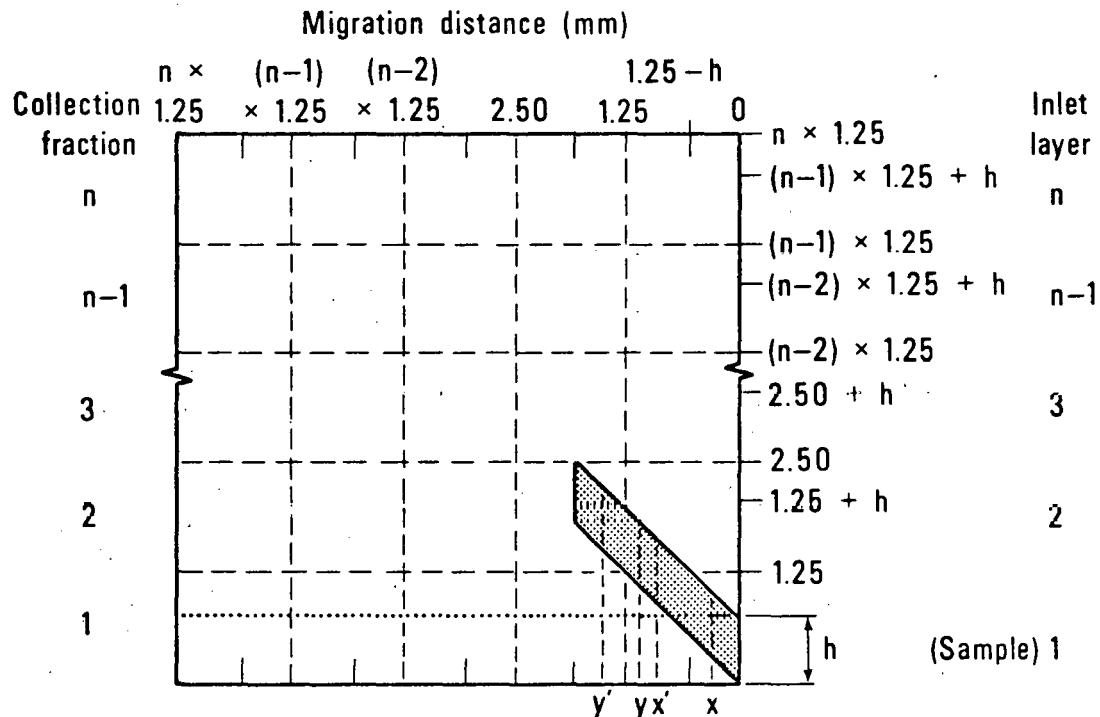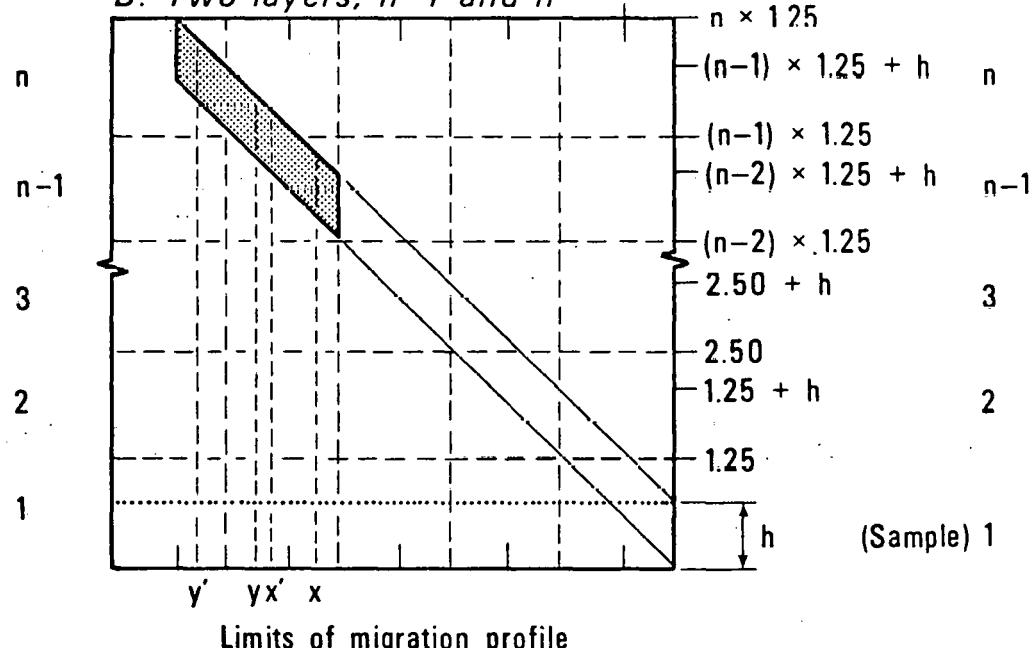
DBL 7011 5975

Fig. 20. Range and distribution diagram for sedimentation-upward electrophoresis migration distances; two-layer spread. A. Exit in layers 1 and 2. B. Exit in layers n-1 and n.

RANGE AND DISTRIBUTION OF
STAFLO "SUE" MIGRATION DISTANCES

A. Three layers; 1, 2 and 3

Migration distance (mm)

Collection fraction

| n ×
1.25 | (n−1)
× 1.25 | (n−2)
× 1.25 | 2.50 | 1.25 | 1.25 − h
0 |

Inlet layer

n × 1.25
(n−1) × 1.25 + h   n
(n−1) × 1.25
(n−2) × 1.25 + h   n−1
3.75
2.50 + h   3
2.50
1.25 + h   2
1.25
h   (Sample) 1

y′ y   x′ x

B. Three layers; n−2, n−1 and n

| n ×
1.25 | (n−1)
× 1.25 | (n−2)
× 1.25 | (n−3)
× 1.25 | 1.25 | 1.25 − h
0 |

n × 1.25
(n−1) × 1.25 + h   n
(n−1) × 1.25
(n−2) × 1.25 + h   n−1
(n−2) × 1.25
(n−3) × 1.25 + h   n−2
(n−3) × 1.25
1.25 + h   2
1.25
h   (Sample) 1

y′ y   x′ x

Limits of migration profile
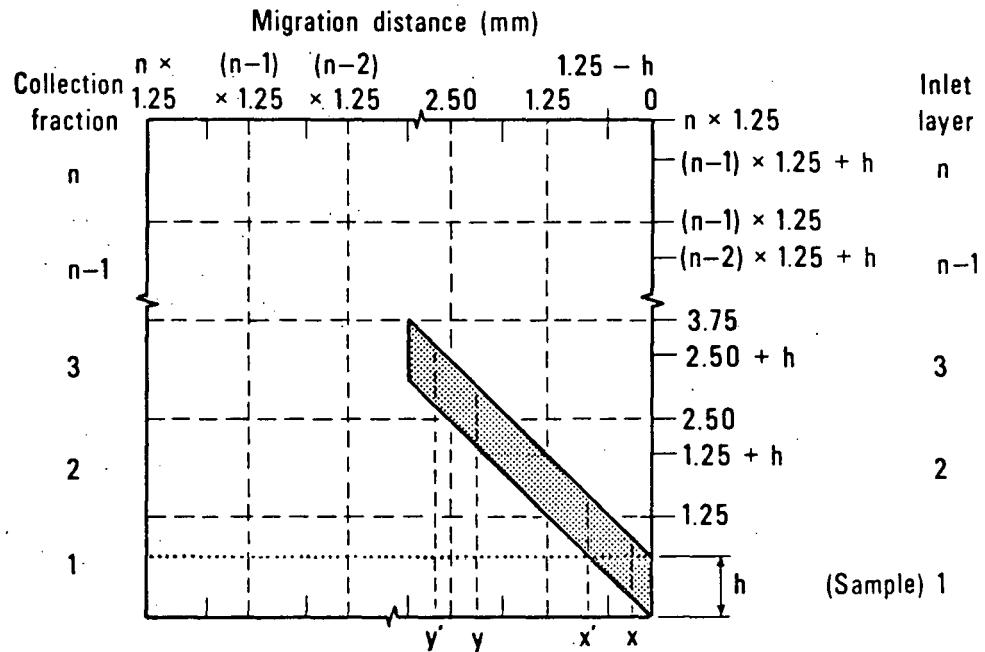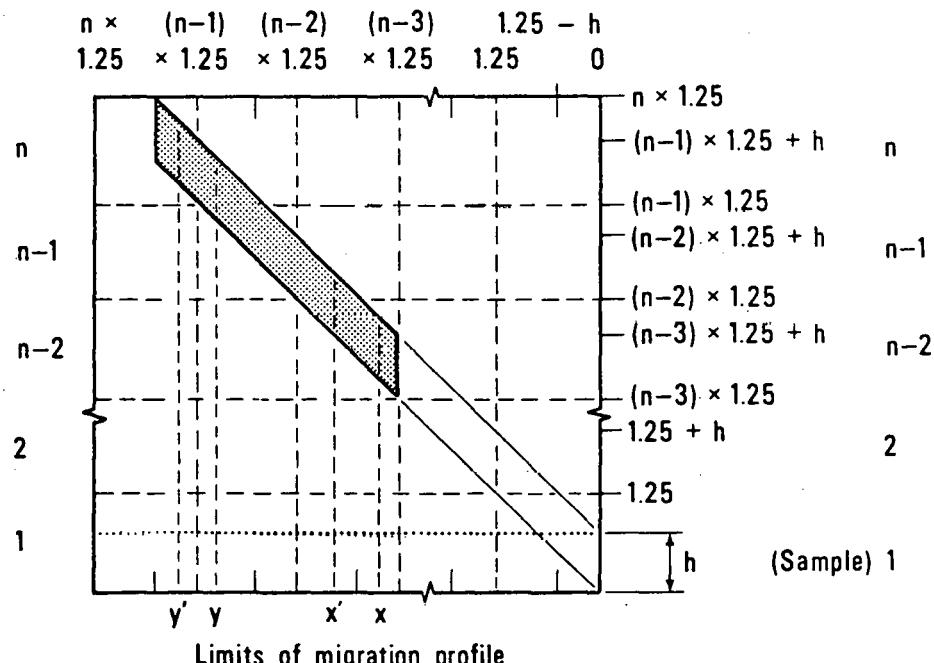
DBL 7011 5976

Fig. 21. Range and distribution diagram for sedimentation-upward-electrophoresis migration distances; three-layer spread. A. Exit in layers 1, 2 and 3. B. Exit in layers n-2, n-1 and n.

**RANGE AND DISTRIBUTION OF
STAFLO "SUE" MIGRATION DISTANCES**
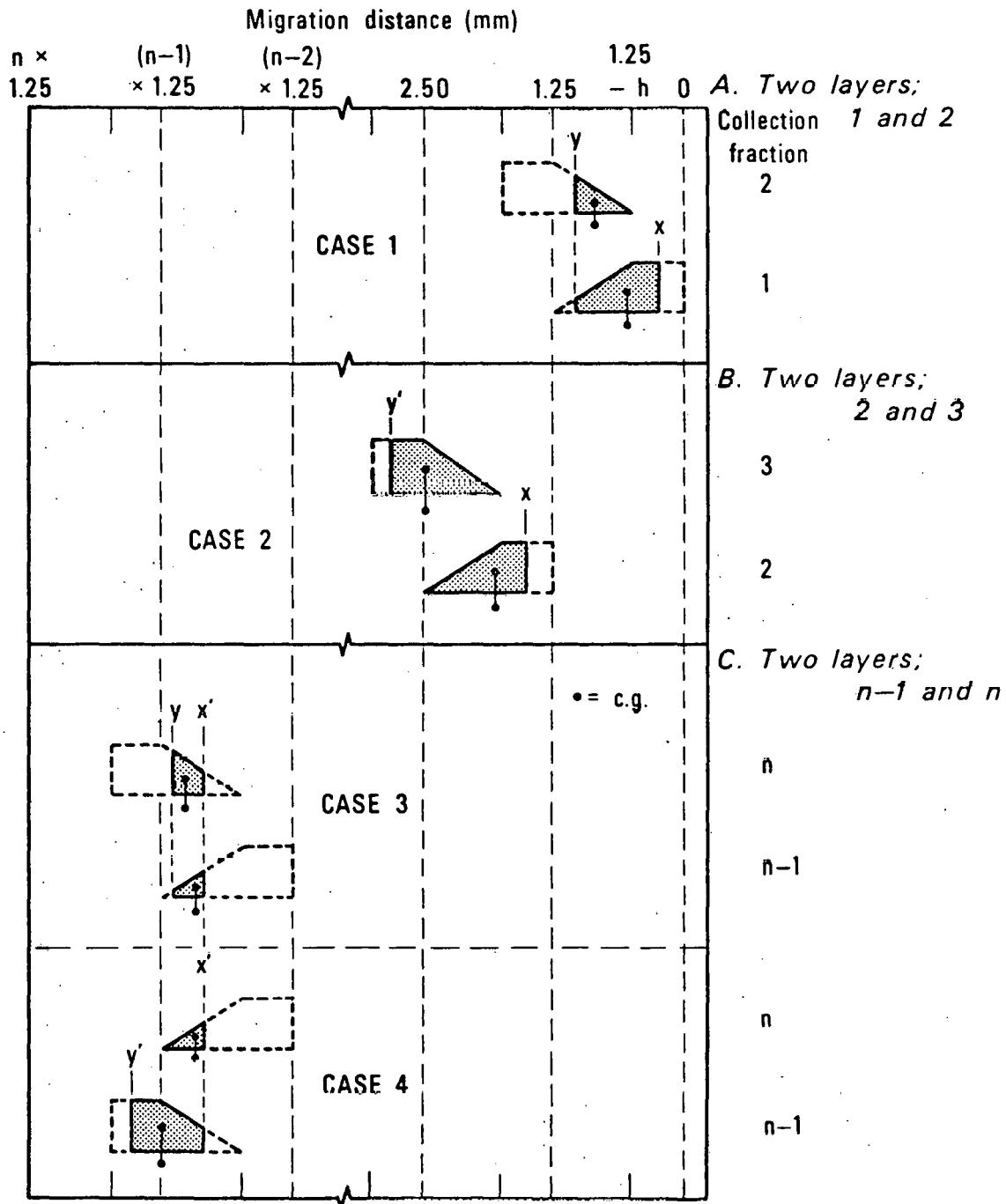
DBL 7011 5977

Fig. 22. Alternative range and distribution diagram for migration distances in sedimentation-upward-electrophoresis experiments; two-layer spread. Dots indicate centers of gravity of the respective areas.

RANGE AND DISTRIBUTION OF
STAFLO "SUE" MIGRATION DISTANCES
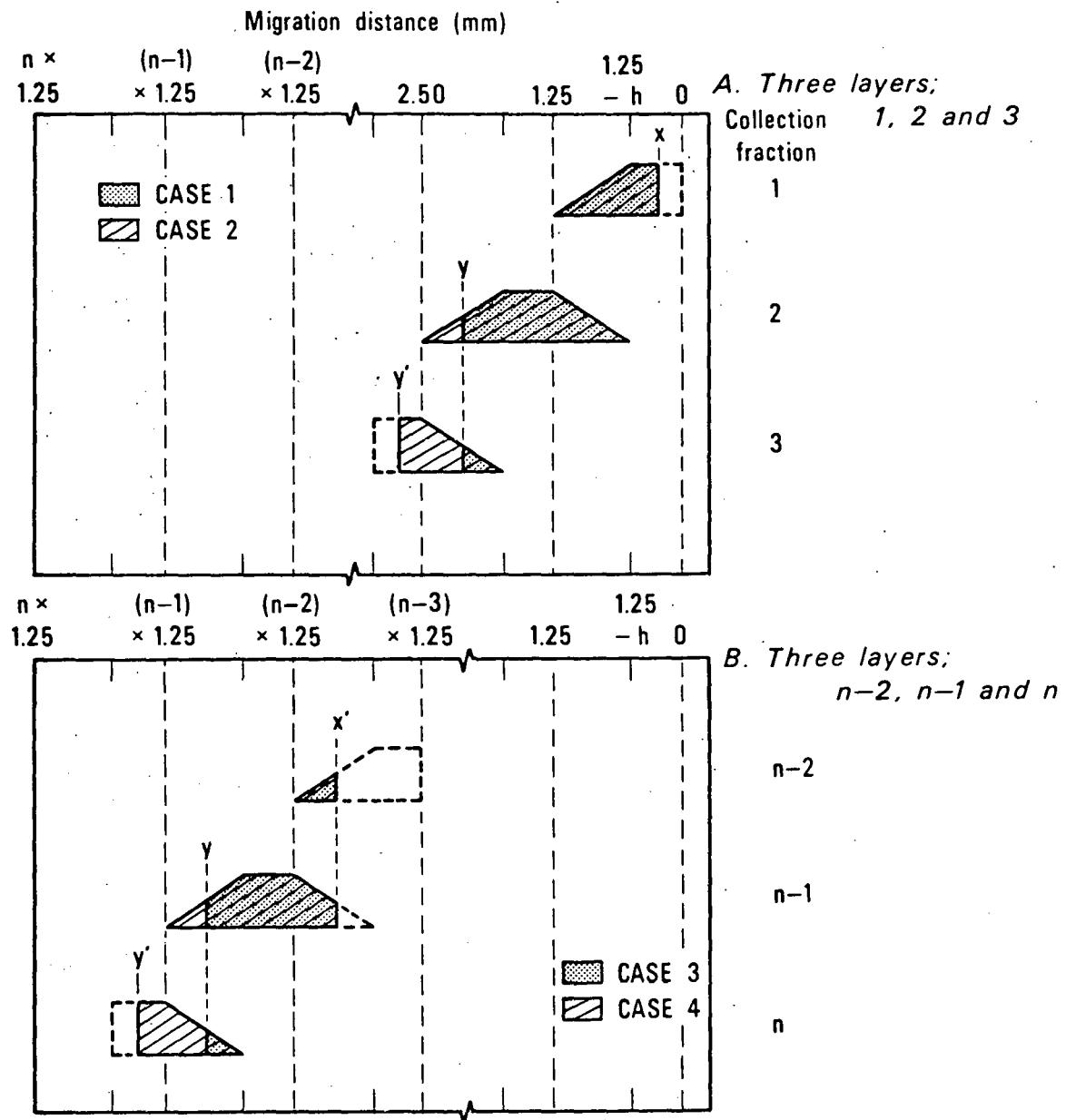
Migration distance (mm)

Fig. 23. Alternative range and distribution diagram for migration

distances in sedimentation-upward-electrophoresis experiments;

three-layer spread. Dots show centers of gravity of the

respective areas.

DBL 7011 5978

est difference between the average migration distances of the 2 collection

fractions. Case 3 gives the least difference. Figure 23 shows that Cases

2 and 3 give the largest and smallest numbers of particles, respectively,

in the extreme fractions of the 3-layer migration pattern.

c. Comparison of Sedimentation-Downward and -Upward-Electrophoresis.

The relationships among the sedimentation, electrophoresis and sedimenta-

tion-electrophoresis migration distances are as follows:

$$\lambda_{SDE_{min}} = \lambda_{E_{min}} + \lambda_{S_{min}} \tag{6}$$

$$\lambda_{SDE_{max}} = \lambda_{E_{max}} + \lambda_{S_{max}} \tag{7}$$

$$\lambda_{SUE_{min}} = \lambda_{E_{min}} - \lambda_{S_{max}} \tag{8}$$

$$\lambda_{SUE_{max}} = \lambda_{E_{max}} - \lambda_{S_{min}} \tag{9}$$

$$\lambda_{SDE_{ave}} = \lambda_{E_{ave}} + \lambda_{S_{ave}} \tag{10}$$

$$\lambda_{SUE_{ave}} = \lambda_{E_{ave}} - \lambda_{S_{ave}} \tag{11}$$

These equations apply to individual collection fractions or to the mi-

gration pattern as a whole.

For spherical objects and for non-spherical particles if the value

of $\lambda_{S_{ave}}$ is not changed by a reorientation of particles upon changing the

direction of the electric field, the average electrophoretic migration

distance can be determined from a combination of upward- and downward-

electrophoresis experiments. Adding equations (10) and (11) gives

$$\lambda_{E_{ave}} = 1/2 \left(\lambda_{SDE_{ave}} + \lambda_{SUE_{ave}} \right) \tag{12}$$

The lines labelled s, S, E·S, E·s, e·S and e·s in Figures 16 and 19 were explained above. With particles having a significant sedimentation rate, a transit time can be selected to give a partial separation of the slowest and fastest sedimenting particles shown by lines s and S in Figures 16 and 19. There will be overlap between the 2 groups as discussed in relation to Figure 15. If a downward electrophoretic migration is superimposed upon the sedimentation pattern as shown in Figure 16, the extreme fractions will contain predominantly e·s and E·S groups. With upward electrophoretic migration as shown in Figure 19, the extreme fractions will contain primarily E·s and e·S groups. In table form, the distribution would be as follows:

Collection Fraction	SDE	SUE
Top	e·s	E·s
Upper Middle	e·S	E·S
Lower Middle	E·s	e·s
Bottom	E·S	e·S

Thus, by conducting SDE and SUE experiments one can get e·s, E·S, E·s and e·S groups of particles for evaluation. Because of the overlap of migration distances discussed above in relation to Figures 15, 22 and 23, it may be desirable to shave the top or bottom of the migration pattern at a flow-divider to get a more homogeneous population of particles for study. It should be noted that only if the sedimentation rate and electrophoretic mobility of the particles are independent, would the segregation of the 4 groups of particles occur as described.

d. Calculation of Electrophoretic Mobility. The minimum, maximum and

average electrophoretic migration distances (λ_E) can be calculated from equations (6)-(11) as appropriate. Using these migration distances, the electrophoretic mobility (μ) can be calculated as follows:

$$\mu = \frac{\nu}{\tilde{E}} = \frac{\lambda_E / t_{E_{min}}}{I/A \cdot \sigma} \qquad \frac{(cm/sec)}{(V/cm)} \qquad (13)$$

where ν is the particle migration rate (cm/sec) and $\tilde{E}$ is the electric field strength (V/cm). I is the electric current (amps), A is the electrode area (cm^2) and $\sigma = \frac{1.46}{R}$ (ohm^{-1} cm^{-1}). R is the resistance of the medium (ohms) and 1.46 cm^{-1} is the cell constant for the conductivity meter in our laboratory.

According to Bier[38], "because nearly all the effect of temperature on mobility is due to the change in viscosity of H_2O, the following relationship can be used to correct the mobility to any temperature as long as is not too large: $\eta_1 \mu_1 = \eta_2 \mu_2$." This relationship can be used to normalize electrophoretic mobilities determined at different temperatures in the same medium or in different media if the viscosities are known.

3. Effects of Motility on Migration and Fractionation.

In early Staflo experiments conducted at temperatures of 6-7°C, motile spermatozoa were observed in the flow-chamber with a microscope. At these temperatures and with pH 6 or greater, the orientation of all spermatozoa, including the motile spermatozoa, appeared to be predominantly vertical with tails upward in the sedimentation and SUE experiments regardless of the electric field strength in the latter. In SDE experiments, the orientation was uniformly vertical with tails pointing downward at pH 7 with high electric field strengths only. In SDE experiments with low electric field strengths, the spermatozoa assumed a variety of orientations as discussed in Chapter VIII.

In order to quantitate the effect of intrinsic motility of spermatozoa in Staflo experiments, one must know the true range of sedimentation
velocities and/or of electrophoretic mobilities in the medium and density
gradient. These can be obtained in experiments conducted at or below
3°C depending upon the medium. Then if the temperature is controlled to
eliminate random swimming, the orientation of the spermatozoa is determined
by gravity and/or the electric field, and the motility vector is parallel
to the sedimentation and/or electrophoretic vectors.

a. <u>Sedimentation Experiments</u>. Figure 24 is a schematic diagram of a
Staflo sedimentation experiment with an intrinsic motility component showing minimum and maximum spermatozoa migration distances, x and y, respectively. The expected maximum sedimentation distance, $\lambda_{S_{max}}$, is assumed to
have been determined in experiments at very low temperatures. Then, from
Figure 24, the maximum contribution of motility to the net migration is
given by the expression:

$$\lambda_{MOT_{max}} = y - \lambda_{S_{max}} \tag{14}$$

In Figure 24, one must be careful to interpret the maximum migration
distance attributable to motility as the distance from $\lambda_{S_{max}}$ to y and not
from zero to y. The sample rarely has 100% motile spermatozoa, but if it
did, the minimum contribution of motility ($\lambda_{MOT_{min}}$) would be observed as
a displacement of x from the expected minimum sedimentation distance
($\lambda_{S_{min}}$) which it represents in Figure 24. The relationship between these
components is:

$$\lambda_{MOT_{min}} = x - \lambda_{S_{min}} \tag{15}$$

b. <u>Sedimentation-Electrophoresis Experiments</u>. Unfortunately, the differential orientation of spermatozoa in an electric field under varying

STAFLO MIGRATION PATTERN
Sedimentation and Intrinsic Motility Components
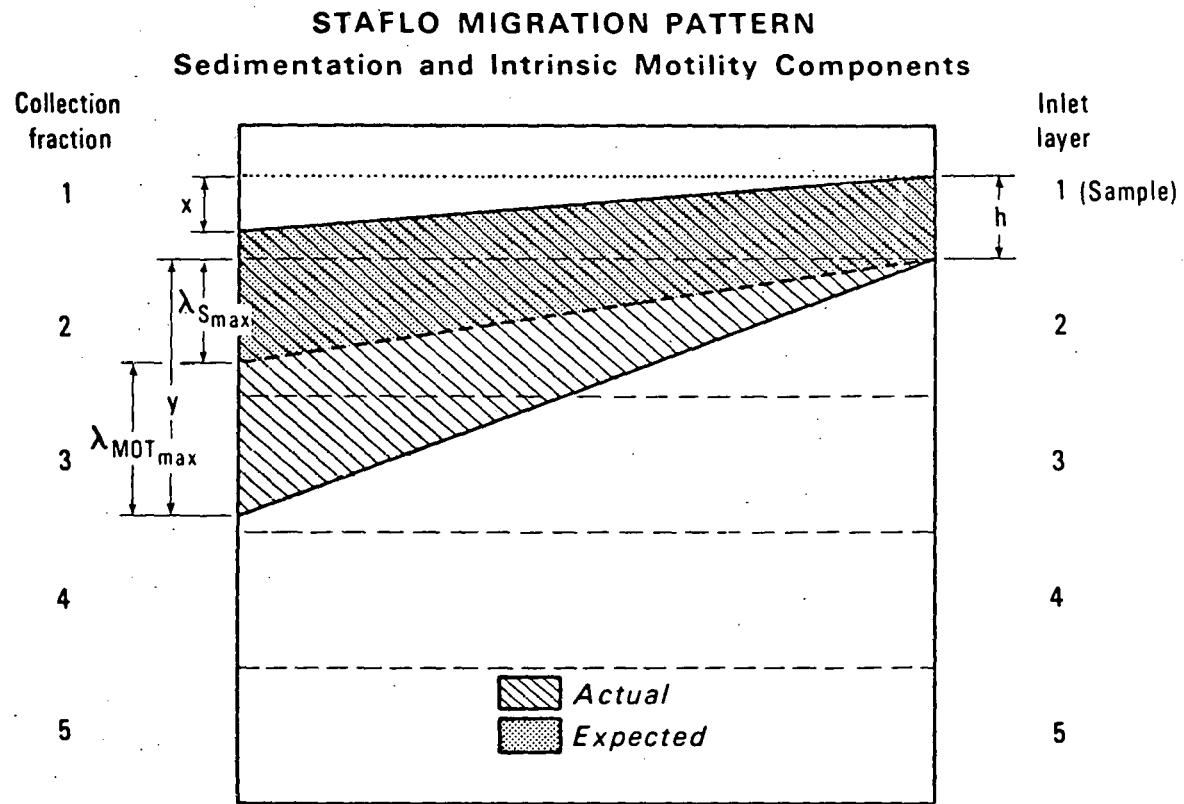
Fig. 24. Schematic diagram of a sedimentation migration pattern showing the effect of intrinsic motility on migration.

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conditions of pH and ionic strength is further complicated by the random orientation of strongly swimming spermatozoa if their intrinsic motility is sufficient to overcome the polarizing effects of the electric field as mentioned by Bangham[18], and Vessilinovitch[233]. However, one can control the head-tail orientation by proper selection of pH and ionic strength and can control the random swimming by using a temperature at which the intrinsic motility is insufficient to change the orientation imposed by gravity and/or the electric field.

Figures 25 and 26 show a motility component to the net migration in addition to the electrophoretic and sedimentation migration distances for SDE and SUE experiments, respectively. In both downward- and upward-electrophoresis under conditions of pH and ionic strength which cause the sperm to be tail anode, the direction of the motility component is opposite that of the electrophoretic component. Because most spermatozoa samples contain a significant number of immotile spermatozoa which would exper-ience the maximum electrophoretic migration, the observed migration pattern would extend from x to y in both figures. The minimum expected electro-phoretic migration distance ($\lambda_{E_{min}}$) in both cases is reduced by the intrinsic motility component $\lambda_{MOT_{max}}$. Thus, if intrinsic motility and electrophoretic mobility are independent, one would get an enrichment of the lower collec-tion fractions with regard to motility in upward-electrophoresis and of the upper collection fractions in downward-electrophoresis experiments. With a range of intrinsic velocities one would expect to find a range of degrees of intrinsic motility among the spermatozoa in the collection fractions with the least motile sperm having migrated the greatest distance in the directions shown in Figures 25 and 26.

Expressions relating intrinsic motility to sedimentation-electrophoresis

STAFLO "SDE" MIGRATION PATTERN

Sedimentation, Electrophoresis and Intrinsic Motility Components
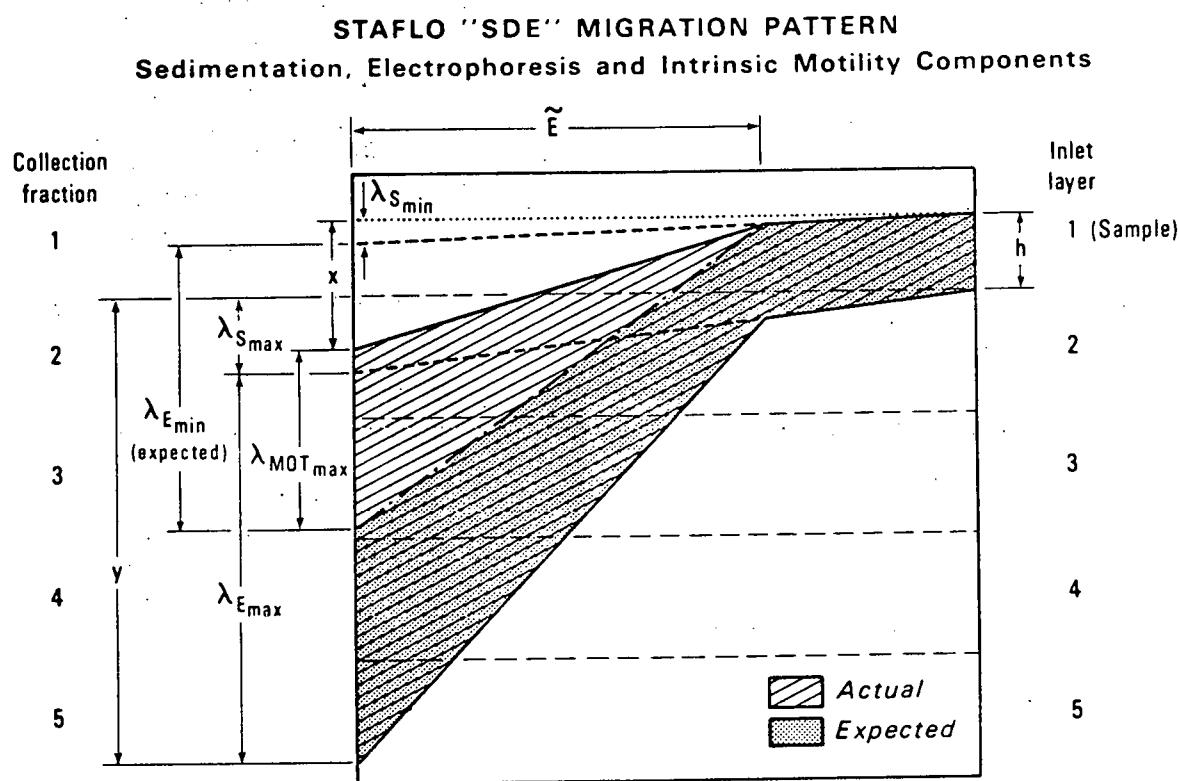
DBL 7011 5980

Fig. 25. Schematic diagram of a sedimentation-downward-electrophoresis migration pattern showing the interrelationships among the sedimentation, electrophoresis and intrinsic motility components.

STAFLO "SUE" MIGRATION PATTERN

Sedimentation, Electrophoresis and Intrinsic Motility Components
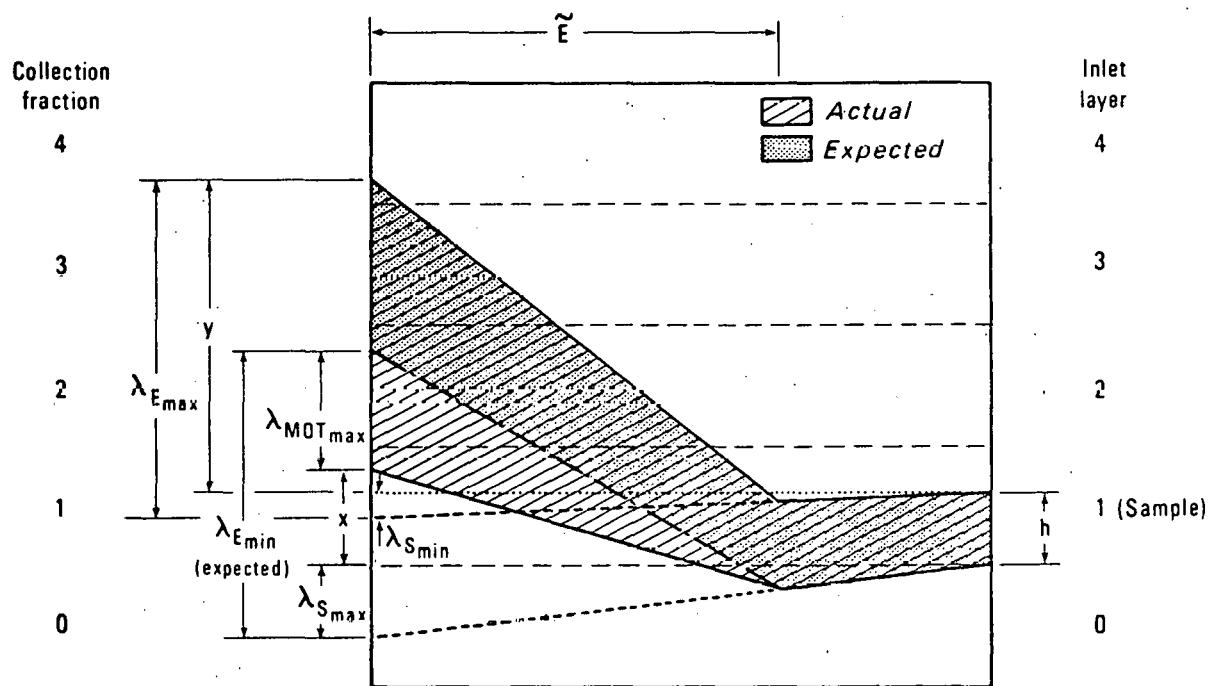
DBL 7011 5981

Fig. 26. Schematic diagram of a sedimentation-upward-electrophoresis migration pattern showing the interrelationships among the sedimentation, electrophoresis and intrinsic motility components.

migration distances are as follows:

Sedimentation-downward-electrophoresis (Figure 25):

$$\lambda_{MOT_{max}} = \lambda_{E_{min}} \text{ (expected)} - x + \lambda_{S_{min}} \tag{16}$$

Sedimentation-upward-electrophoresis (Figure 26):

$$\lambda_{MOT_{max}} = \lambda_{E_{min}} \text{ (expected)} - x - \lambda_{S_{max}} \tag{17}$$

4. <u>Significance of This Method of Analysis.</u>

From the several theoretical analyses developed in this chapter, certain conclusions can be drawn.

Figure 15B shows that for most particle populations, a 3-layer spread of the migration pattern is the minimum from which one can obtain collection fractions with completely different populations. Thus, if possible, one should attempt to achieve at least a 3-layer spread of the pattern. However, Figure 15A shows that the difference between the collection fractions in a 2-layer spread can be quite meaningful if the pattern is spread over the entire 2 layers. This is determined largely by the relative height of the sample stream at the inlet (h) compared to the layer thickness. Therefore, if the physical or biological properties of the sample limit the spread to 2 layers, one should use the "thin sample technique"[263] and select operating conditions that will give the widest spread within those 2 layers.

Figures 16 and 19 show that, in sedimentation-electrophoresis experiments, the sedimentation and electrophoretic migration components can be separately identified if one of the 2 properties is known. Similarly, Figures 24-26 demonstrate the interrelationships among the intrinsic motility and sedimentation and/or electrophoretic migration vectors. The

effects of motility on sedimentation and on sedimentation-electrophoresis experiments are discussed in Chapters IV and VIII, respectively.

Separation of the fastest electrophoresing-slowest sedimenting (E·s) particles and of particles possessing the other combinations of sedimentation-electrophoretic properties (E·S, e·S and e·s) from a population are discussed in Section C2c. An initial attempt to use this analysis to separate these sub-populations of semen spermatozoa is reported in Chapter VIII.

The most important assumption in deriving the equations presented in Appendix E is that the distribution of migration properties among the population is uniform. Migration distance range and distribution diagrams of the form shown in Figures 15, 22 and 23 provide a visual method for comparing actual and hypothetical distributions of properties among populations. Such a comparison is made in Chapter VIII, where the actual distribution of spermatozoa among the collection fractions in Sedimentation Experiment 12-26-67 is compared to the expected distributions of (hypothetical) uniform, bimodal and normal populations of cells with the same range of sedimentation velocities.

Within their stated limitations, the equations in Appendix E provide an accurate method of calculating Staflo migration distances. Representative calculations using these equations and the guideline in sub-sections 1 and 2, above, for sedimentation and sedimentation-electrophoresis experiments are given in Appendices F and G, respectively.

A computer program, using the equations in the text and in Appendix E would greatly simplify calculations of the sedimentation velocities or electrophoretic mobilities of individual collection fractions and of the average for the migration pattern as a whole. The equations in Appendix E

can be extended to migration patterns encompassing 4 or more adjacent layers. In addition, the equations for patterns extending over 2, 3 or more adjacent layers can be generalized so that the inlet position need not be specified as it was in the assumptions in this chapter. Both of these extensions should be made in adapting the equations to a computer program to give even greater flexibility and simplicity in quantitating the migration properties of particles studied in Staflo experiments.

This method of analysis of the migration properties of a hetero-geneous mixture of particles should also be applicable to a variety of fractionation techniques; for example, the sedimentation column used by Bhattacharya[35], by Schilling[205] and by Bedford and Bibeau[29]. Other applicable techniques include rate and zonal centrifugation, and column, paper and starch-gel electrophoresis.

D. Summary

In addition to describing the Staflo apparatus and its many modes of operation, this chapter significantly extends the Staflo method in the direction of quantitation of design and interpretation of experiments. It presents the concept of range and distribution diagrams for particle migration distances (e.g. Figures 13 and 15). These diagrams are used in developing the equations in Appendix E. The alternate form for presenting the range and distribution information (e.g. Figures 15, 22 and 23) clarifies the requirements for optimum fractionation of migration patterns and provides a method of analyzing the distribution of migration properties among particle populations. The remaining contribution is a method of identifying individual components of complex migration patterns involving separate identification of 2 or more of the properties of sedimentation rate, electrophoretic mobility and intrinsic motility, when they occur together in various combinations.

IV. EARLY STAFLO SEDIMENTATION EXPERIMENTS: RABBIT SPERMATOZOA

Contents

A. Introduction

The first Staflo study of spermatozoa was a series of sedimentation
experiments with rabbit spermatozoa using the 1 x 12 flow-cell. The in-
terest in the sedimentation properties of rabbit spermatozoa was stimulated
by Bhattacharya's report of significant control of the sex ratio of off-
spring from the insemination of rabbit spermatozoa fractionated in a column

sedimentation device[35]. In addition, Gordon reported dimorphism of spermatozoa ejaculate populations which was not related to the electrophoretic properties of the spermatozoa[92]. At the time of these studies, Schilling's report of the partial control of the sex ratio of offspring from the insemination of fractionated bull spermatozoa[205] and the report by Bedford and Bibeau showing no difference in the sex ratio from the insemination of fast and slow sedimenting rabbit spermatozoa[29] had not been published.

Ancillary purposes of these early experiments were to develop and improve procedures for handling spermatozoa in all phases of Staflo experiments and to explore the effects of the numerous experimental variables (Table I) affecting biophysical studies of spermatozoa. At the same time, an evaluation of media for the preservation of motility and fertilizing capacity of spermatozoa was being conducted (Appendix D).

As will be evident in the results, the maintenance of motility and fertilizing capacity was very poor in most of these experiments. Thus, the most interesting biological assay system, the sex ratio of offspring, could not be correlated with other biological results and the physical properties in these studies. However, these experiments demonstrate a number of important interrelationships among the biological and physical properties of spermatozoa and the numerous experimental variables listed in Table I. Of special interest is the effect of the intrinsic motility of spermatozoa upon "sedimentation -migration"rates.

B. Materials and Methods

1. Sample Preparation

Spermatozoa samples were obtained from mature Dutch Belt rabbits using the artificial vagina described in Appendix C. Each ejaculate was diluted immediately by adding 2.0 ml of medium (see below) at 37°C

to the collection vial. The suspension was cooled to room temperature in an insulating nest of glass wool. A 0.1 ml aliquot of this sample was diluted 1:10 for counting and for evaluation of the percentage and degree of motility. While the sample was being cooled slowly to the temperature of the experiment, it was diluted in several stages to the desired concentration. In order to obtain a minimum of 15 ml containing 20×10^6 spermatozoa/ml, it was usually necessary to pool ejaculates from 2 or more rabbits. After cooling and dilution, the spermatozoa suspension was subjected to Staflo sedimentation.

2. Staflo Apparatus

The 1 x 12 flow-cell (designated No. VII C), described in Chapter III, was used in this series of experiments. The spermatozoa suspension was introduced into inlet 4, using the same size syringe (20 ml) as used for the other inlet solutions. Thus, the height of the sample stream at the inlet was 1.25 mm. A typical migration pattern for this series of experiments is illustrated in Figure 27, where the spermatozoa suspension enters via inlet 4 and spreads over layers 4-7. In all experiments a set-up period equivalent to 2 times the average steady-state transit time through the flow-cell was allowed before collection of the fractionated spermatozoa was begun.

3. Medium and Density Gradient

A sucrose density gradient was used in 4 of the 7 sets of experiments described in this chapter and a dextran density gradient was used in the other 3. These experiments are hereafter referred to as "sucrose" or "dextran" experiments, respectively. In the sucrose experiments, the primary medium (medium S) consisted of 1/3 Hanks' solution and 2/3 isotonic saline supplemented with 0.1% fructose. The flowing layers, numbered 1-12

STAFLO FLOW-CELL SHOWING SPERMATOZOA MIGRATION PATTERN
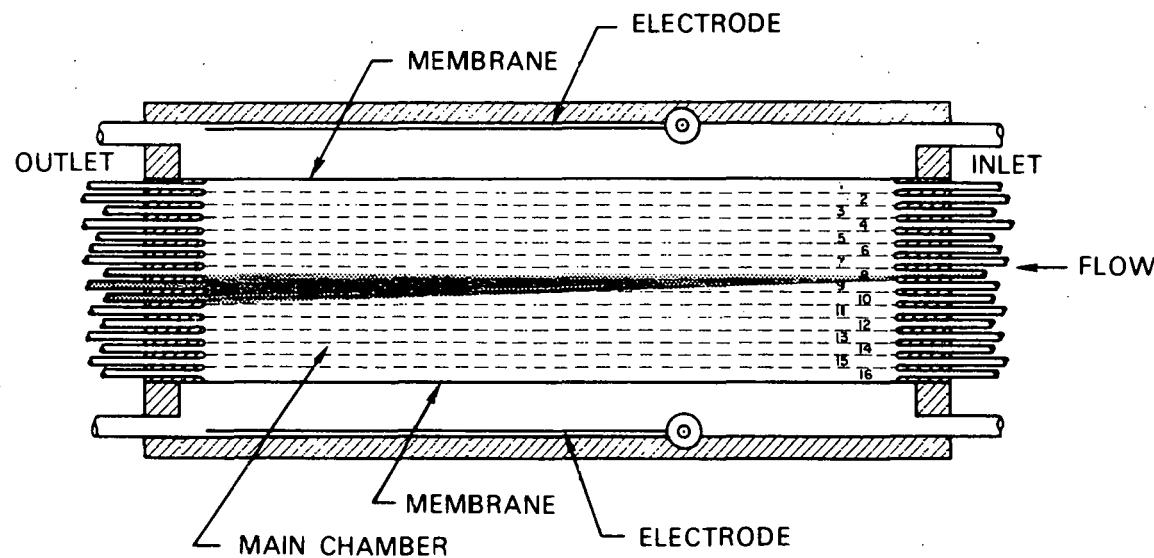
DBL 688-5418

Fig. 27. Schematic diagram of Staflo flow-cell showing typical rabbit spermatozoa sedimentation migration pattern at $7^{\circ}C$; sucrose density gradient.

from top to bottom, contained sucrose in weight/volume percent as follows: No. 1, 0% (distilled water); Nos. 2 and 3, 0% (medium S); No. 4, 0% (spermatozoa suspended in medium S); No. 5, 0.800%; No. 6, 0.875%; No. 7, 0.950%; No. 8, 1.025%; No. 9, 1.100%; Nos. 10-12, 3.0%. The viscosity, osmolarity and specific gravity of the solutions in layers 3-9 are shown in Figure 28 for both the sucrose and dextran density gradients. Under the conditions employed in these experiments, few spermatozoa migrated below the seventh (at 7°C) or ninth (at 15°C) outlets. The "density barrier" in layers 10-12 was designed to stop spermatozoa or other particles that migrated that distance in order to minimize contamination of the membrane over the bottom electrode compartment.

In the dextran experiments, the primary medium (medium D) consisted of a mixture of 10% 0.154 M glycine and 90% medium S. The layers contained dextran (MW 73,000) in weight/volume percent as follows: No. 1, 0% (distilled water); Nos. 2 and 3, 0% (medium D); No. 4, 0% (spermatozoa suspended in medium D); No. 5, 0.70%; No. 6, 0.75%; No. 7, 0.80%; No. 8, 0.85%; No. 9, 0.90%; Nos. 10-12, 3.0%.

4. Temperature Control

In the experiments conducted at 4°C or above, the temperature of the solutions in the syringe on the pump rack was controlled by enclosing the pump rack in a plastic canopy and cooling with dry ice. The flow-cell was cooled with dry ice in a doorless refrigerator in the sucrose experiments and in a lucite box in the dextran experiments.

The experiments at 2.5°C were conducted in a newly constructed refrigerated room which was large enough for the entire apparatus, accessory equipment and several investigators.

5. Observation and Evaluation of Spermatozoa

CHARACTERISTICS OF DEXTRAN AND SUCROSE GRADIENTS
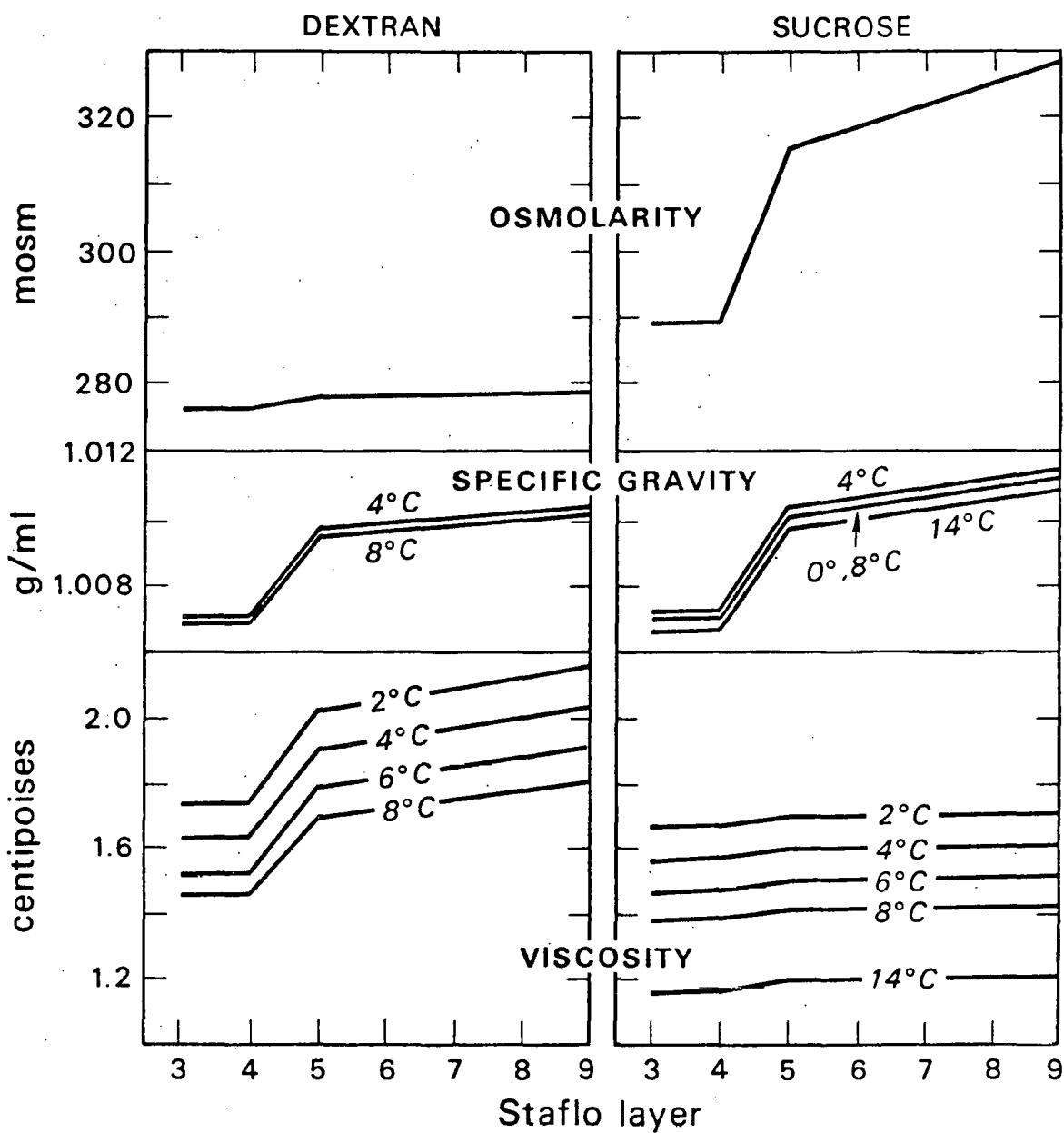
DBL 688-5422

Fig. 28. Characteristics of dextran and of sucrose density gradients for early sedimentation experiments.

The migration pattern was observed with the "slit lamp technique"[263] and observations of individual spermatozoa and the microscopic steady-state pattern were made with a portable microscope at magnifications up to 640 X. This portable microscope was a prototype for the Staflo microscope described in Chapter III. After collection, the spermatozoa concentration and motility in each fraction was determined with a hemocytometer and the viability was evaluated by nigrosin-eosin staining as described in Appendix B. The categories of spermatozoa in the nigrosin-eosin stained preparations are as follows: unstained (U) or viable, morphologically normal but stained (MNS) and stained (S) or non-viable.

C. Results

1. Fractionation in a Sucrose Density Gradient.

Experiments were conducted at 4 temperatures in the following sequence: 7°C, 15°C, 4°C and 2.5°C. The results are presented here and are compared in section D.

a. 7°C. The results of a typical experiment (10-21) at 7°C with a sucrose density gradient and a spermatozoa concentration of 20×10^6/ml are shown in Figure 29. The most striking feature is the absolute enrichment in fractions 5, 6 and 7 with regard to viable spermatozoa relative to the unfractionated sample both before and after the experiment. The curve for stained spermatozoa is almost a mirror image of the curve for viable spermatozoa. The determination of motility was made 90 minutes after collection stopped and shows an increase from fraction 4 to fraction 5. The concentrations shown in Figure 29 for collection fractions 4 - 7 represent 35.6%, 50.4%, 12.7% and 1.3% of the collected spermatozoa, respectively. It may be helpful to refer to Figure 27 to visualize the spermatozoa migration pattern.

SPERM SEPARATION
Run 10-21 (7°C, sucrose)
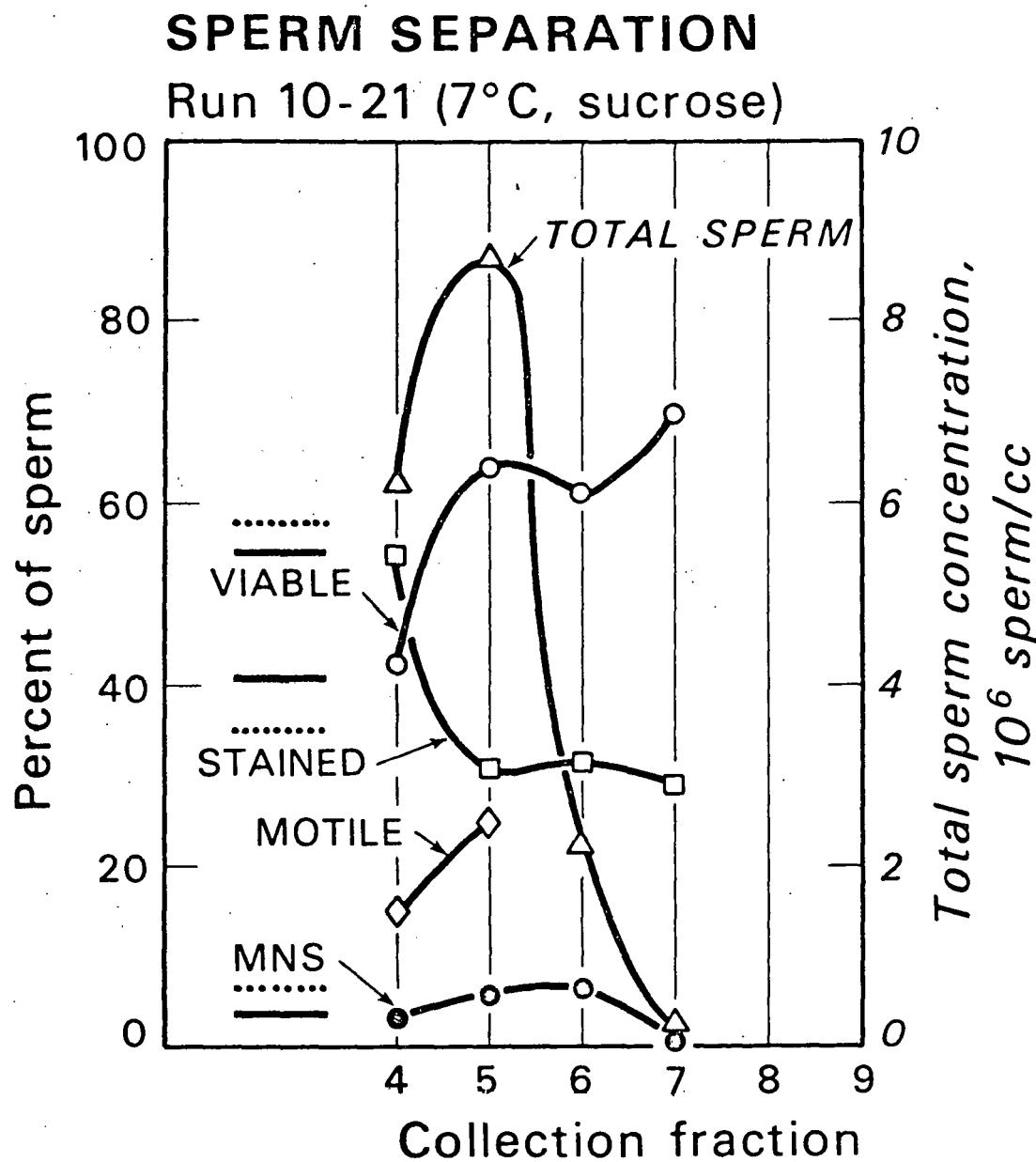
Fig. 29. Results of Sedimentation Experiment 10-21 with rabbit spermatozoa at 7°C; sucrose density gradient. Also shown are the characteristics of the unfractionated sample before (••••••••) and after (————) the experiment.

The results of this experiment and 4 others run at 7°C with the same sucrose density gradient and spermatozoa concentrations ranging from 19-26 x 10^6/ml are shown in Figure 30. The spermatozoa samples consisted of an ejaculate from rabbit H in all experiments mixed with ejaculates from rabbit G in 3 experiments and from rabbits O and P in 1 experiment each. Samples were of approximately equal initial viability and motility. Despite great changes in spermatozoa viability during the course of some experiments, the corresponding curves for viable and stained spermatozoa in Figure 30 show the same qualitative features seen in Figure 29. A consistent feature of the motility curves is their qualitative similarity to their respective total sperm distribution curves. The viability consistently increases from fraction 6 to 7 while both spermatozoa concentration and motility decrease. Included among and indistinguishable from the curves of total sperm distribution is the result of one experiment (shown by the arrow) conducted under the same conditions with a spermatozoa concentration of about 20 x 10^6/ml, all of which were immotile upon ejaculation.

The general features of the sets of curves in Figure 30 are clearly seen in Figure 31 which shows the averages for all 5 experiments. The relative composition of stained spermatozoa (U, MNS and S) in each fraction can be determined from this figure.

Nominal sedimentation rates can be calculated for the spermatozoa in each collection fraction using a layer thickness of 1.25 mm and an average steady-state transit time of 23.3 minutes. All spermatozoa entered the main chamber via inlet 4. Spermatozoa leaving via outlet 4 must have sedimented between 0 and 1.25 mm. For these cells, the average extreme sedimentation rate is approximately 1.08 mm/hr. This and sedimentation

SPERM SEPARATION
5 runs (7°C, sucrose)
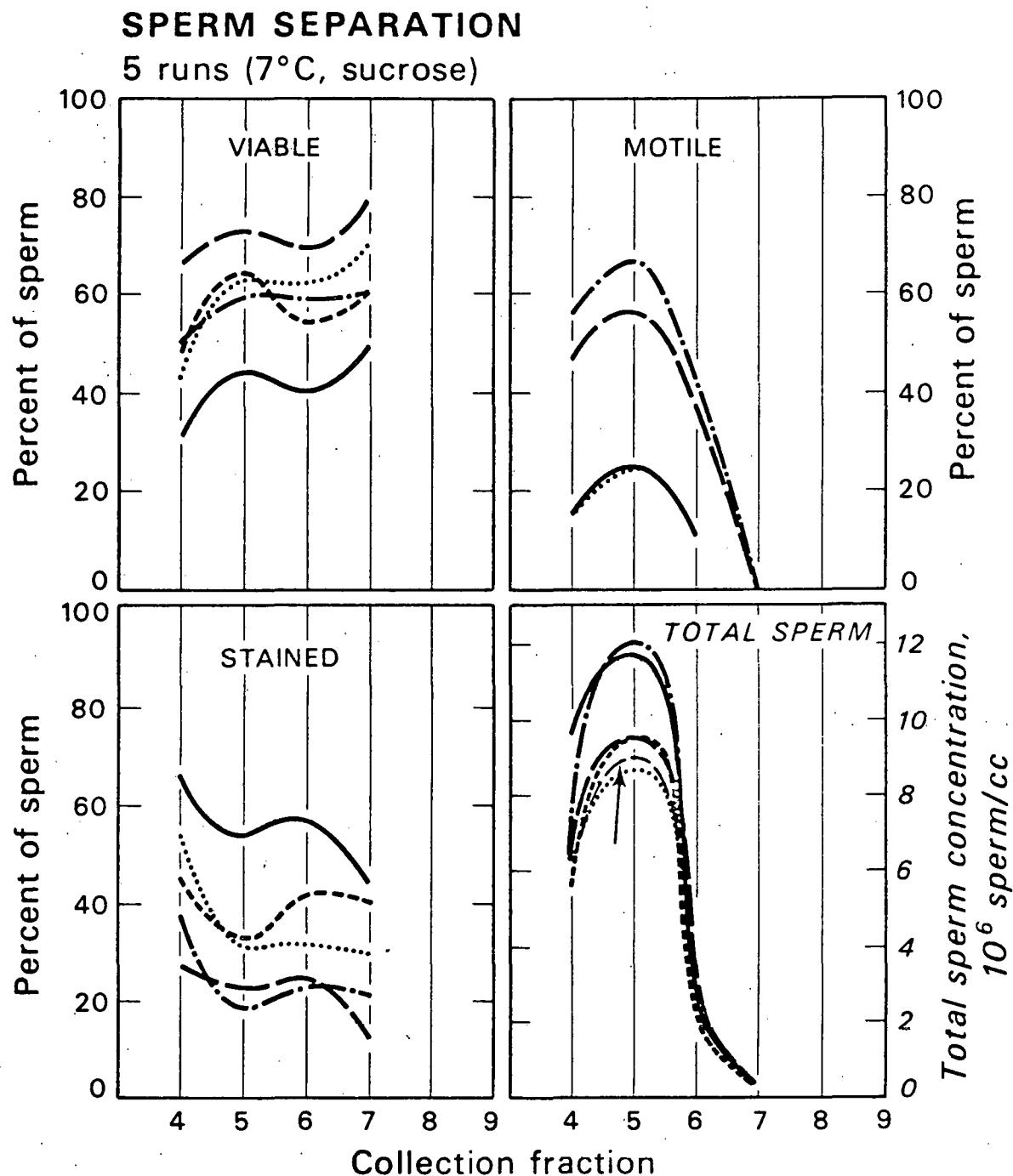
Fig. 30. Individual results of five sedimentation experiments with rabbit spermatozoa at 7°C; sucrose density gradient. Key for experiments: 9-15 (————); 9-22 (- - -); 10-5 (— —); 10-12 (—·—·); 10-21 (·······).

SPERM SEPARATION
Average of 5 runs
(7°C, sucrose)
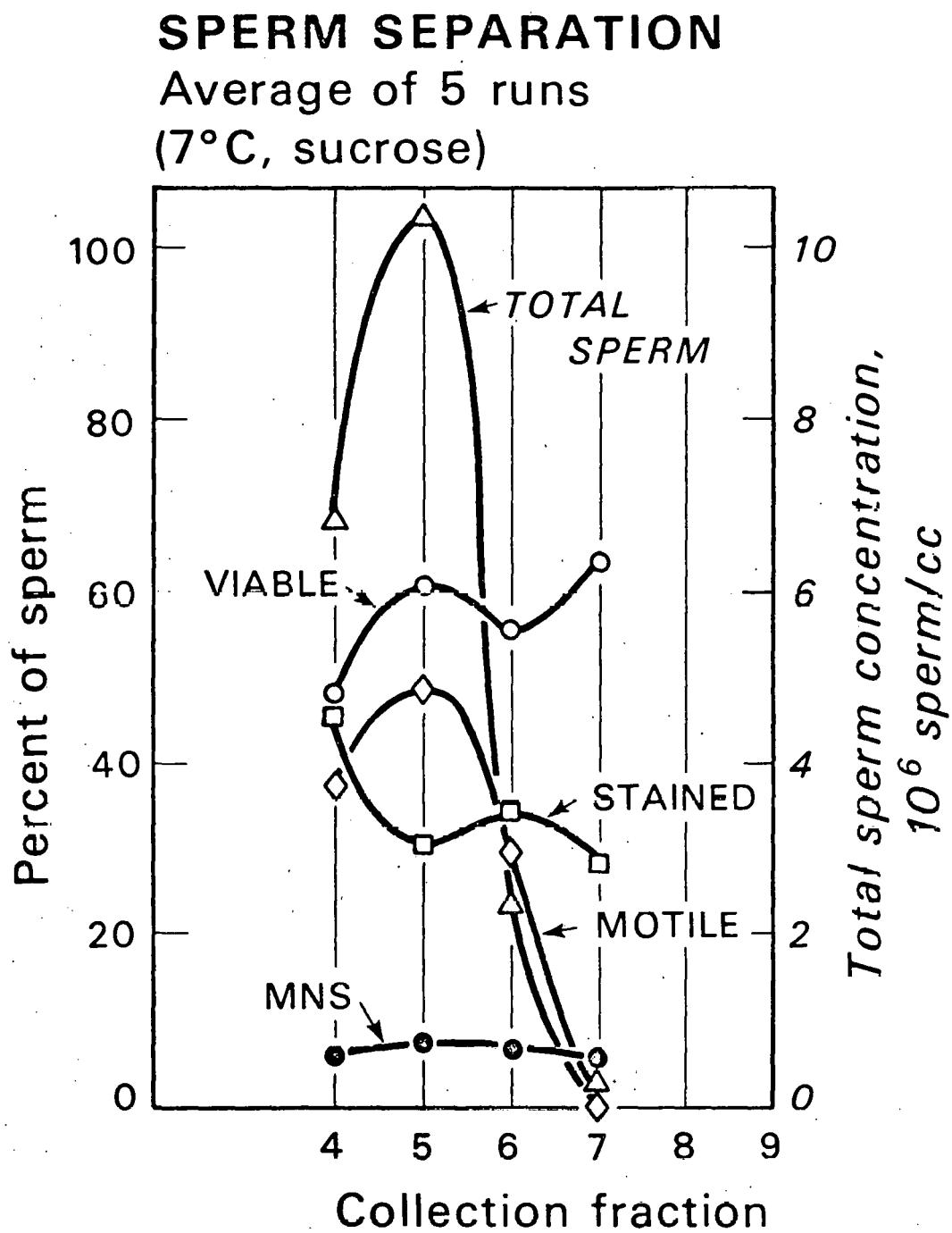
DBL 7011 5984

Fig. 31. Average results of five sedimentation experiments with rabbit spermatozoa at 7°C; sucrose density gradient.

rates for spermatozoa in the other collection tubes were calculated with the equations in Chapter III and Appendix E. They are summarized in Table III. The percentages of spermatozoa in collection fractions 4-7 ranged from 31.5-38.6%; 47.6-55.9%, 11.4-12.7% and 0.9-1.4%, respectively, in the 5 experiments, demonstrating good reproducibility.

Microscopic observations in the flow-cell showed that about 90% of the spermatozoa sedimented vertically with heads down and the remainder were obliquely oriented with their heads below the horizontal.

The evaluation of motility generally was made 60-90 minutes after collection stopped. At this time, 5-10% of the spermatozoa in fraction 4 showed vigorous forward motion, whereas the remainder of the motility was primarily tail-wagging. Even though the spermatozoa in fraction 5 generally had a higher percentage of motility, the degree thereof was less than in fraction 4.

b. 15°C. The average composition of the collection fractions in two experiments and the percentage of motile spermatozoa in each fraction for one experiment are shown in Figure 32A. Again, the curves for viable and stained spermatozoa change in opposite directions. Because of the limited data on motility, there is probably no significance to the second peak in motility in fraction 8. In Figure 32B, the average distribution of sperm in these 2 experiments at 15°C is shown with the average for the 5 experiments at 7°C (from Figure 31). There is a significantly greater number of sperm in fractions 6, 7 and 8 at 15°C than at 7°C. The cell concentrations at 15°C were 25 x 10^6 and 28 x 10^6 spermatozoa/ml and were mixtures of ejaculates from rabbits G, O and P. Slight "cluster sedimentation" was observed in both experiments. Actively swimming spermatozoa were observed microscopically in layers 4 through 10 during the collection period in both

Table III. Average Migration Velocities: Intrinsic Motility-Sedimentation, for
Rabbit Spermatozoa; Sucrose Density Gradient; 2.5, 4, 7 and 15°C

Temp	Collection Fraction	Percent of Sperm Collected	Avr. Migration Velocity		Avr. Migration Rate[a]
			$(cm/sec \times 10^{-4})$	(mm/hr)	
2.5°C (3 runs)	4	55.7	0.30	1.08	0.31
	5	42.0	0.71	2.56	0.73
	6	2.3	1.02	3.67	1.04
4°C (1 run)	4	43.7	0.30	1.08	0.31
	5	47.9	0.90	3.24	0.92
	6	7.9	1.53	5.51	1.56
	7	0.5	1.84	6.62	1.88
7°C (5 runs)	4	34.0	0.30	1.08	0.31
	5	52.5	0.90	3.24	0.92
	6	12.3	1.71	6.16	1.75
	7	1.2	1.91	6.87	1.95
15°C (2 runs)	4	19.2	0.30	1.08	0.31
	5	42.4	0.90	3.24	0.92
	6	23.3	1.79	6.44	1.83
	7	10.8	2.69	9.68	2.74
	8	3.4	3.32	11.9	3.39
	9	0.9	3.63	13.1	3.70

a. units of 10^6 Svedbergs; 1 Svedberg = 10^{-13} seconds
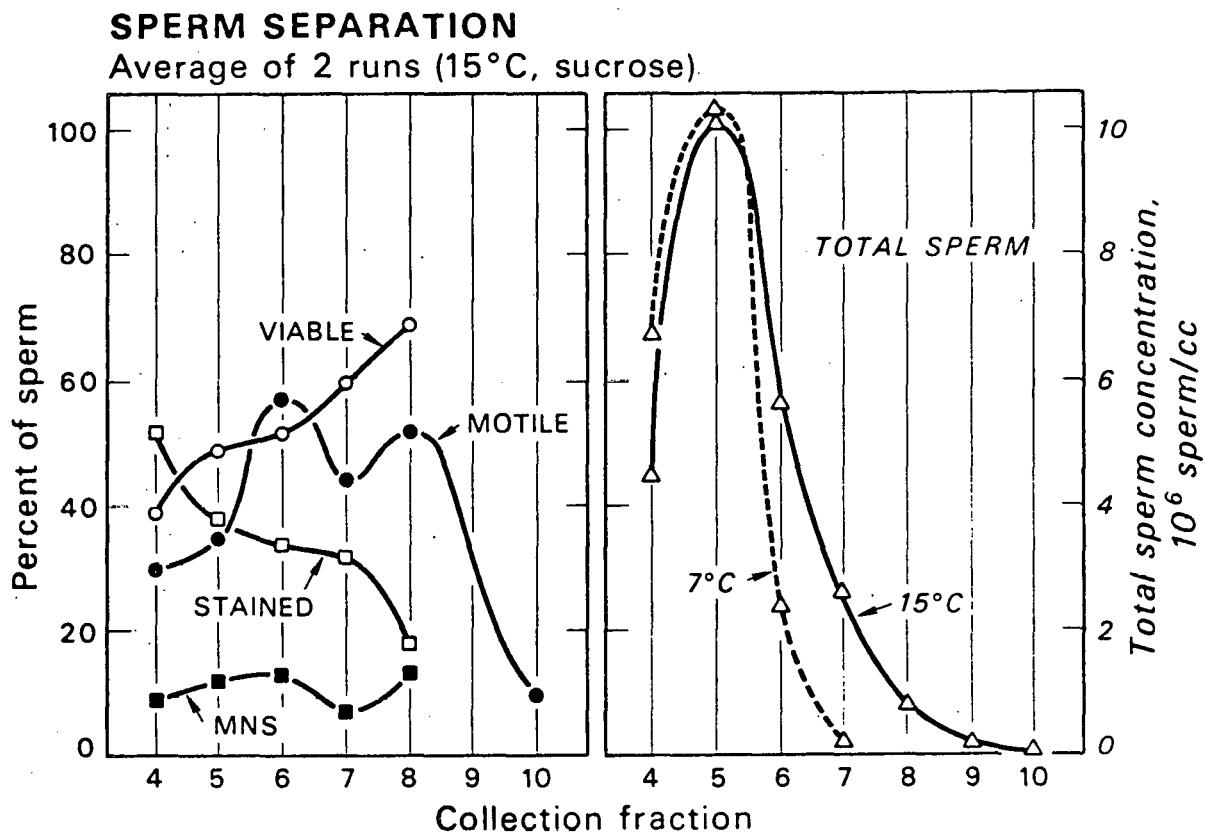
Fig. 32. Average results of two sedimentation experiments at 15°C and
a comparison of the average total rabbit spermatozoa distribution
at 15°C with that from five experiments at 7°C; sucrose density
gradient.

experiments.

c. <u>4°C</u>. The results of a single experiment at 4°C are shown in Figure 33. Comparing Figure 33 with Figure 31, it is seen that at 4°C, a greater proportion of spermatozoa remained in layer 4 and fewer reached layer 6. As at 7°C, a higher percentage of spermatozoa were motile and viable in fraction 5 compared to fraction 4.

d. <u>2.5°C</u>. The individual results of 3 experiments at 2.5°C with a sucrose density gradient are shown in Figure 34 and the average values for all 3 experiments are shown in Figure 35. At room temperature, there were no motile spermatozoa in the initial samples in Experiments 12-17-66 and 12-18-66. In Experiment 12-19-66, only 20% of the spermatozoa initially showed moderate tail-wagging with no forward progress. As shown in Figure 34, only 2% of the total spermatozoa were in fraction 6 and they were of lower viability than those in either fraction 4 or 5. Even though no motility was observed microscopically in the flow-cell in Experiment 12-19-66, the total spermatozoa distribution for that experiment differs from the 2 others in Figure 34 and is similar to that observed in experiments at 4°C and 7°C. Figure 35 shows a slight rise in viability going from fraction 4 to 5, as in experiments at higher temperatures, and a marked drop in viability from fraction 5 to 6.

e. <u>Calculation of Sedimentation Velocities</u>. Using the fractionation-migration principles outlined in Chapter III, the intrinsic motility-sedimentation velocities were calculated for the rabbit spermatozoa in each of the collection fractions in the experiments at 2.5°C, 4°C, and 15°C. These results are presented in Table II with the values for the experiments at 7°C.

2. <u>Fractionation in a Dextran Density Gradient</u>

SPERM SEPARATION
Run 11-15 (4°C, sucrose)
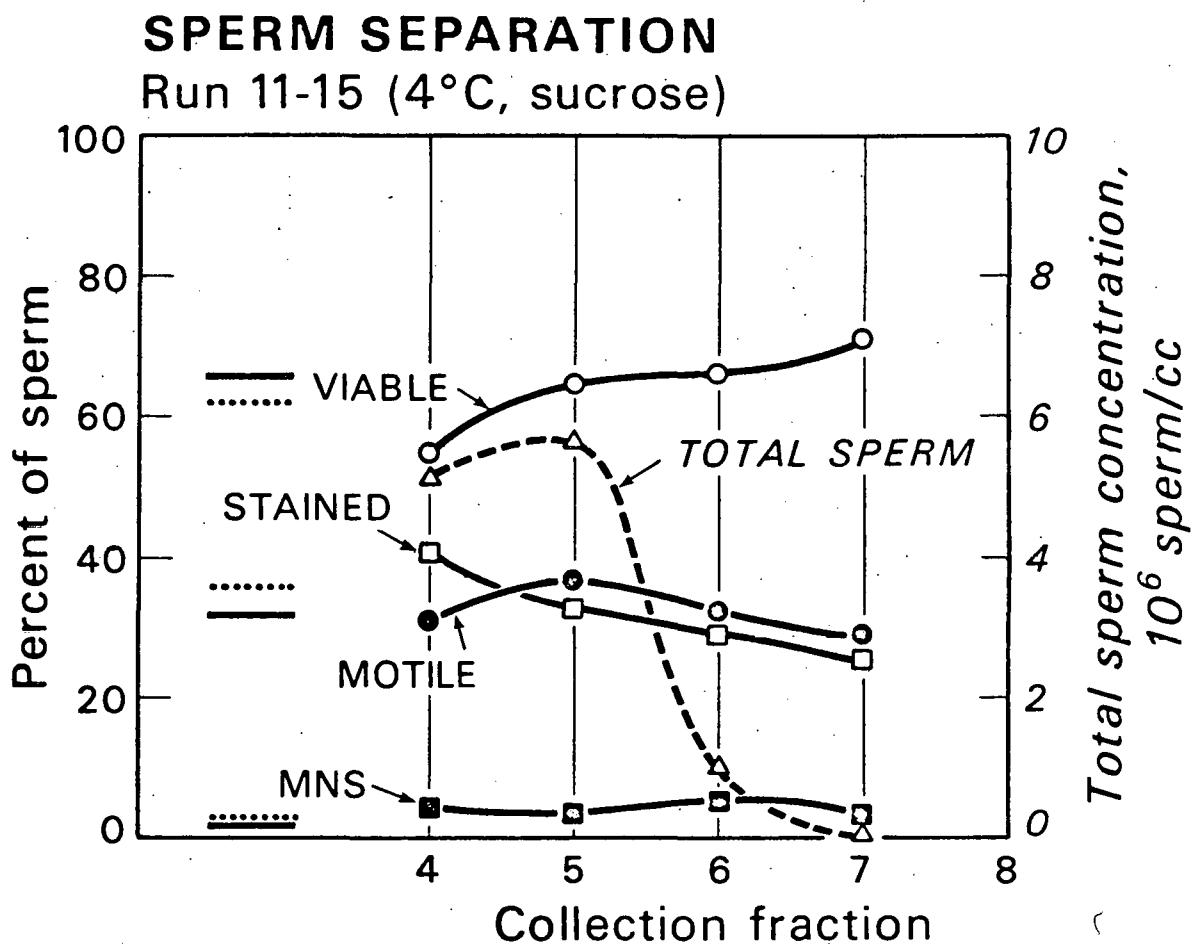
Fig. 33. Results of one sedimentation experiment with rabbit sperm-
atozoa at 4°C; sucrose density gradient. Characteristics of the
unfractionated sample: before (————) and after (•••••••) the
experiment.

DBL 7011 5986

SPERM SEPARATION
3 runs (2.5°C, sucrose)
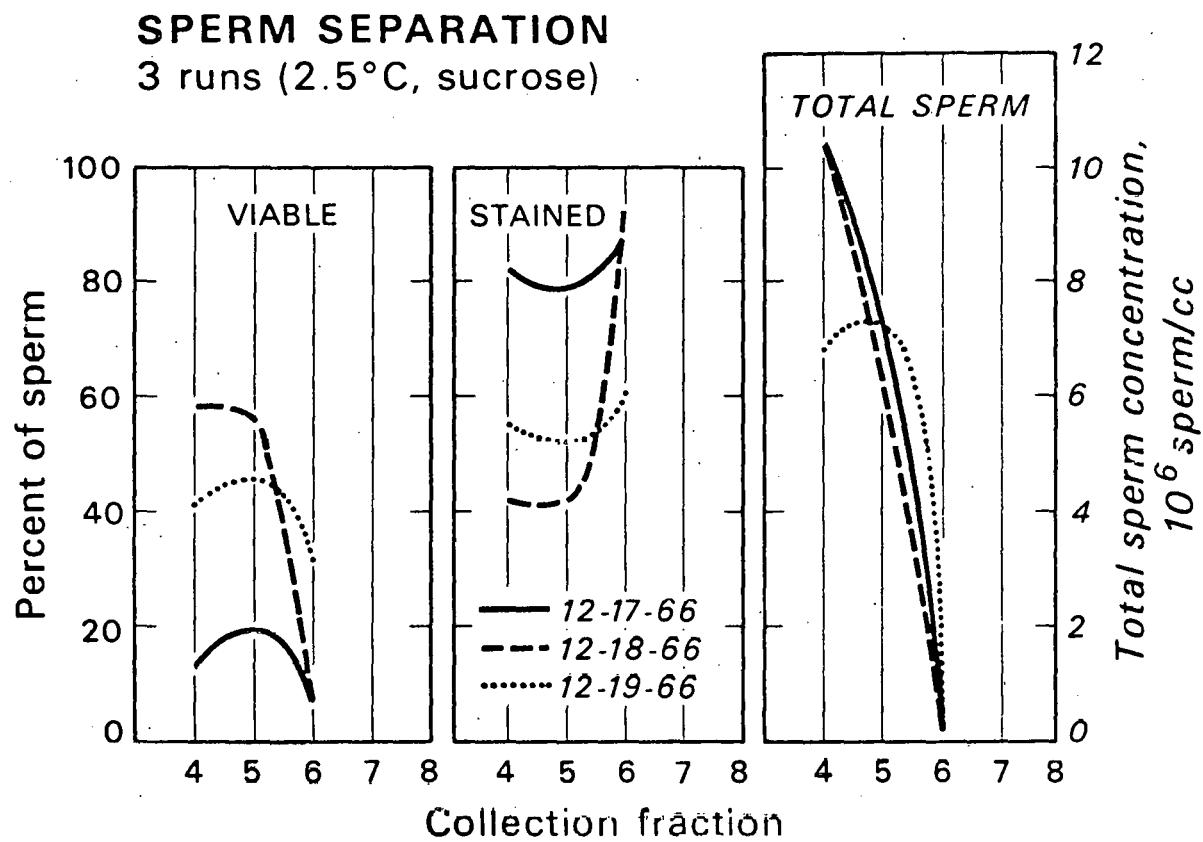
DBL 7011 5987

Fig. 34. Individual results of three sedimentation experiments with rabbit spermatozoa at 2.5°C; sucrose density gradient.

SPERM SEPARATION
Average of 3 runs (2.5°C, sucrose)
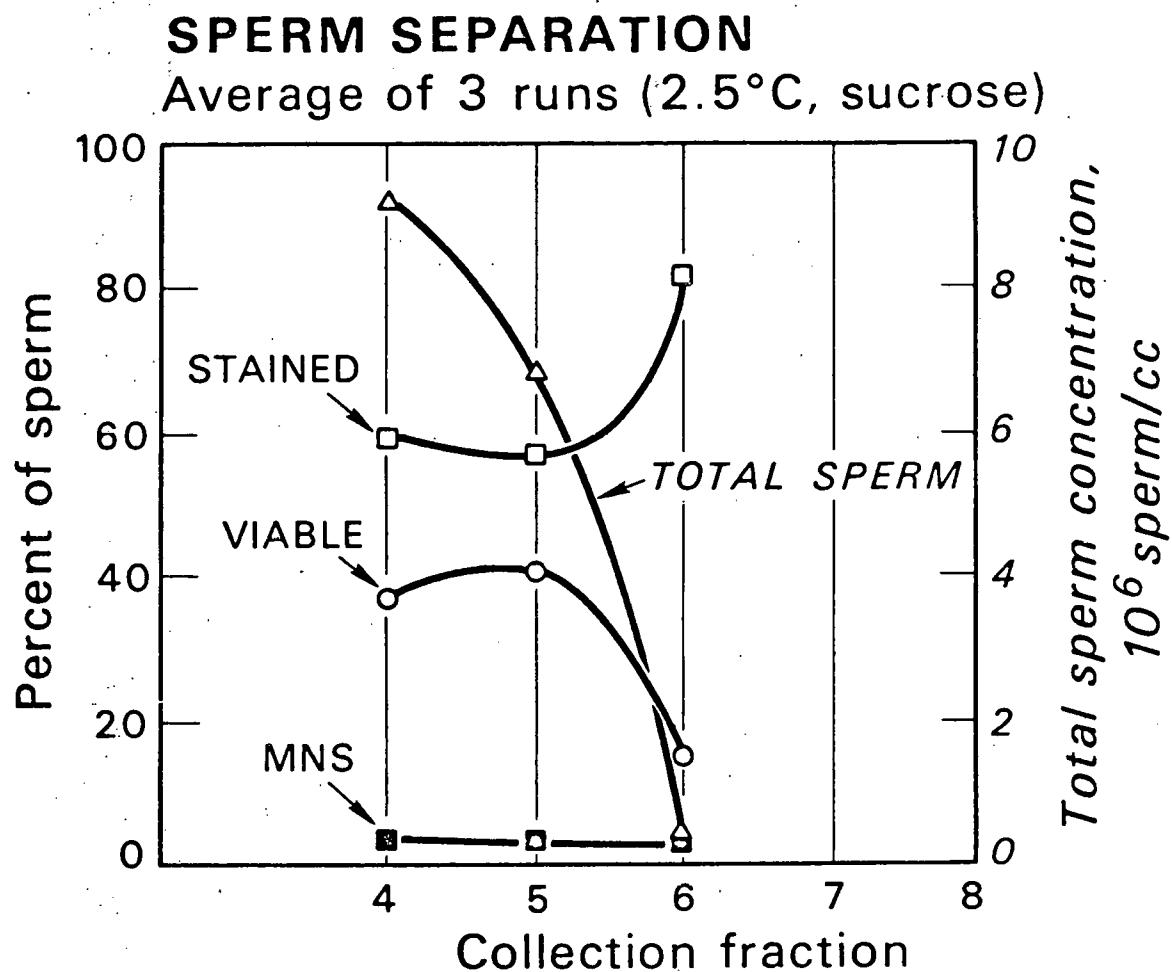
DBL 7011 5988

Fig. 35. Average results of three sedimentation experiments with rabbit spermatozoa at 2.5°C; sucrose density gradient.

Experiments were conducted only at temperatures of 7°C, 4°C and 2.5°C in this density gradient.

a. <u>7°C</u>. The results of 2 experiments at 7°C using a glycine supplemented medium and dextran density gradient with a 27 minute steady-state transit time are shown in Figure 36. Ejaculates from rabbits Chi, O and P were diluted to concentrations of 18×10^6 and 22×10^6 spermatozoa/ml for Experiments 1-4 and 1-14, respectively. In Figure 36A, there is an increase in viability from fractions 4-6. In contrast, the percent motility decreases from fraction 5 to 6, again showing that the loss of motility precedes the loss of viability. The curves for total spermatozoa distribution and motility in Figure 36 are qualitatively similar. A high percentage of the spermatozoa in the flow-cell showed slight tail-wagging. In both experiments, over 25% of the spermatozoa in fraction 4 and a few percent in fractions 5 and 6 showed vigorous forward movement. The activity of the spermatozoa in the collection fractions in this glycine supplemented medium was much greater than ever observed in the collection fractions in the sucrose experiments.

b. <u>4°C</u>. A single experiment was conducted with the glycine-dextran medium at 4°C with a spermatozoa sample of fair initial quality. Eighty percent of the spermatozoa exited the flow-chamber from layer 4 (the inlet layer), 19% exited from layer 5 and only 1% of the spermatozoa exited in layer 6.

c. <u>2.5°C</u>. After construction of a refrigerated room, 2 experiments were conducted at 2.5°C using the same dextran density gradient, a spermatozoa concentration of 20×10^6/ml and an average transit time of 66 minutes. A 10 ml syringe was used to introduce the spermatozoa suspension; thus, the sample stream at the inlet was only 0.75 mm high. At the outlet, the sample stream was 1.55 mm high but spermatozoa were observed with a micro-

SPERM SEPARATION
2 runs (7°C, Dextran-glycine)
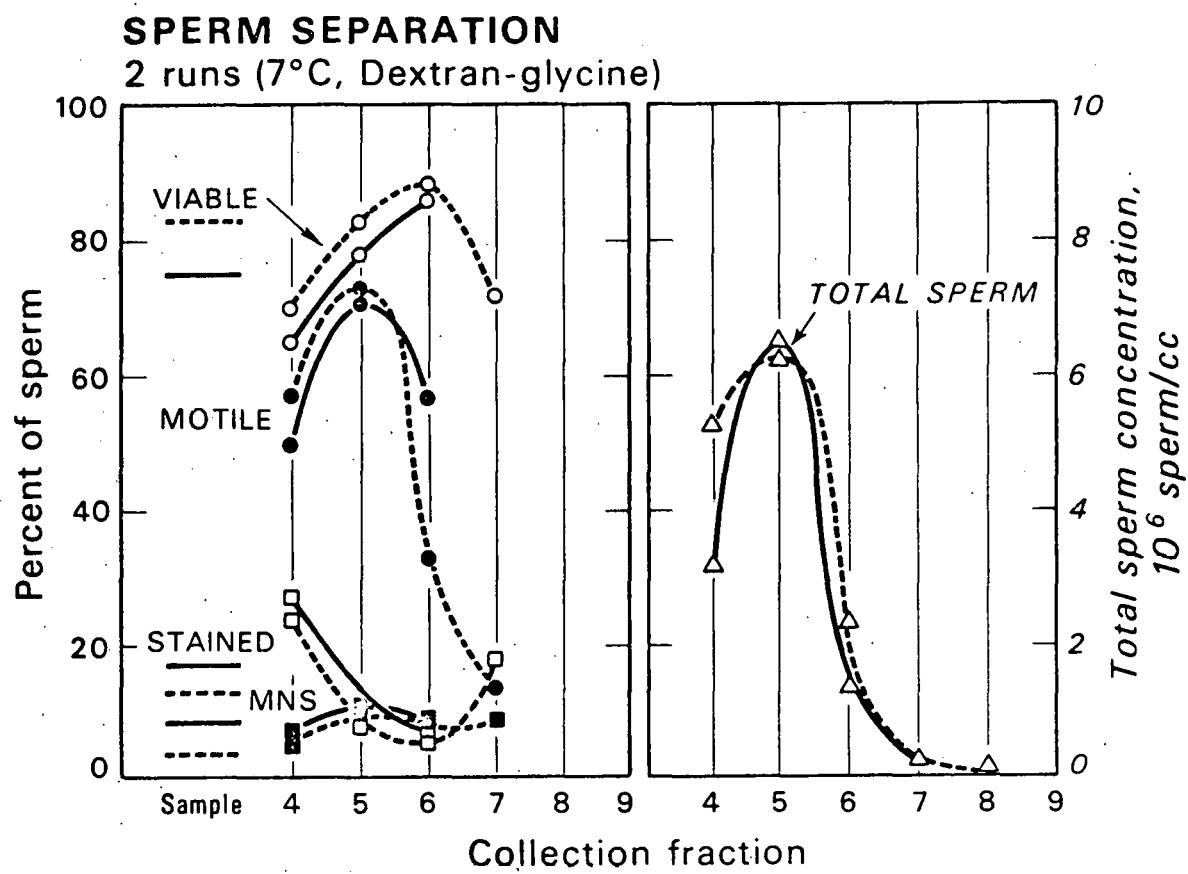
DBL.7011 5989

Fig. 36. Individual results of two sedimentation experiments with rabbit spermatozoa at 7°C; dextran density gradient. Initial sample values are also given. Key: Exp. 1-4 (————); Exp. 1-14 (- - - -).

scope to be in the bottom 0.95 mm only. Thus, the minimum migration distance of the spermatozoa was 0.60 mm and the maximum migration distance from the bottom of the sample stream at the inlet to the bottom of the sample stream at the outlet was 0.80 mm. Using these migration distances and the average transit time of 1.1 hours, calculations give minimum, maximum and average sedimentation rates of 0.82, 1.09 and 0.97 mm/hr, respectively. Because the spread of the spermatozoa migration pattern was less than the thickness of 1 Staflo layer, the fractionation was not meaningful in these 2 experiments.

d. Calculation of Sedimentation Velocities. Using the fractionation-migration principles discussed in Chapter III, the average intrinsic motility-sedimentation rates were calculated for the spermatozoa in each of the collection fractions in the experiments at 2.5°C, 4°C and 7°C. These rates are presented in Table IV in units of cm/sec x 10^{-4}, mm/hr and Svedbergs.

3. Artificial Insemination.

Despite inseminating a large number of rabbits with spermatozoa from the collection fractions in many of the experiments presented above, only 4 litters were obtained. In the 4°C sucrose experiment, both rabbits inseminated with spermatozoa from fraction 4 gave litters, one of 4 males and 3 females, and the other, 1 male and 4 females. Insemination of spermatozoa from fraction 5 in that experiment gave no offspring. In 2 dextran experiments, one each at 4°C and 2.5°C, with 80% and 95% of the spermatozoa in fraction 4, respectively, insemination of spermatozoa from those collection fractions gave 2 litters of 3 females and 2 males each. This small number of offspring from several different experiments was insufficient for correlation with the physical results.

Table IV. Average Migration Velocities: Intrinsic Motility-Sedimentation, for Rabbit Spermatozoa in a Dextran Density Gradient at 2.5, 4 and 7°C

Temp	Collection Fraction	Percent of Sperm Collected	Avr. Migration Velocity		Avr. Migration Rate[a]
			(cm/sec x 10^{-4})	(mm/hr)	
2.5°C (2 runs)	–	100	0.27	0.97	0.27
4°C (1 run)	4	80	0.22	0.78	0.22
	5	19	0.48	1.73	0.49
	6	1	0.71	2.56	0.73
7°C (2 runs)	4	32.7	0.26	0.94	0.27
	5	51.3	0.77	2.77	0.79
	6	14.1	1.34	4.82	1.37
	7	1.3	1.60	5.76	1.63

a. units of 10^6 Svedbergs; 1 Svedberg = 10^{-13} seconds

D. Discussion

The similarity of the shapes of the curves in Figure 30, and the small range of values for the average distribution of spermatozoa among the collection fractions are demonstrations of experimental reproducibility under specified conditions. However, this series of experiments has shown that in the multi-variable Staflo system, a large number of parameters influence the sedimentation-migration pattern of spermatozoa. These, in turn, affect the biological results and must be considered in planning experiments. Variables of importance include the initial sample quality (the ratio of viable to stained spermatozoa and the degree of motility) and durability (a measure of the loss of viability and motility during the experiment), the spermatozoa concentration, and the temperature. Related factors include the average steady-state transit time, and the medium and density gradient. After considering the influence of these variables on the experiments, a comparison will be made with the results of other investigators on the sedimentation of rabbit spermatozoa.

1. Variables Affecting Sedimentation Experiments.

a. Quality of the Spermatozoa Sample. In addition to great variations in the initial quality of ejaculates from the same rabbit on different days, or different rabbits on the same day, ejaculates of the same apparent initial quality varied in their ability to withstand the stresses in Staflo experiments. This unpredictability of spermatozoa quality is discussed in more detail in Chapter IX.

In this series of experiments, the variable durability of spermatozoa samples is illustrated in the sucrose experiments using the percentage of viable (unstained) spermatozoa for comparison. In Experiments 9-15 and 9-22 (Figure 30) and in the 2 experiments at 15°C (Figure 32), the percentages

of viable spermatozoa in the collection fractions were approximately 20% less than in the corresponding initial samples. In the other sucrose experiments, there was essentially no degradation of spermatozoa quality during the experiment as judged by nigrosin-eosin staining.

Using the degree of motility after collection relative to the initial sample as a criterion, the durability of the spermatozoa sample in all of the sucrose experiments was poor. The relatively higher degree of motility of the spermatozoa in the collection fractions in the dextran experiments may have been a result of (1) a beneficial effect of the glycine or dextran, or (2) more durable spermatozoa samples obtained at a different time of year.

The poor results from the insemination of spermatozoa from the collection fractions in this series of experiments is believed to be due primarily to dilution in media not suitable for the preservation of motility and fertilizing capacity. In retrospect, in most experiments, the spermatozoa in the collection fractions had such a poor degree of motility that insemination was not warranted.

b. <u>Spermatozoa Concentration</u>. The spermatozoa concentration affects both the migration pattern and the quality of spermatozoa in the collection fractions at the termination of the experiment. Based upon the results of the 7°C-sucrose experiments, the migration pattern appears independent of concentration over the range 19-26 x 10^6 spermatozoa/ml. In the 15°C-sucrose experiments, slight "cluster sedimentation" was observed with spermatozoa concentrations of 25 x 10^6 and 28 x 10^6/ml. The critical concentration of yeast cells referred to in Mel's discussion of "cluster" sedimentation[158] is 26-27 x 10^6/ml, suggesting a similar threshold for the 2 types of cells in similar low density-low viscosity media.

Other experiments in which there is a suggestion that "cluster sedimentation" occurred are the experiment at 7°C with immotile sperm (arrow in Figure 30) and Experiment 12-19-66, at 2.5°C, where the total sperm distribution resembles that at higher temperatures. In the latter experiment, it is unlikely that there was sufficient motility to explain the increased migration.

The effect of low spermatozoa concentrations (dilution effect) on the quality of the spermatozoa at the termination of the experiment is also illustrated in this series of experiments. In all sucrose experiments at 7°C, the percentage of motile spermatozoa decreased from fractions 5-7 with changes in concentration from over 8×10^6/ml in fraction 5 to about 2×10^6 and 0.5×10^6/ml in fractions 6 and 7, respectively. A similar loss of motility was also observed in the 7°C dextran experiments (Figure 36) in suspensions of less than about 4×10^6 spermatozoa/ml and is attributed primarily to dilution. This dilution effect has been reported by others to occur at concentrations of less than 4×10^6 spermatozoa/ml and to be prevented by certain macromolecules such as gum-arabic[125]. The recent studies by Quinn, White and Wirrick showed that dilution of ram and bull spermatozoa caused an influx of sodium and an efflux of potassium, calcium and magnesium from the cells, and was associated with loss of motility[193]. The macromolecule dextran may have had a beneficial effect in the dextran density gradient experiments as noted in "a", above.

Because the spermatozoa concentrations in fractions 4 and 5 were greater than 4×10^6/ml in all experiments except in the Dextran Experiment 1-4, there was no dilution effect in those fractions. The percentage of motile spermatozoa in fraction 5 was greater than in fraction 4 in all experiments; however, the spermatozoa in fraction 4 had a higher degree of motility in

all cases. The presence of seminal plasma in fraction 4 is the most likely explanation for the higher degree of motility in that fraction.

In all of the sucrose experiments at temperatures of 4°C or higher, and in all the dextran experiments at 7°C (except for fraction 7 in Experiment 1-14), the percentage of viable spermatozoa increased with increasing migration distance below layer 5. This is in contrast to the loss of motility observed with decreasing spermatozoa concentrations in those same fractions. This shows that at least during a minimum period averaging 36 minutes at low concentrations, the loss of motility preceded the loss of viability, judged by nigrosin-eosin staining. It is postulated that at these temperatures, the best spermatozoa judged by degree of intrinsic motility swam downward, thereby giving an increase in the percentage of unstained (viable) spermatozoa in the lower fractions.

c. <u>Temperature and Motility</u>. The temperature affects the sedimentation migration pattern primarily through its influence on motility. Figure 37 is a comparison of the total spermatozoa distribution for sucrose experiments at 2.5°C, 4°C, 7°C and 15°C. It shows a significant increase in the percentage of the total number of spermatozoa appearing in the lower collection fractions at the higher temperatures. Because the spermatozoa used in the 3 experiments at 2.5°C were of poor initial quality and no motility was observed in the flow-chamber, the migration patterns observed in those experiments are believed to be free of a motility component. In the experiment at 4°C, spermatozoa were observed to have a rolling motion in the flow-cell and had sufficient motility to move forward slightly in a hemocytometer at that temperature. No motility was observed microscopically in the flow-cell during the 7°C sucrose experiments, but the optical system was in a very preliminary stage of development at the time of those

SPERM SEPARATION
Total distribution at various temperatures
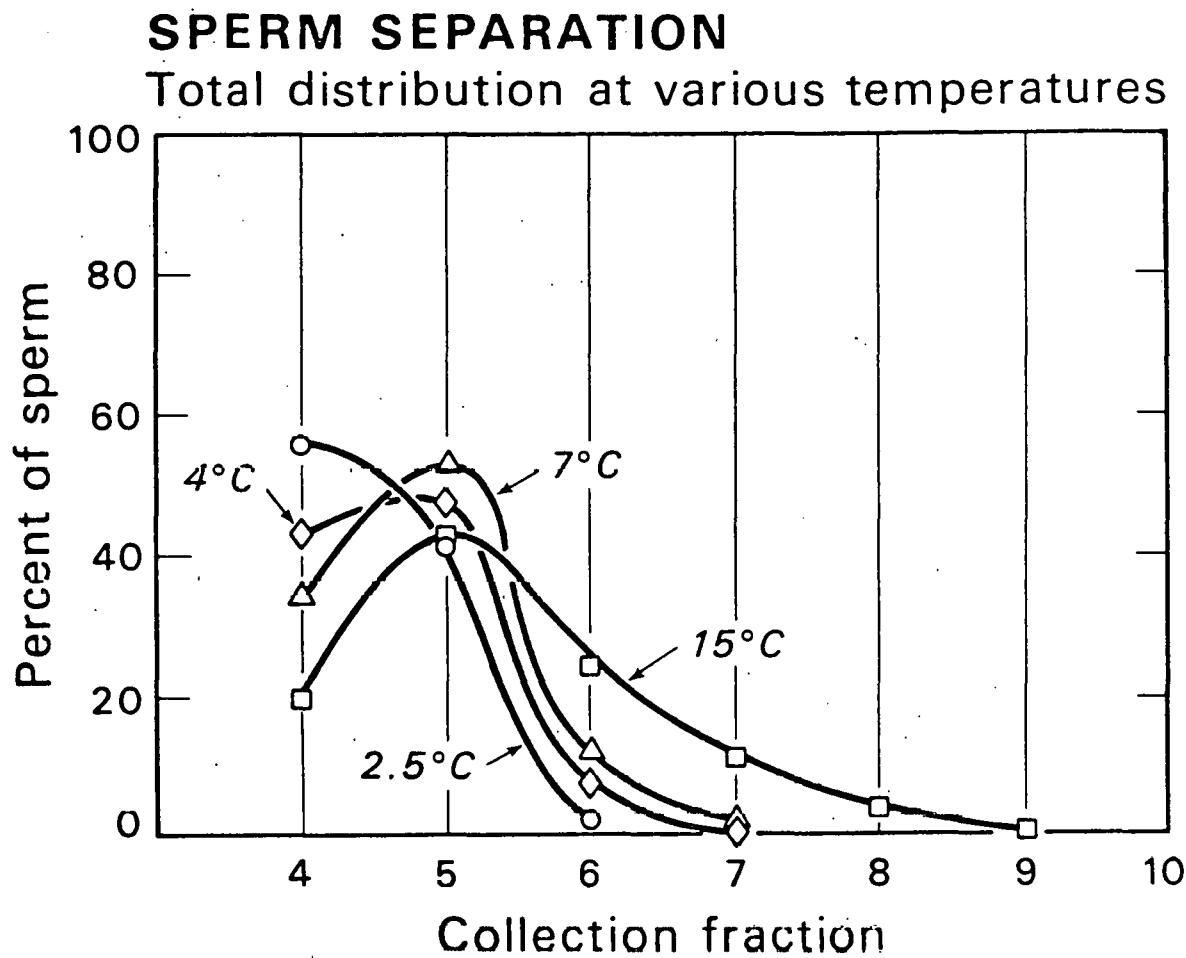
DBL 7011 5990

Fig. 37. Average distribution of rabbit spermatozoa among the collection fractions in sedimentation experiments at 2.5°C, 4°C, 7°C and 15°C; sucrose density gradient.

experiments. Thus, motility could have contributed to the migration patterns in the 7°C experiments. A high percentage of actively swimming spermatozoa was observed microscopically in the flow-cell in both experiments at 15°C. Thus, even though there was slight "cluster sedimentation" in both experiments, the greater migration at 15°C is attributed primarily to the intrinsic motility of the spermatozoa at that temperature.

Figure 38 is a comparison of the different-temperature sucrose experiments using the migration distance diagrams discussed in Chapter III. I believe that the experiments at 2.5°C represent true sedimentation. Thus, the observed and expected distribution of spermatozoa at that temperature would be the same. As shown in Figure 28, the viscosity of the sucrose density gradient changes from 1.675 centipoises at 2.5°C to 1.175 centipoises at 15°C. Using these differences in viscosity and the distribution at 2.5°C, the expected distributions at 4°C, 7°C and 15°C were calculated. They are shown in Figure 38 along with the actual distributions of spermatozoa in the 4 sets of experiments. Even though the decrease in viscosity contributes more to the distribution of the spermatozoa than one might expect, the migration is much greater than can be explained by viscosity alone. Therefore, the greater migration is attributed to the intrinsic motility of the spermatozoa at the respective temperatures.

The effect of motility upon the distribution of spermatozoa in the dextran experiments is similar to that observed in the sucrose experiments. In both dextran experiments at 2.5°C, the spermatozoa were of poor initial quality and the migration pattern was not influenced by motility. Thus, the observed and expected migration distances are considered to be the same at 2.5°C. At 4°C, a small percentage of the spermatozoa showed a slight degree of tail-wagging in the flow-cell. At 7°C, a high percentage of

EFFECT OF MOTILITY
Sucrose
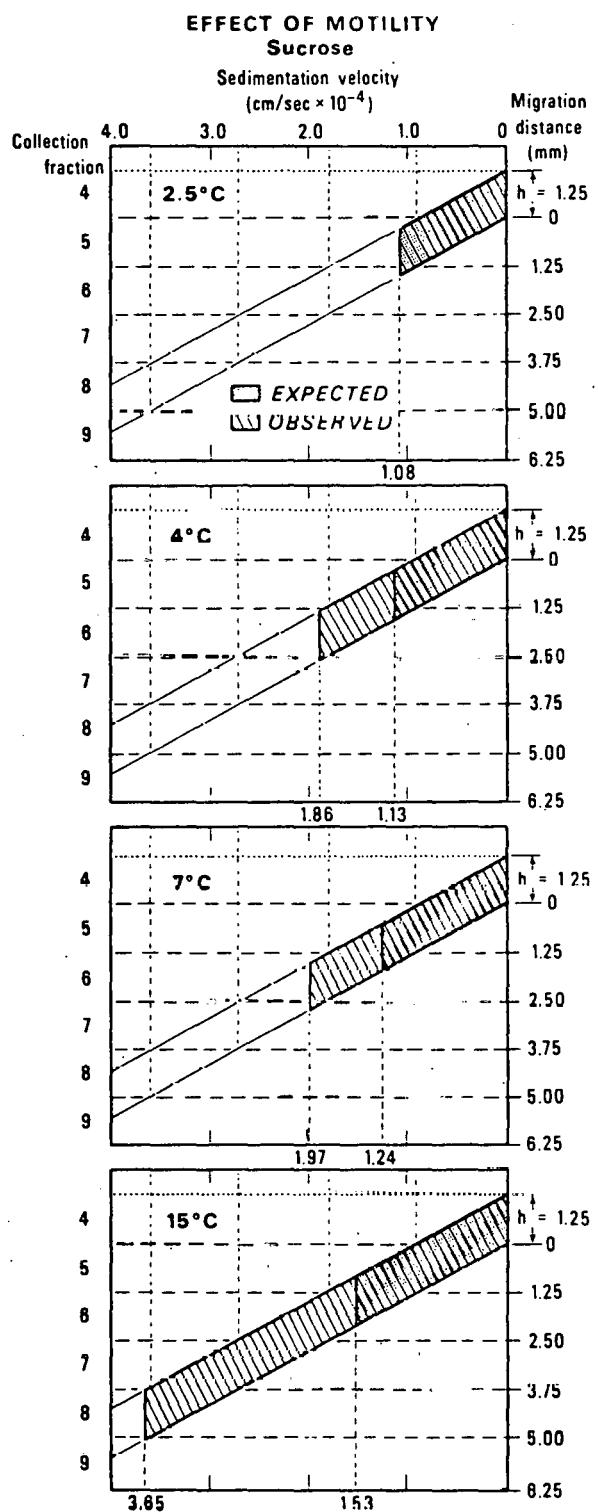
Fig. 38. Comparison of expected and actual distributions of rabbit spermatozoa in sucrose density gradient sedimentation experiments at various temperatures.

spermatozoa in the flow-cell showed gentle rolling or tail-wagging. The effects of the motility on the migration distances are shown in Figure 39. Using the known differences in viscosity at the 2 temperatures, the expected distributions of spermatozoa at 4°C and 7°C were calculated and are shown in Figure 39 with the respective observed migration distances. The contribution of motility at the higher temperatures in the dextran experiments is even more pronounced than in the sucrose experiments.

The observations of motility of varying degrees in the flow-cell at 4°C and at the higher temperatures supports the hypothesis that the difference between the expected and observed migration distances in Figures 38 and 39 are due to motility. However, as noted earlier, one experiment conducted at 7°C with immotile spermatozoa gave a distribution similar to the average of the 5 experiments conducted with spermatozoa samples of fair initial quality. This suggests that with migration distances as small as those achieved in most of these experiments, "cluster sedimentation" can have a significant relative influence on the migration pattern.

2. Comparisons with Results of Other Investigators.

In the sucrose density gradient at 2.5°C, the sedimentation velocity ranged from 0-3.86 mm/hr assuming that the 2.3% of spermatozoa in fraction 6 had sedimented a maximum distance of 1.50 mm (this is probably a generous estimate). At this temperature, the viscosity and specific gravity of the medium in layer 5 are 1.70 cp and 1.010, respectively.

Calculations based upon the data reported by Bhattacharya[35] give a "sedimentation velocity" range of 0-5 mm/hr for rabbit spermatozoa in his apparatus at 0°C. The medium had a viscosity of 75 centipoises and a specific gravity of 1.0304. This estimate of the sedimentation velocity of rabbit spermatozoa in his medium is greater than that in my sucrose

EFFECT OF MOTILITY
Glycine-dextran medium
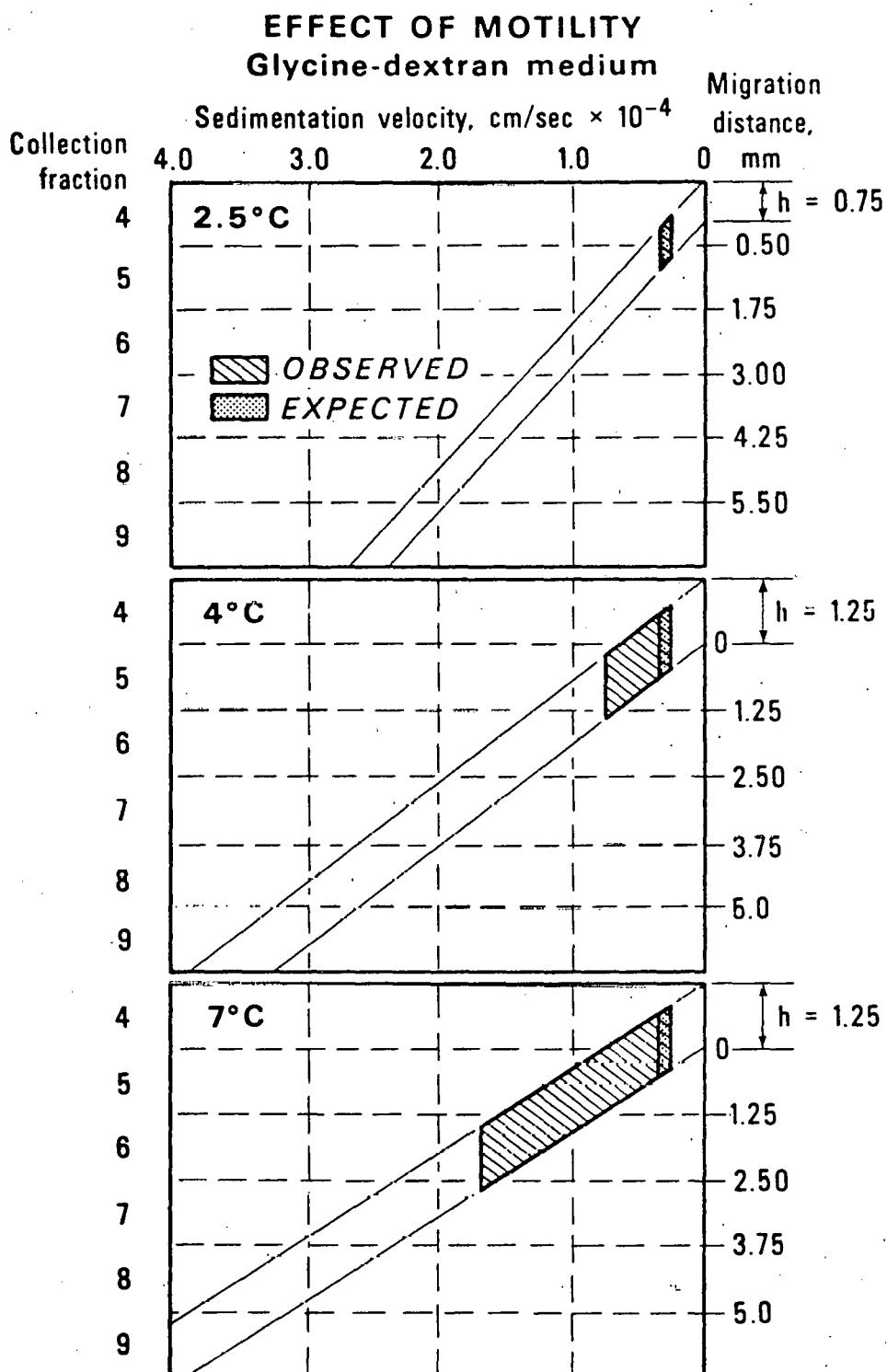
Fig. 39. Comparison of expected and actual distributions of rabbit spermatozoa in dextran density gradient sedimentation experiments at various temperatures.

DBL 689-5467

density gradient even though the specific gravity and viscosity, in particular, were much higher in his apparatus.

Schilling reported large sedimentation distances for bull spermatozoa in a similar device in 60 minutes at 0°C, in a medium with a viscosity of 7-10 centipoises and a specific gravity of 1.037-1.044[205]. If interpretation of his report is correct, the maximum "sedimentation velocities" in his apparatus would have been 50-60 mm/hr.

Bedford and Bibeau reported rabbit spermatozoa sedimentation experiments at 2°C in a medium with a viscosity of 6.7 cp and a specific gravity of 1.048[29]. The sedimentation distances are not reported but the apparent "sedimentation velocities" appear to be comparable to those in Schilling's experiments even though rabbit and bull spermatozoa were used in the respective studies.

Compared to Bhattacharya's results, the proportionate increase in sedimentation velocity in the experiments by Schilling and by Bedford and Bibeau is consistent with the lower viscosity of their media. Thus, the relative differences between the sedimentation velocities observed in my experiments and those of the three other groups of experiments are comparable. All three are incompatible with my results and suggest that more than true sedimentation occurred in their experiments.

The other investigators layered spermatozoa at concentrations of 200-300 x 10^6/ml on top of their media in a burette. Even though the greater viscosities and specific gravities of their media would raise the threshold for "cluster sedimentation" above the 25-30 x 10^6 spermatozoa/ml threshold of my sucrose density gradient, that phenomenon is considered the most likely explanation of the higher "sedimentation velocities" in

their experiments. Because of the low temperatures used by those investigators, there should have been no contribution of motility to the migration.

As noted in Chapter II, Bhattacharya obtained a significant difference in the sex of offspring from the extreme fractions of his column sedimentation device[35]. Schilling had less success[205], and Bedford and Bibeau reported none[29]. It is interesting to speculate that "cluster sedimentation" accentuated the sedimentation of the faster migrating species[158], while at the same time giving a false high sedimentation velocity for the spermatozoa. Even though this phenomenon may have given a differential distribution of slow and fast sedimenting spermatozoa the separation of the X- and Y-chromosome bearing spermatozoa by Bhattacharya and by Schilling is more likely related to the aqueous polymer two-phase system described by Albertsson[270] or to some undefined cell-cell interactions related to certain constituents of the medium.

E. Summary and Conclusions

The 7°C sucrose experiments demonstrate good reproducibility under the same operating conditions (e.g., medium, pH, density gradient and average transit time) with spermatozoa concentrations ranging from 19-26 x 10^6/ml. The differences among the different-temperature experiments with both sucrose and dextran density gradients are attributed primarily to the intrinsic motility of the spermatozoa. In addition to this variable, the spermatozoa concentration is an important factor, especially at temperatures below 2.5°C where motility is negligible in the sucrose and dextran media.

In these low density-low viscosity media, the upper limit of the cell concentration is approximately 25 x 10^6/ml, above which "cluster sedimen-

tation"was observed with the slit lamp technique. Examples were given where it is believed that "cluster sedimentation", in certain experiments at 2.5°C and 7°C, gave distributions similar to those expected with motile spermatozoa. Thus, depending upon the medium and density gradient, the threshold for "cluster sedimentation"will determine the maximum desirable spermatozoa concentration. At the other extreme, the motility and fertilizing capacity were not preserved adequately at concentrations below 4×10^6 spermatozoa/ml in these media. In certain fractions the presence of diluted seminal plasma enhanced the preservation of motility, but not of fertilizing capacity. In view of the dilution inherent in multi-layer fractionation of any kind, the "dilution effect" dictates using an initial sample of the highest possible concentration. Thus, one is faced with the contradictory objectives of maintaining the vital functions of the spermatozoa without compromising the ideal migration pattern. The resolution of these problems is described in later chapters.

At the higher temperatures, "cluster sedimentation"still should generally be avoided but the key factors are the direction and magnitude of the intrinsic motility vector, which depend upon the temperature, medium and quality of spermatozoa. Since the microscopic optical system available for the 7°C and 15°C sucrose experiments was very primitive and unreliable, the degree of motility in the flow-chamber in those experiments was not well defined. The orientation of spermatozoa at both temperatures appeared to be primarily downward. In the later dextran experiments at temperatures of 7°C and lower, the spermatozoa were seen to be oriented primarily downward which suggests that the same was the case for the 7°C sucrose experiments.

The relative enrichment of the lower collection fractions in many

experiments, with respect to motility and viability, is attributed to intrinsic motility. This interpretation of the differential distribution was made difficult by the fact that motility was lost prior to viability. However, in the 2.5°C sucrose experiments, where there was no motility in the flow-chamber, there was no difference between the two "high concentration" fractions with respect to viable spermatozoa. This suggests that the differential fractionation of viable spermatozoa at higher temperatures was due to motility. This is consistent with Bhattacharya's report that there was no difference in the relative distribution of live and dead spermatozoa in his "sedimentation" device.

Figures 37 and 38 are 2 ways of displaying temperature-dependent migration information. The range and distribution diagram (Figure 38) indicates a clear distinction between the expected and actual spermatozoa distributions at these temperatures and demonstrates the feasibility of quantitatively defining the intrinsic motility of spermatozoa as a function of temperature, for the population as a whole. This would be of particular interest in fractionations with media capable of preserving both motility and fertilizing capacity. Under these conditions, the temperature dependence of the degree of motility could be correlated with the migration distance. In addition, it would also be possible to determine the fertilizing capacity of the spermatozoa, and the sex ratio of offspring derived therefrom, e.g., as a function of the relative degrees of intrinsic motility.

The difficulty of dealing quantitatively with "cluster sedimentation" argues for modifying the cell concentration, medium and/or density gradient to avoid the problem[158,263]. The much greater "sedimentation velocities" observed by other investigators are no doubt due to "cluster sedimentation" or sedimentation processes other than sedimentation of individual cells.

It does not appear that the separation of X- and Y-chromosome bearing rabbit (Bhattacharya[35]) and bull (Schilling[205]) spermatozoa is a simple function of differential sedimentation of the two types of spermatozoa.

V. EARLY STAFLO-ELECTROPHORESIS EXPERIMENTS:
RABBIT AND FOWL SPERMATOZOA

Contents

A. Introduction

Studies by others of the electrophoretic properties of spermatozoa were summarized in Chapter II. This included the work by Schröder[206,207] and by Gordon[91,92] showing a variation from the normal sex ratio upon the insemination of anodic and cathodic fractions of spermatozoa separated in an electrophoretic apparatus. However, their work was not confirmed in studies by many other investigators[18,174,233]. Bedford demonstrated changes in the electrophoretic properties of spermatozoa during maturation[24], while Bangham showed changes in the relative surface charge distribution between heads and tails of spermatozoa in media of different ionic strength and at different pH's[18]. He also found differences in the electrophoretic mobility under varying conditions of pH and ionic strength[18].

This chapter is devoted to a series of experiments conducted during a transition phase in the development of improved Staflo equipment and techniques. While experiments with the 1 x 12 flow-cell were providing knowledge on the Staflo electrophoresis properties of spermatozoa and the requirements for handling spermatozoa IN VITRO, work was in progress concurrently, on the following: (1) the construction of a 3 x 16 Staflo apparatus to provide greater resolution in fractionation; (2) the development of new buffers to preserve the viability, motility and fertilizing capacity of the spermatozoa, as well as giving greater pH stability in the Staflo apparatus; (3) the design and construction of a microscope to be used with the Staflo apparatus for better observation of spermatozoa within the flow-chamber and (4) the construction of a refrigerated room to provide accurate temperature control during experiments.

Because many of the spermatozoa samples used in the series of
1 x 12 flow-cell experiments had high initial quality, these experiments
demonstrate many of the interrelationships among the biophysical proper-
ties of spermatozoa and the experimental variables, especially the effects
of motility and temperature on the distribution of spermatozoa in the flow-
chamber. Subsequent experiments provide information on the characteristics
of the 3 x 16 Staflo apparatus, the orientation of spermatozoa in the
flow-chamber, and the electrophoretic mobility of both rabbit and fowl
spermatozoa during electrophoresis in upward and downward directions.

B. Materials and Methods

1. Sample Preparation

Spermatozoa samples were obtained from mature Dutch Belt rabbits using
the artificial vagina described in Appendix C. Each ejaculate was diluted
immediately by adding 2.0 ml of medium (see below) at 37°C to the collec-
tion vial and then was allowed to cool to room temperature in an insulating
nest of glass wool. A 0.1 ml aliquot of this sample was diluted to 1.0 ml
and evaluated for percent and degree of motility as described in Appendix
B. After the sample had been cooled slowly in a water bath to the experi-
ment temperature, it was diluted to approximately 20×10^6 spermatozoa/ml.
In order to obtain a sample suspension of at least 15 ml at that concen-
tration, it was usually necessary to pool ejaculates from 2 or more rabbits.
The cooled, diluted spermatozoa suspension then was subjected to Staflo
electrophoresis. All work with the spermatozoa was accomplished using
minimal lighting in order to reduce the deleterious effects of visible
light[176].

2. Staflo Apparatus

The 1 x 12 and 3 x 16 flow-cell experiments were conducted with flow-cell

number VII-C and XI, respectively. The characteristics of these flow-cells
and the Staflo microscope are described in Chapter III.

3. Media and Density Gradients

a. 1 x 12 Flow-Cell Experiments. The 1 x 12 flow-cell experiments were
conducted with a Hanks-glycine primary medium using a dextran density grad-
ient. The Hanks-glycine electrophoresis medium consisted of 100 ml of Hanks
solution, 10 ml of 0.35% $NaHCO_3$, 9.25 g glycine and 25.0 g glucose diluted
to 1,000 ml with distilled water, including adjustment of pH with 1.0 N NaOH.
The density gradient consisted of dextran (MW, 73,000, Sigma Chemical Company)
in weight/volume percent in the Staflo layers as follows: No. 1, 0%; No. 2,
0.1%; No. 3, 0.3%; No. 4, 0.6%; No. 5, 0.7%; No. 6, 0.8%; No. 7, 0.9%; No. 8,
1.0%; No. 9, 1.0%; Nos. 10-12, 2%. The top electrode compartment contained
Hanks-glycine medium without dextran, while the bottom electrode compart-
ment contained the same medium with 2% dextran. Even though this density
gradient supported a suspension of up to 30×10^6 spermatozoa/ml in layer 9,
lower cell concentrations were used to avoid "cluster sedimentation" des-
cribed in Chapter IV.

b. 3 x 16 Staflo Experiments. Low ionic strength glycine-phosphate and
glycine-citrate buffers were used in this series of experiments.

(1) Glycine-phosphate buffer. The glycine phosphate buffer consisted
of 2.20 g $Na_2HPO_4 \cdot 12\ H_2O$, 0.20 g NaCl, 0.20 g $NaHCO_3$, 0.016 g KH_2PO_4,
9.25 g glycine, 23.0 g fructose, 10^6 units of penicillin G, all diluted
to 1,000 ml with distilled water. pH adjustments were made with concen-
trated HCl.

(2) Glycine-citrate buffer. The glycine-citrate buffer consisted of
2.0 g sodium citrate dihydrate, 9.25 g glycine, 3.0 g fructose, 0.25 ml
10% citric acid solution, 48.6 g sucrose, 10^6 units penicillin G diluted

to 1,000 ml with distilled water. pH was approximately 6.94.

Different density gradients consisting of dextran (MW, 73,000, Sigma Chemical Company) or sucrose were used in the 3 x 16 flow-cell experiments, and, therefore, are described in the appropriate sections below.

4. Temperature Control

In the 1 x 12 flow-cell experiments conducted at 4°C or above, the temperature of the solutions in the feed syringes was controlled by enclosing the pump rack in a plastic canopy and cooling with dry ice. The flow-cell was housed in a lucite box and also cooled with dry ice. The experiments at 2.5°C were conducted in a newly constructed refrigerated room which was large enough for the entire apparatus, accessory equipment and several investigators.

All experiments with the 3 x 16 Staflo apparatus were conducted in the refrigerated room. Measurements with a temperature recorder (Varian Associates) showed that under the maximum electric field conditions used in this series of experiments, the average and maximum temperature rises near the outlet of the flow-cell were 0.5°C and 1.0°C, respectively.

5. Observation and Evaluation of Spermatozoa

The gross electrophoresis pattern was observed by eye using the slit lamp technique[229,263]. Observations of individual spermatozoa and the microscopic steady-state pattern were made with the optical equipment described in Chapter III. After collection, the concentration and degree of motility of the spermatozoa in each collection fraction were determined by counting with a hemocytometer. Vital staining of spermatozoa in each fraction was accomplished with the nigrosin-eosin staining technique described in Appendix B.

C. Results

1. **1 x 12 Flow-Cell Experiments.**

The characteristics of the spermatozoa sample and Staflo operating

parameters for the 1 x 12 flow-cell upward-electrophoresis experiments are

presented in Table V. Except for Experiments 12-21-65-1 & 2, which were

conducted at 2.5°C in a refrigerated room, all experiments were conducted

with marginal temperature control. In all experiments, spermatozoa were

introduced into layer 9 with electrophoresis in an upward direction. Be-

cause geometrical considerations prevented the use of the slit lamp,

quantitative measurements of the migration pattern were not made.

a. **Experiments at 5, 6.25 and 7.5 V/cm.**

(1) **5 V/cm**. Figure 40 presents the individual results of the first

4 experiments in Table V. As shown in this figure, the total sperm distri-

bution was upward out of layer 9 except in Experiment 4-29-65. In that

experiment, the higher percentage of spermatozoa remaining in layer 9 might

be attributed either to "cluster" sedimentation, because the spermatozoa

concentration was 30×10^6/ml, and/or to a high degree of intrinsic motility

at 7°C. The upward distribution was most pronounced in Experiment 5-6-65

which was conducted at 5°C with the poorest quality spermatozoa sample.

The distribution of spermatozoa in Experiment 5-13-65 at 5°C was similar to

that in Experiment 5-6-65. The upward distribution in Experiment 5-20-65

at 7°C was less than in the 2 experiments at 5°C, also suggesting that

there is less upward migration at the higher temperature.

With regard to the distribution of viable spermatozoa, the least

difference among the collection fractions was observed in Experiment

5-6-65 at 5°C. In Experiment 4-29-65 at 7°C, using high quality spermatozoa,

there was a higher percentage of viable spermatozoa in fractions 8 and 9

Table V. Spermatozoa Sample and Staflo Operating Parameters for
1 x 12 Flow-Cell Upward-Electrophoresis Experiments

Experiment No.	Spermatozoa Sample			Staflo Operating Parameters					
	Rabbit(s)	Initial Quality	Initial Sperm Concentration (Units of 10^6/ml)	pH	Temp. (°C)	τ_{ave} (min)	$\tau_{\tilde{E}}$ (min)	L (cm)	$\tilde{E}$ (V/cm)
4-29-65	O & Psi	70% motile	30	6.5	7	20	13.3	20	5
5-6-65	P & Psi	40% motile	23	6.5	5	20.5	13.7	20	5
5-13-65	P	Excellent	22	6.5	5	20	13.3	20	5
5-20-65	O & Chi	Excellent	22	6.5	7	20	13.3	20	5
7-28-65	O & Psi	80% motile	16	7.0	5	20.5	13.7	20	6.25
8-4-65	O & Chi	Excellent	15	7.0	3.5	20.5	13.7	20	6.25
6-23-65	Chi	80% motile	14	6.5	6	17	11.3	20	7.5
7-14-65	P	60% motile	13	6.5	4	17	11.3	20	7.5
7-21-65	Psi	Very poor	13	6.5	4	17	11.3	20	7.5
7-23-65	P	85% motile	13	6.5	5	17	11.3	20	7.5
12-21-65-1	Chi	Very poor	11	7.0	2.5	20	13.3	20	6.25
12-21-65-2	Chi	Very poor	9	7.0	2.5	18	12.0	20	7.5

SPERM ELECTROPHORESIS, 1 × 12 STAFLO
4 RUNS AT 5.0 V/cm
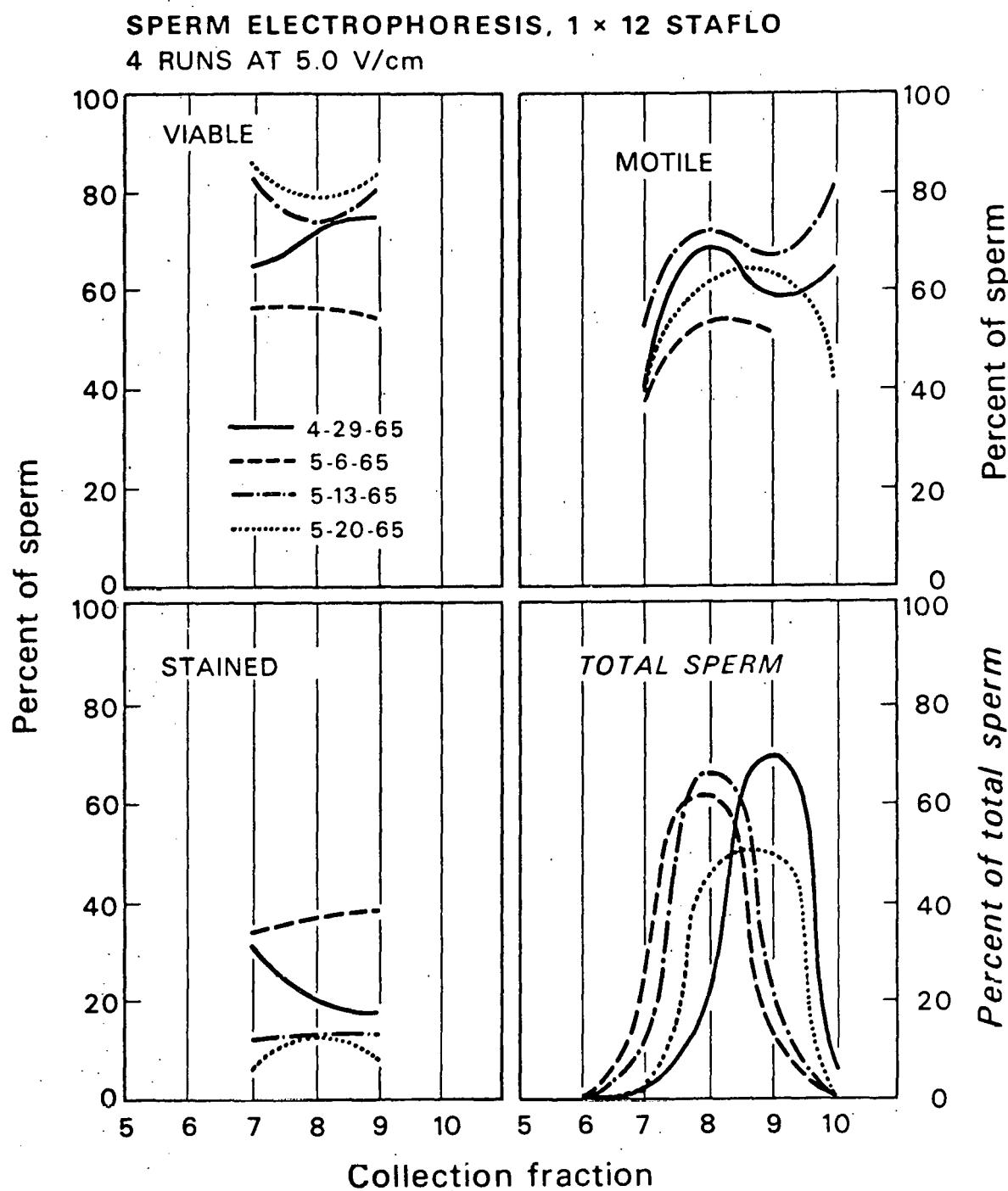
DBL 688-5379

Fig. 40. Individual results of four upward-electrophoresis experiments
with rabbit spermatozoa at 5 V/cm; 1 x 12 Staflo apparatus.

compared to fraction 7. In the other 2 experiments with samples of comparable initial quality, the distribution of viable spermatozoa was similar despite the difference in temperature (5 vs. 7°C). The distribution of stained spermatozoa was essentially a mirror image of that of the viable spermatozoa.

Except for a lower percentage of motile spermatozoa in fraction 7 compared to fractions 8 and 9, Figure 40 shows no definite pattern in the distribution of motile spermatozoa among the collection fractions. In 2 experiments, the percentages of motile spermatozoa in fractions 8 and 9 are approximately the same, while in the other 2 experiments, there is a higher percentage of motile spermatozoa in fraction 8. In Experiment 4-29-65, approximately 5% of the total number of spermatozoa migrated downward into layer 10 against the electrophoretic force, suggesting a strong intrinsic motility component. This variability in the distribution of motile spermatozoa and the influence of intrinsic motility on the distribution of other categories of spermatozoa are considered in detail in the discussion.

In Experiment 5-6-65, a few spermatozoa in the flow-cell were oriented horizontally, while over 95% were oriented with head down and tail upward in the direction of the positive electrode. A high percentage of the spermatozoa had vigorous tail-wagging, but the significance of this degree of motility in terms of migration against the electric field could not be assessed with the microscope. At the conclusion of the experiment, the degree of motility of the spermatozoa in the collection fractions was relatively poor. Thus, the degree of motility observed in the collection fractions at the end of the experiment does not correlate well with the apparent interaction between the intrinsic motility and electrophoretic

force in the flow-cell. This illustrates the need for microscopic obser-
vations in the flow-chamber during experiments.

(2) 6.25 V/cm. Figure 41 presents the results of 2 experiments at 6.25
V/cm. The distribution of the various categories of spermatozoa is similar
in the 2 experiments despite the difference in temperatures (3.5°C and
5.0°C). Despite the lower percentage of viable spermatozoa in fraction
9 compared to 8, in both experiments, there is an unequivocal increase in
the percentage of motile spermatozoa going from fraction 7 through 10.

In Experiment 7-28-65, observations with the microscope showed that
in layer 7, all spermatozoa were oriented vertically with the tail toward
the top (positive) electrode and that some spermatozoa had tail-wagging.
In layer 9, most spermatozoa were oriented vertically, while a few were
in a horizontal position. Nearly all spermatozoa in layer 9 showed some
degree of motility at 5°C. In both experiments, seminal plasma was dis-
tributed primarily in layers 7 and 8 with only large "blobs of junk" in
layer 9.

(3) 7.5 V/cm. Figure 42 presents the individual results of 4 experiments
at 7.5 V/cm. There is a slightly greater upward total sperm distribution
in Experiments 7-14-65 and 7-21-65, both of which were conducted at 4°C
with poorer quality spermatozoa samples than in the other 2 experiments.
The least migration was observed in Experiment 6-23-65 conducted at 6°C
with the spermatozoa sample having the highest percentages of viable and
motile spermatozoa at the conclusion of the experiment.

There is little reproducibility in the distribution of viable and
motile spermatozoa among the collection fractions. Despite the low cell
concentrations, viable spermatozoa were observed in fractions 6 and motile
spermatozoa were observed in fractions 6 and 5. There is some similarity
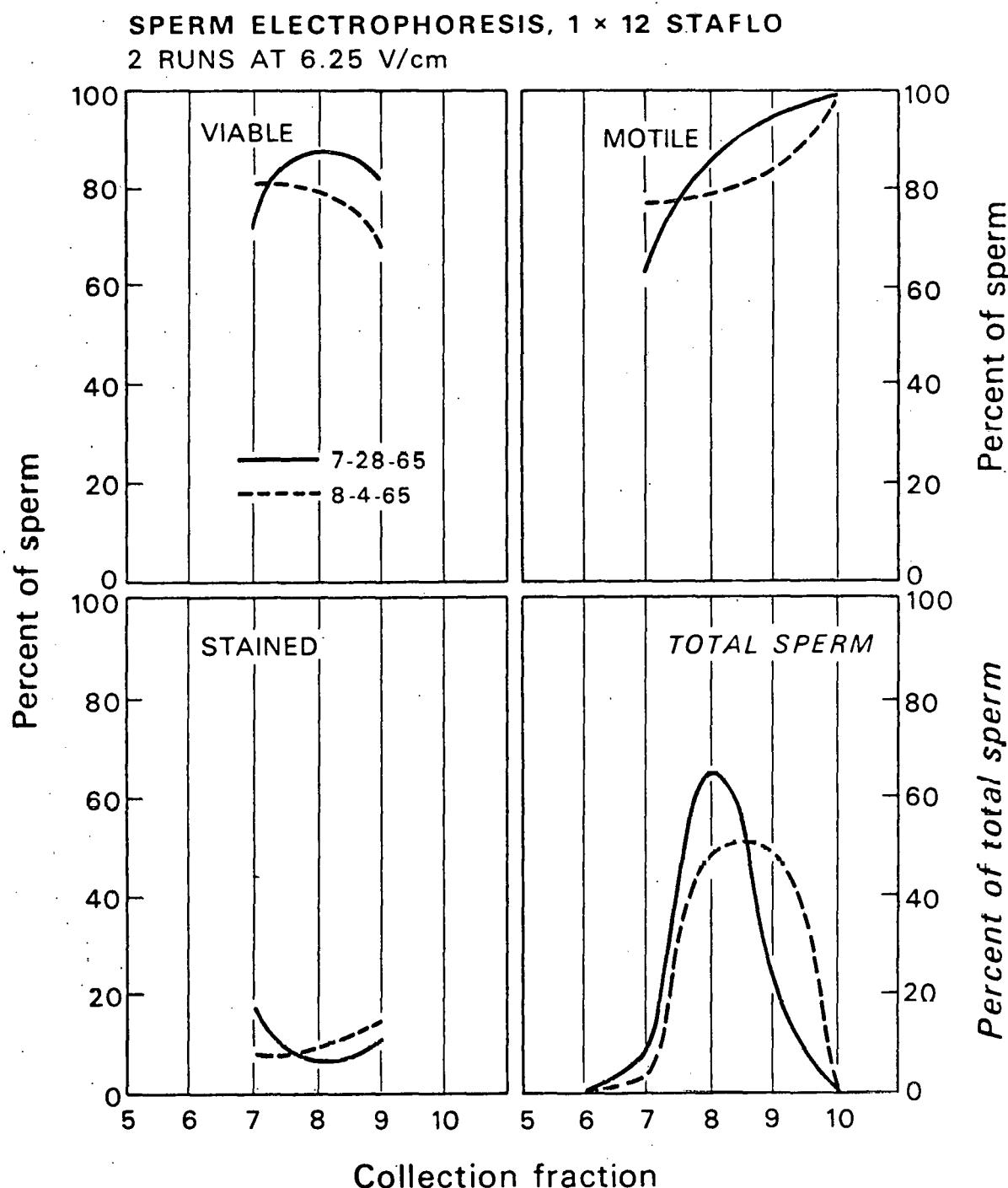
Fig. 41. Individual results of two upward-electrophoresis experiments with rabbit spermatozoa at 6.25 V/cm; 1 x 12 Staflo apparatus.
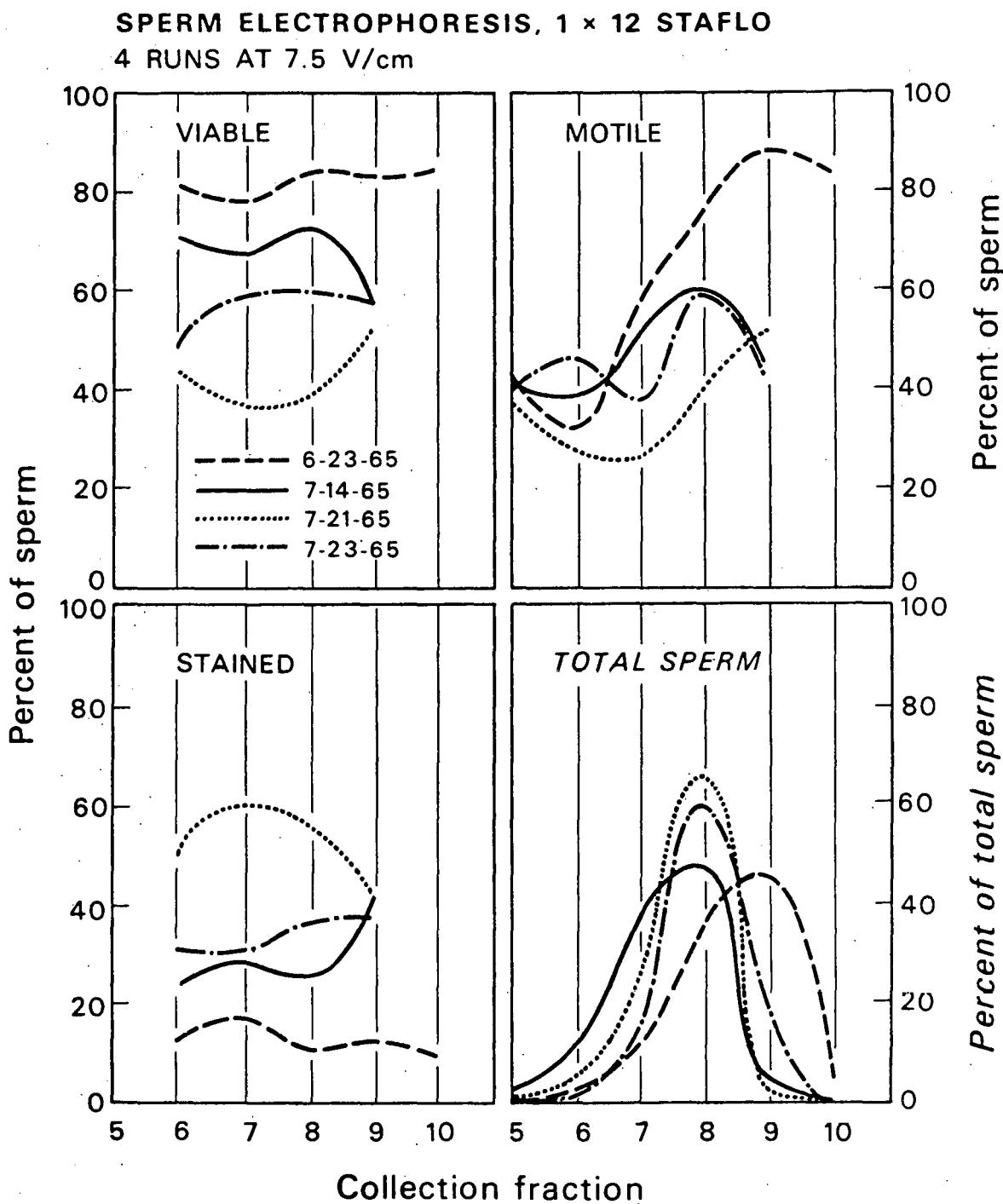
Fig. 42. Individual results of four upward-electrophoresis experiments with rabbit spermatozoa at 7.5 V/cm; 1 x 12 Staflo apparatus.

between the distribution of viable and motile spermatozoa in the respective experiments. The distribution of stained spermatozoa mirrors that of viable spermatozoa.

(4) <u>Experiments at 6.25 and 7.5 V/cm and a Temperature of 2.5°C.</u> After construction of a refrigerated room, upward electrophoresis experiments were conducted at 6.25 and 7.5 V/cm at a temperature of 2.5°C. In both experiments, the initial quality of the spermatozoa samples was poorer than in previous experiments and the degree of motility observed in the collection fractions at the termination of the experiments was extremely poor. The results are presented in Figure 43 and show a slightly greater upward total migration of spermatozoa at an electric field strength of 7.5 V/cm.

As shown in Figure 43, there is a slight increase in the percentage of viable spermatozoa going from collection fraction 7 to 9, while the distribution of stained spermatozoa is a mirror image thereof. In both experiments, there is a higher percentage of motile spermatozoa in fraction 7 than in either fraction 8 or 9. This distribution of motile spermatozoa is the opposite of that observed in the series of experiments at higher temperatures at both electric field strengths as shown in Figures 41 and 42.

In the experiment at 6.25 V/cm, essentially 100% of the spermatozoa near the wall in the single cell suspension were oriented vertically with their tails towards the top (positive) electrode. The spermatozoa showed a slight degree of twisting at the lowest levels of the migration pattern. Spermatozoa in the collection fractions initially had a fair degree of motility which was observed to decrease rapidly over a period of 30 minutes. In the experiment at 7.5 V/cm, swaying spermatozoa were observed in the lower part of the migration pattern. Before the experiment started, only 12 of 900 spermatozoa showed feeble tail-wagging in a hemocytometer at 2°C.

SPERM ELECTROPHORESIS, 1 × 12 STAFLO
2 RUNS AT 2.5°C
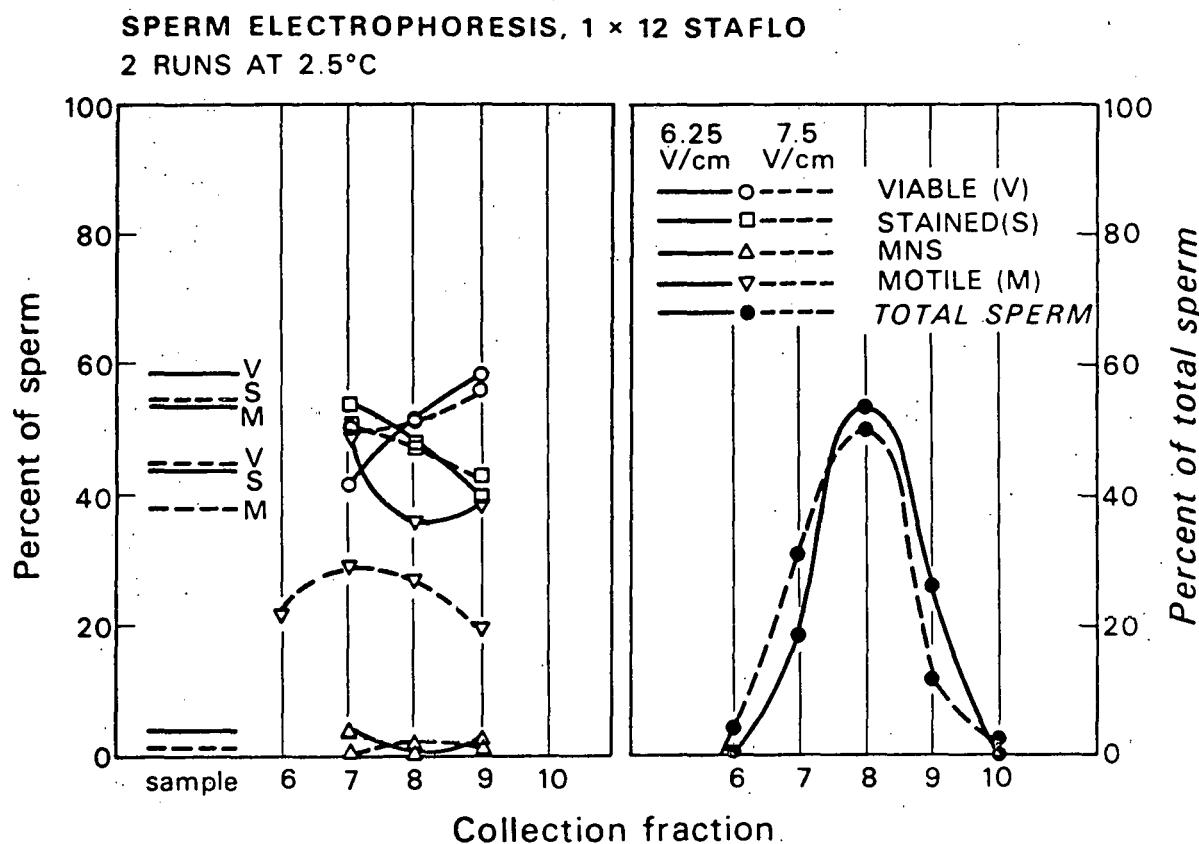
DBL 688-5384

Fig. 43. Comparison of results of upward-electrophoresis experiments with rabbit spermatozoa at 6.25 and 7.5 V/cm, at 2.5°C; 1 x 12 Staflo apparatus.

The swaying motion is attributed to the slightly higher temperature in the flow-chamber due to the electric field.

b. <u>Comparison of Different-Field Strength Experiments</u>. Figures 44, 45 and 46 present average values for the total distribution, and the distribution of several categories of spermatozoa among the collection fractions for the upward-electrophoresis experiments at 5, 6.25 and 7.5 V/cm shown in Figures 40, 41 and 42, respectively. Compared to the experiments at 5 V/cm, there is a slight increase in the upward distribution of spermatozoa among the collection fractions at 6.25 V/cm and a definite upward shift at 7.5 V/cm. There is little difference among the collection fractions with regard to the distribution of viable spermatozoa at 5 and 7.5 V/cm, while at 6.25 V/cm, there is a higher percentage of viable spermatozoa in fraction 8 than in 7 or 9. In Figures 44-46, the percentage of motility in fraction 7 is lower than in fractions 8 and 9. The differential distribution of motile spermatozoa is most pronounced in the experiments at 6.25 V/cm where a much higher percentage of motility was found in fraction 9 compared to 8. In all 3 figures, there are marked differences in the distribution of viable and motile spermatozoa. The distribution of morphologically normal but stained (MNS) spermatozoa is essentially the same in all collection fractions at 5 and 7.5 V/cm but is slightly higher in fractions 7 and 9 at 6.25 V/cm, thereby accentuating the differential distribution of viable spermatozoa in that group of experiments.

In many of the early electrophoresis experiments, the quality of spermatozoa at the end of the experiment appeared to be adequate for artificial insemination. However, only one insemination of dozens resulted in offspring. In Experiment 7-28-65, the migration pattern for which is shown in Figure 41, insemination of spermatozoa from fraction 8 gave 4 male offspring from the same female. Insemination of spermatozoa from

SPERM ELECTROPHORESIS, 1 × 12 STAFLO
AVERAGE OF 4 RUNS AT 5.0 V/cm
20.5 min TRANSIT
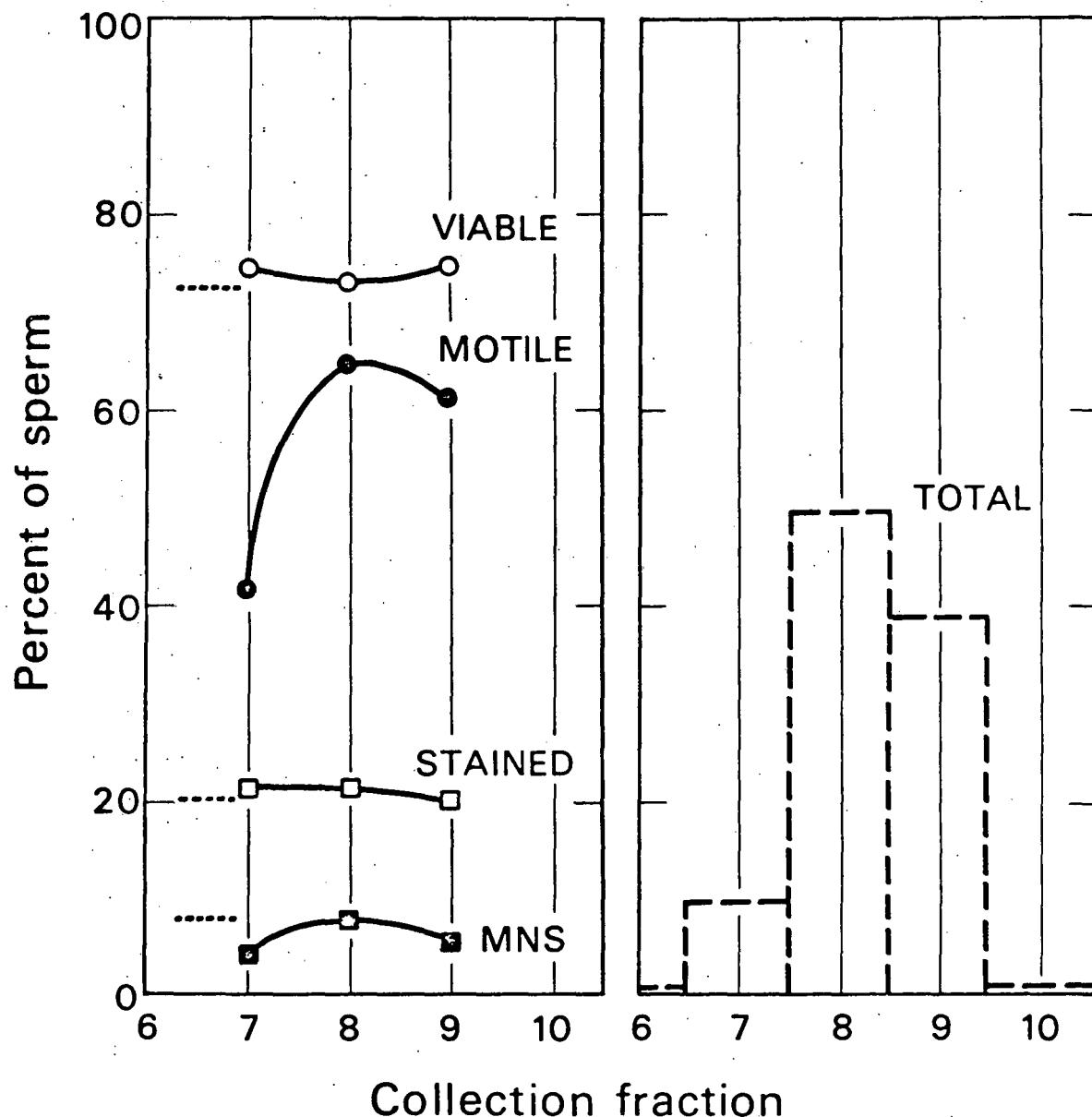
DBL 688-5410

Fig. 44. Average results for four upward-electrophoresis experiments with rabbit spermatozoa at 5 V/cm; 1 x 12 Staflo appratus.

Fig. 45. Average results for two upward-electrophoresis experiments with rabbit spermatozoa at 6.25 V/cm; 1 x 12 Staflo apparatus.
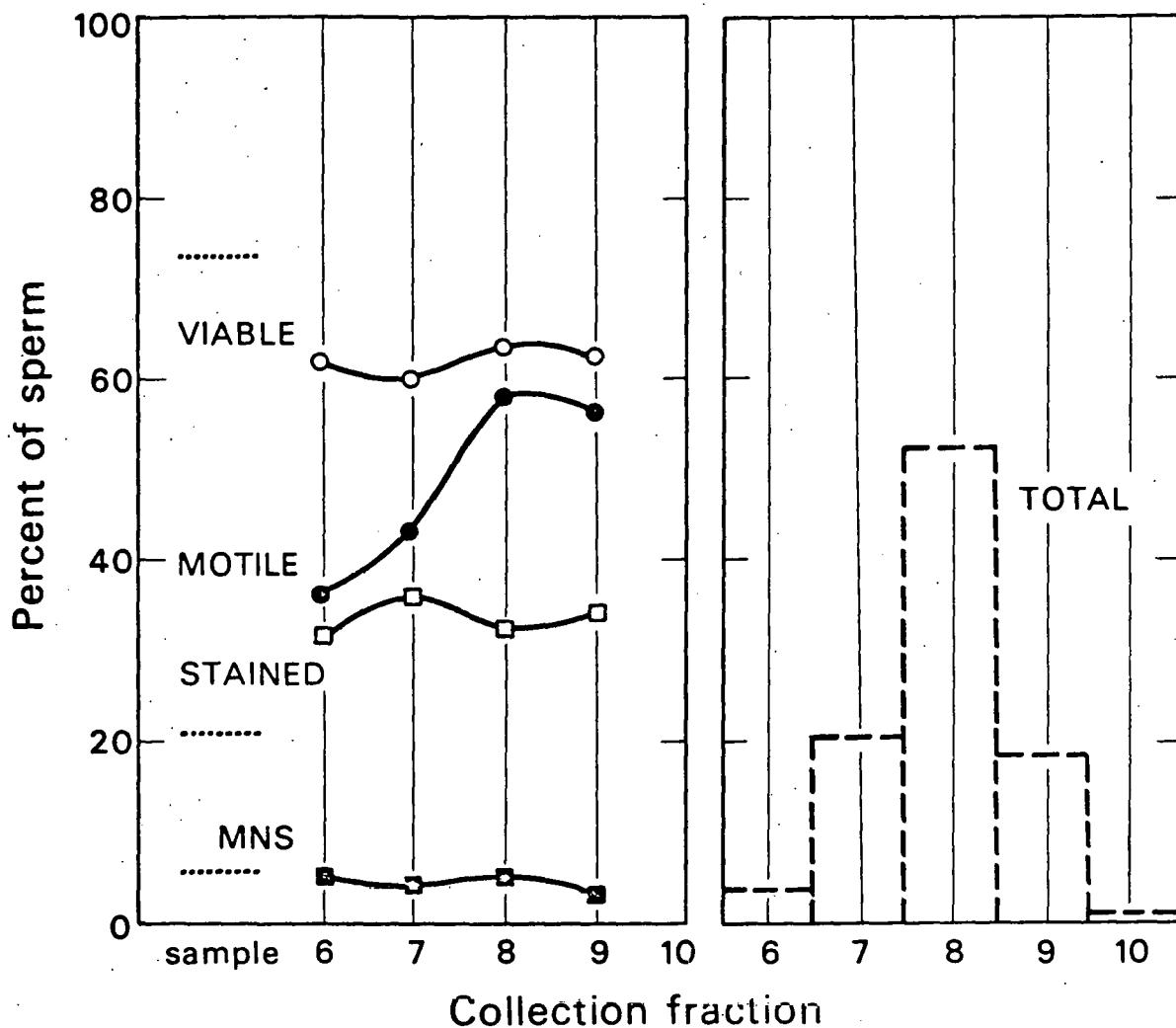
Fig. 46. Average results for four upward-electrophoresis experiments with rabbit spermatozoa at 7.5 V/cm; 1 x 12 Staflo apparatus.

fraction 9 gave no offspring.

2. 3 x 16 Flow-Cell Experiments.

As mentioned in Chapter III, greater resolution in the fractionation

of migration patterns is achieved with the 3 x 16 flow-cell. This section

reports the results of several preliminary experiments using the 3 x 16

flow-cell and new electrophoresis media providing greater pH stability and

better preservation of the vital functions of spermatozoa. The availability

of a refrigerated room facilitated the use of the slit lamp technique for

macroscopic observations and the Staflo microscope for the observation of

individual spermatozoa in the flow-chamber.

In addition to the preliminary evaluation of the 3 x 16 flow-cell,

results are presented below for: (1) upward electrophoresis of rabbit

spermatozoa at 8 V/cm with a dextran density gradient in a glycine-phos-

phate buffer, (2) downward electrophoresis of rabbit and fowl spermatozoa

with a dextran density gradient in the glycine-phosphate buffer, and (3)

upward electrophoresis of rabbit and fowl spermatozoa with a sucrose den-

sity gradient in the glycine-citrate buffer.

a. **Preliminary Flow-Cell Evaluation.** Figure 47 shows the average dis-

tribution of the total number and different categories of spermatozoa among

the centerline collection fractions in 2 experiments at 8 V/cm, 3.5°C, pH 7,

an average transit time of 21.5 minutes, and an average concentration of

25×10^6/ml. The density gradient, prepared in glycine-phosphate-buffer,

consisted of dextran (MW, 73,000) in weight/volume percent in the Staflo

layers as follows: No. 1, 0%; No. 2, 0.1%; No. 3, 0.2%; No. 4, 0.3%; No. 5,

0.4%; No. 6, 0.5%; No. 7, 0.6%; No. 8, 0.7%; No. 9, 0.8%; No. 10, 0.9%;

No. 11, 1.25%; No. 12, 1.5%; Nos. 13 and 14, 1.75% and Nos. 15 and 16, 2.0%.

The top and bottom electrode compartments contained the same medium and

percent dextran as layers 1 and 16, respectively.

SPERM ELECTROPHORESIS, 3 × 16 STAFLO
AVERAGE OF 2 RUNS AT 8 V/cm, 3.5°C
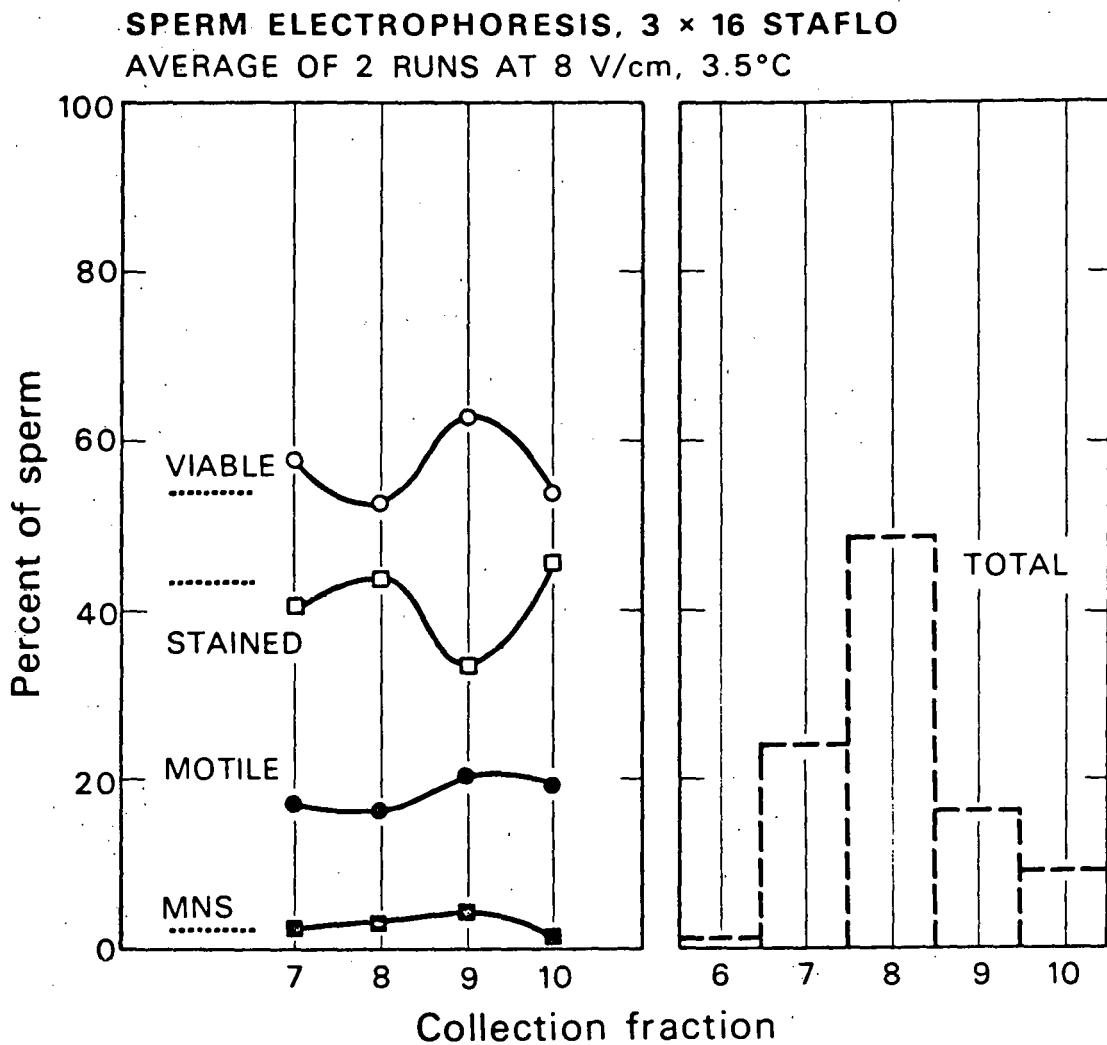
DBL 688-5407

Fig. 47. Average results for two upward-electrophoresis experiments
with rabbit spermatozoa at 8 V/cm; 3 x 16 Staflo apparatus.

In both of these experiments, the spermatozoa were introduced into layer 10 with electrophoresis in an upward direction causing approximately 75% of the spermatozoa to migrate from layer 10 through layer 9 into layers 8, 7 and 6. The highest percentage of viable spermatozoa was found in fraction 9, one layer below that having the highest spermatozoa concentration. This is the direction in which motile spermatozoa would have been expected to swim against the electric field. However, the initial degree of motility of the spermatozoa was relatively poor, and the difference in motility among the spermatozoa in the collection fractions was not significant. The distribution of stained spermatozoa is a mirror image of the distribution of viable spermatozoa, while that of MNS spermatozoa is essentially constant from fractions 7-10.

In both experiments, all spermatozoa within the region of the electric field were oriented vertically with the tail towards the top (positive) electrode. Table VI shows the differential distribution of mature spermatozoa, spermatozoa with cytoplasmic droplets, heads, and tails among the centerline collection fractions in one of the two experiments. The average migration appears to be greatest for tails and least for heads, and to be slightly greater for mature spermatozoa than for those with cytoplasmic droplets. The distribution of the spermatozoa with the cytoplasmic droplets appears to be slightly more uniform than that of the mature spermatozoa.

The vertical and horizontal distributions of spermatozoa in the 3 x 16 flow-chamber are given in Table VII. Table VII A gives the spermatozoa concentrations in all 3 horizontal channels of layers 7-10. As shown in Table VII B, the horizontal distribution of spermatozoa in the layers is somewhat symmetrical. 48.4% of the spermatozoa exited the flow-chamber in the centerline. This is slightly lower than the prediction by Tippetts

Table VI. Differential Distribution of Mature Sperm, Sperm with Cytoplasmic Droplets (CPD), and Sperm Heads and Tails in Upward-Electrophoresis Experiment 2-28-66, 3.5°C.

Collection Fraction	Mature Spermatozoa		Sperm with CPD		Sperm Heads		Sperm Tails	
	No.[a]	%	No.[a]	%	No.[a]	%	No.[a]	%
6	0.7	0.03	0	0	0	0	5.0	5.7
7	396	17.0	26.7	18.9	7.0	13.5	31.5	35.9
8	1160	49.9	58.7	41.5	18.3	35.3	33.4	38.0
9	475	20.4	32.1	22.7	16.5	31.9	12.5	14.2
10[b]	294	12.6	23.8	16.9	10.0	19.3	5.5	6.2
11	0		0		0		0	

a. in units of 10^4 sperm/ml; b. sample inlet layer

Table VII.   Two-Dimensional Distribution of Spermatozoa in
Upward-Electrophoresis Experiment 2-28-66 in the 3 x 16
Staflo Flow-Cell, 3.5°C.

Staflo Layer	Collection Fraction			
	Left	Centerline	Right	Total
A. Sperm Concentration (units of 10^6/ml)				
6	0	0	0	0.003
7	1.24	1.78	1.50	4.52
8	1.77	5.21	1.77	8.75
9	1.24	2.02	1.54	4.80
10	0.60	1.32	0.58	2.50
11	0	0	0	0
B. Percent of Sperm in Rows (Horizontal)				
7	27.5	39.4	33.1	100
8	20.3	59.4	20.3	100
9	25.8	42.1	32.1	100
10	24.0	52.8	23.2	100
Average	24.4	48.4	27.2	100
C. Percent of Sperm in Columns (Vertical)				
7	25.6	17.3	27.8	21.9
8	36.5	50.4	32.8	42.6
9	25.6	19.5	28.6	23.3
10	12.3	12.8	10.8	12.2
Total	100	100	100	100

that 62% of particles should exit from the central channel of each layer[263].

The vertical distribution given in Table VII C shows less variation on the

2 sides of the flow-chamber than in the centerline. A slightly higher

percentage of spermatozoa is found on the sides of layer 7 than in the

centerline of that layer. This would be expected from the parabolic migra-

tion pattern giving a slower longitudinal flow of the medium along the

sides of the flow-chamber.[229,263]

The pH stability of the glycine-phosphate buffer under the conditions

of these 3 x 16 flow-cell upward-electrophoresis experiments was excellent.

With an electric field strength of 8 V/cm, an average transit time of 21

minutes, and at 3.5°C, the maximum pH change in the layers of interest

(4-11) was 0.1 pH units or less.

b. Downward-Electrophoresis: Rabbit and Fowl Spermatozoa

(1) Rabbit Spermatozoa. Two downward-electrophoresis experiments using

the glycine-phosphate buffer were conducted with rabbit spermatozoa intro-

duced into layer 6 over 1% dextran, with increments of 0.2% dextran in

successively lower layers constituting the density gradient. The operating

conditions were an electric field strength of 10 V/cm using the last 25 cm

of the flow-chamber, a spermatozoa concentration of 15×10^6/ml, a chamber

temperature of 6°C and average transit times of 22 and 34 minutes. As

shown in Table VIII, the longer transit time resulted in both a greater

spread of the migration pattern and a greater downward distribution of the

total number of spermatozoa. In both experiments, there is a suggestion

of a normal distribution of electrophoretic mobilities, the averages of

which are calculated to be 0.62×10^{-4} and 0.60×10^{-4} (cm/sec)/(V/cm) for

the 22 and 34 minute transit times, respectively. When the electrophoretic

mobilities are corrected, as suggested by Bier[38], to that expected at 3°C

Table VIII. Distribution of Rabbit Spermatozoa in Centerline Collection Fractions with Downward-Electrophoresis in a Dextran Density Gradient, Average Transit Times of 22 and 34 Minutes, 10 V/cm; 6°C.

Collection Fraction	Percent of Sperm Collected	
	τ ave = 22 minutes	τ ave = 34 minutes
5	0	0
6	3.2	0.3
7	3.1	0.3
8	6.4	1.6
9	59.8	19.5
10	27.4	32.2
11	0.1	33.3
12	0	12.8
13	0	0.03

in this glycine-phosphate buffer, with a sucrose concentration of 1%, one gets mobility values of 0.82×10^{-4} and 0.79×10^{-4} (cm/sec)/(V/cm), respectively. Because the sedimentation rate of rabbit spermatozoa in a medium containing 1% or greater dextran is very low, the contribution of sedimentation to these values is believed to be very slight.

Observations with the Staflo microscope showed almost all spermatozoa to be oriented vertically with the tail pointing downward in the direction of the bottom (positive) electrode. Because the initial quality of the spermatozoa sample was very poor, no motility was observed in the flow-chamber. After warming to room temperature, the spermatozoa in the collection fractions showed less than 10% tail-wagging. The migration profile in both experiments was parabolic and the mild degree of "cluster sedimention" observed at both transit times is not believed to have affected the distribution of spermatozoa significantly.

In another experiment using the same density gradient, a severe degree of convection was observed[263]. At 15 cm from the inlet, the migration profile was a neat parabola. At 20 cm, migration of the spermatozoa downward along both sides of the flow-chamber and then into the centerline formed a tube-like profile. At 25 cm, the profile again was essentially parabolic with marked central sharpening and it remained that way over the last 5 cm of the flow-chamber. These changes in the migration pattern illustrate the importance of examining the migration profile at frequent intervals throughout the chamber length by the slit lamp technique[229,263].

(2) _Fowl Spermatozoa._ A downward-electrophoresis experiment using the glycine-phosphate buffer was conducted with fowl spermatozoa introduced into layer 5 over 2% dextran with increments of 0.2% dextran in successively lower layers constituting the density gradient. With an electric field

strength of 12 V/cm, an average transit time of 21 minutes and a flow-chamber temperature of 6.8°C, the spermatozoa were distributed over layers 9-12 as follows: No. 9, 4.3%; No. 10, 24.6%; No. 11, 62.1% and No. 12, 8.7%. Approximately 50% of the spermatozoa in each collection fraction were motile with Grade II motility in fraction 11 and Grade I in the other 3 fractions. At this time, the initial sample had Grade III motility.

Under the conditions given above, the average electrophoretic mobility of fowl spermatozoa was calculated to be 1.14×10^{-4} (cm/sec)/(V/cm) at 6.8°C. When a correction is made for viscosity, the average electrophoretic mobility is estimated to be 1.68×10^{-4} (cm/sec)/(V/cm) in this glycine-phosphate buffer with a sucrose concentration of 1% at 3°C.

c. Upward-Electrophoresis: Rabbit and Fowl Spermatozoa. In one pair of separate experiments using the glycine-citrate buffer, rabbit and fowl spermatozoa were introduced into layer 12 in 3.3% sucrose and were electro-phoresed upward through a density gradient formed by decrements of 0.3% sucrose per layer. Operating conditions included an electric field strength of 12 V/cm over the last 20 cm of the flow-chamber, an average transit time of approximately 21 minutes, a temperature of 6°C, and spermatozoa concentrations of 20×10^6/ml and 30×10^6/ml for rabbit and fowl spermatozoa, respectively. Under these conditions, the electrophoretic mobilities for rabbit and fowl spermatozoa were 0.99×10^{-4} and 1.75×10^{-4} (cm/sec)/(V/cm), respectively. When corrected for viscosity, the electrophoretic mobilities at 3°C in the same primary medium with only 1% sucrose were 0.95×10^{-4} and 1.68×10^{-4} (cm/sec)/(V/cm) for rabbit and fowl spermatozoa, respectively.

With the microscope, it appeared that all spermatozoa of both species were oriented vertically with the tails pointing toward the top (positive) electrode. Most fowl spermatozoa had a significant degree of motility in

the flow-chamber and, therefore, the motility may have given a decrease in the net migration distance observed for those spermatozoa. The initial quality of the rabbit spermatozoa was very poor and the effect of intrinsic motility on the migration of that species is believed to be negligible in this experiment.

In another pair of separate experiments, using the glycine-citrate buffer, rabbit and fowl spermatozoa were introduced into layer 12 in 3.8% sucrose and electrophoresed upward through a density gradient with decrements of 0.5% sucrose per layer. Operating conditions included an electric field strength of 12 V/cm over the last 20 cm of the flow-chamber, an average transit time of 19.3 minutes, and pH 6.94. Under these conditions, the average electrophoretic mobility of the rabbit spermatozoa was 0.78 x 10^{-4}(cm/sec)/(V/cm) at 5.4°C and that of the fowl spermatozoa was 1.58 x 10^{-4}(cm/sec)/(V/cm) at 4.3°C. When corrections for viscosity were made, the estimated electrophoretic mobilities at 3°C in the same medium with 1% sucrose were 0.76 x 10^{-4} and 1.58 x 10^{-4}(cm/sec)/(V/cm) for the rabbit and fowl spermatozoa, respectively.

Table IX shows the distribution of tail-wagging and capped rabbit spermatozoa among the collection fractions in this experiment. Although there were no significant differences among the percentages of capped spermatozoa in the fractions, a much higher percentage of tail-wagging was observed among the spermatozoa in fraction 9 than in 10 or 11. The initial sample had been stored in egg yolk and had a high percentage of tail-wagging, but extremely poor forward motion, even at room temperature. These results suggest a greater electrophoretic mobility for tail-wagging rabbit spermatozoa than for viable spermatozoa without that property.

Approximately 90% of the fowl spermatozoa in this experiment exited

Table IX. Distribution of Tail-Wagging and Capped Rabbit Spermatozoa

in Upward-Electrophoresis Experiment 5-14-67; 5.4°C.

Collection Fraction	Total Sperm Collected (%)	Tail-Wagging (%)	Capped (%)
9	24.5	50	90
10	59.0	40	95
11	16.5	32	94
12[a]	0	–	–

a. Sample inlet layer

the flow-chamber in layer 7. The spermatozoa in this layer had a very high percentage and high degree of motility, whereas approximately 99% of the spermatozoa in fraction 6 (concentration 10 x 10^6/ml) were immotile. At 4.3°C, a "sea of motion" was observed in the flow-chamber with the microscope. Thus, the separation of motile and immotile fowl spermatozoa is believed to be due primarily to intrinsic motility. This may explain the slight difference in the average electrophoretic mobility observed in the 2 upward-electrophoresis experiments with fowl spermatozoa. The average electrophoretic mobility of the immotile fowl spermatozoa which exited the flow-chamber in layer 6 is estimated to be 1.67 x 10^{-4} (cm/sec)/ (V/cm) compared to an average of 1.58 x 10^{-4}(cm/sec)/(V/cm) for the sample as a whole at 3°C in a 1% sucrose solution.

D. Discussion

1. Factors Influencing the Distribution of Spermatozoa in the Flow-Chamber

The most important lesson to be gained from the experiments reported in this chapter is an understanding of the factors influencing the distribution of spermatozoa in the flow-chamber. The lack of reproducibility among the 1 x 12 flow-cell experiments introduces many of the problems related to that apparatus and to work with spermatozoa. For convenience, this section of the discussion is divided into sections concerned with the Staflo apparatus and the intrinsic motility of spermatozoa.

a. _Staflo Apparatus_. The characteristics of the 3 x 16 flow-cell, which underwent its initial evaluation in this series of experiments, were discussed in the Results section. The following remarks pertaining to density gradient stability, pH stability and the magnitude of the electric field pertain equally well to both the 1 x 12 and 3 x 16 flow-cells.

Because of the limited means for observation in the flow-chamber during

the 1 x 12 flow-cell experiments, the role of "cluster sedimentation" in those experiments could not be assessed, but is believed to be slight. In several of the later experiments, a few small clumps of spermatozoa were observed with the microscope in the lower part but not the upper part of the migration pattern. Similar observations were made in the sedimentation experiments reported in Chapters IV and VIII. A specific example of convection, in which spermatozoa migrated from the sides of the migration profile into the center of the flow-chamber, was described in the results. Because this increases the apparent net migration in the centerline of the flow-chamber, this phenomenon, as well as that of "cluster sedimentation", distorts the migration profile. By influencing the net migration of the spermatozoa, both of these processes interfere with the interpretation of the fractionation. Thus, heterogeneity of the population could be masked by these irregularities in migration.

The excellent pH stability of the glycine-phosphate buffer was demonstrated in the 3 x 16 flow-cell experiments. Because of the effects of pH on electrophoretic mobility and on the viability and intrinsic motility of spermatozoa, it is essential to have good pH stability in the Staflo apparatus. Within limits, changes in the flow-cell temperature and in the pH of the medium due to increased electric field strength can be compensated for by increasing the electrode wash flow rate. pH changes were greater with the medium used in the 1 x 12 flow-cell experiments than with the glycine-phosphate buffer and also may have influenced the distribution of spermatozoa slightly in those experiments. No temperature measurements were made in the 1 x 12 flow-cell and it is probable that the poor reproducibility in that series of experiments, especially at 7.5 V/cm, is also due in part to temperature differences among experiments.

b. <u>Intrinsic Motility</u>. With the same medium, density gradient and pH, the effect of intrinsic motility upon the distribution of spermatozoa in the Staflo apparatus is influenced by temperature and by the orientation and quality of the spermatozoa. Many examples of these interrelationships are presented in Section C.1.a above.

(1) <u>Temperature</u>. The magnitude of the intrinsic motility of individual spermatozoa can be controlled by temperature regulation. The degree of motility at any specific temperature depends strongly upon the suspension medium as reported by Bhattacharya[35]. At temperatures of 2°C or lower, there was no motility in any of the low ionic strength buffers used in this series of experiments. In the experiments at 6.25 and 7.5 V/cm, at 2.5°C, the initial quality of the spermatozoa was quite poor, and motility should have played no part in the distribution of the spermatozoa. Thus, in those 2 experiments, there was not a higher percentage of motile spermatozoa in the lower collection tubes as observed in the experiments at higher temperatures (e.g., Figure 41) under the same conditions of electric field strength. On the contrary, at 2.5°C, spermatozoa in the upper collection fractions had a higher percentage of motility (Figure 43).

(2) <u>Orientation</u>. In all of the electrophoretic media used in the experiments reported in this chapter at pH 6-7, and at an electric field strength of 5 V/cm or greater, all spermatozoa were oriented vertically in the electric field with the tails pointing toward the positive electrode. The orientation was not affected by the direction of migration. The differential distribution of the component parts of spermatozoa in one of the early 3 x 16 flow-cell experiments confirms the presence of a relatively greater net negative charge on the tail of the spermatozoa. The suggestion in Table VI of a lesser migration for the spermatozoa with cytoplasmic

droplets, which are considered a sign of the final stage of maturation, is consistent with Bedford's report of a lower electrophoretic mobility for epididymal than for mature spermatozoa[24].

Under the proper conditions of temperature and electric field strength, one can use the tail anode property to orient the intrinsic motility vector. As shown in Experiment 4-29-65 at 5 V/cm (Figure 40), the conditions of temperature and electric field were such that 5% of the spermatozoa migrated into layer 10, the layer below the inlet stream.

As reported in Chapter IV, the spermatozoa generally assume a head-down orientation in a 1g field. Because of the similar conditions of transit time and temperature in the 1 x 12 flow-cell experiments, the contribution of sedimentation to the migration should have been approximately the same in all experiments. The dextran density gradients used in some of the 3 x 16 flow-cell experiments greatly retarded the sedimentation of spermatozoa and, therefore, the sedimentation component can probably be ignored in those experiments.

(3) Quality. The ability to control the magnitude and orientation of the intrinsic motility vector is hypothetical unless the initial spermatozoa quality is excellent. The same protocol was followed in ejaculate collection and handling in all of the experiments in this series, and, therefore, should not have caused unnatural differences in the initial quality of spermatozoa samples. The characteristics by which quality is defined include the percentage of motility, the degree of motility of individual spermatozoa and the durability of the spermatozoa. The latter implies the survival of the vital functions (motility, viability and fertilizing capacity) during the experiments.

It is rare to obtain an ejaculate in which all of the spermatozoa have

forward motion. Therefore, in electrophoresis experiments, a certain percentage of the sample will undergo the maximum electrophoretic or sedimentation-electrophoretic migration. In order to observe significant effects on the net migration of the sample, a substantial fraction of the population must have a high degree of forward motion. Some of the differences among the 1 x 12 flow-cell experiments can be attributed to the variable percentages of motile spermatozoa in the initial samples. The degree of motility is important for an initial sample with only tail-wagging at room temperature is going to have no motility at lower temperatures. In Experiment 4-29-66 in the 1 x 12 flow-cell series, the spermatozoa had a high degree of intrinsic motility and a significant downward migration of motile spermatozoa was evident from the distribution thereof.

The importance of the durability of spermatozoa, or the preservation of intrinsic motility, viability and fertilizing capacity, was well illustrated in these experiments. As observed in the early sedimentation experiments, the spermatozoa lost their intrinsic motility prior to losing viability, judged by a nigrosin-eosin staining technique. In most cases, there was a great difference between the initial quality of the spermatozoa sample and the quality of spermatozoa in the collection fractions immediately after the experiment. When the spermatozoa in the collection fractions initially did have some degree of motility, it was often lost within 30 minutes after termination of the experiment. Unfortunately, this loss of motility prevented analysis of the interrelationships among the motility, temperature and electric field strengths in the flow-chamber. In addition, the rapid loss of motility was associated with an irreversible loss of fertilizing capacity, demonstrated by the poor fertilization rate achieved in the 1 x 12 flow-cell experiments. Because the spermatozoa were insem-inated 1-2 hours after the female was mated with a vasectomized buck, the

spermatozoa would have had to remain motile IN VIVO for 8-9 hours in order
to fertilize ova. However, the loss of fertilizing capacity was observed
with spermatozoa samples in concentrations greater than 4×10^6/ml in the
1 x 12 flow-cell experiments, suggesting a deficiency in the medium rather
than a dilution effect. Thus, the loss of fertilizing capacity may not
be just a simple reflection of the loss of motility.

The preservation of the motility and viability of spermatozoa was
greatly enhanced by the glycine-phosphate buffer. Observations on the
evaluation of this buffer are presented in Appendix D and in Chapter VI.
In the early sedimentation experiments, there appeared to be an enhance-
ment of motility and viability by the seminal plasma in collection fraction
4. There is a suggestion of a similar effect in many of the 1 x 12 flow-
cell experiments in which the seminal plasma migrated into layers 8 and 7.
Although seminal plasma may be beneficial from the point of view of pre-
serving vital functions of the spermatozoa, in high concentrations it
would, unfortunately, change the ionic strength of the medium.

2. Electrophoretic Mobility Comparisons

The results of the upward and downward-electrophoresis experiments
with rabbit and fowl spermatozoa using the 3 x 16 flow-cell are summarized
in Table X. In all of the experiments with rabbit spermatozoa, the intrin-
sic motility was very poor and should not have affected the electrophoretic
mobility measurements. Fowl spermatozoa, however, had a high percentage
and degree of motility at room temperature in all experiments, and the
calculated average electrophoretic mobility, therefore, may be slightly
low.

In Table X, it is seen that under similar experimental conditions,
the electrophoretic mobility of fowl spermatozoa is approximately 2 times

Table X. Average Electrophoretic Mobilities of Rabbit and Fowl Spermatozoa Normalized to a Density Gradient of 1% Sucrose at $3^{\circ}C$.

Medium and Density Gradient	Electrophoresis Direction	Temp	Electrophoretic Mobility [Units of 10^{-4}(cm/sec)/(V/cm)]	
			Rabbit	Fowl
Glycine-phosphate buffer;	Downward	$6^{\circ}C$	0.82	-
dextran density gradient	Downward	$6^{\circ}C$	0.79	-
	Downward	$6.8^{\circ}C$	<u>-</u>	<u>1.68</u>
Average			0.81	1.68
Glycine-citrate buffer;	Upward	$6^{\circ}C$	0.95	1.68
sucrose density gradient	Upward	$5.4^{\circ}C$	0.76	-
	Upward	$4.3^{\circ}C$	<u>-</u>	<u>1.58</u>
Average			0.86	1.63

that of rabbit spermatozoa. Additional measurements of electrophoretic mobility of both rabbit and fowl spermatozoa are reported in subsequent chapters and confirm this impression.

Considering the use of different buffers and different density gradient materials, the average values of the electrophoretic mobility for rabbit spermatozoa in upward and downward directions agree within the limits of the measurement in the Staflo apparatus. Although only one downward electrophoresis experiment was conducted with fowl spermatozoa, the agreement between that value and the average of the 2 upward-electrophoresis experiments, as shown in Table X, is also very good. These values are normalized to that expected in a density gradient containing 1% sucrose at 3°C for comparison with other electrophoresis and sedimentation results reported in Chapter VIII.

E. Summary and Conclusions

The different-electric field groups of 1 x 12 flow-cell upward electrophoresis experiments were characterized by a lack of reproducibility which is explained, in part at least, by variations both in temperature and in the quality of spermatozoa in individual experiments. Because the initial spermatozoa concentrations were 20.5×10^6/ml or less in all experiments except one (Experiment 4-29-65), there should have been no "cluster sedimentation" with this one possible exception.

The lack of reproducibility within, and differences among, groups of experiments prevented drawing firm conclusions about the distribution of viable spermatozoa among the collection fractions as judged by nigrosin-eosin staining. As was also observed in the early sedimentation experiments (Chapter IV), the rapid loss of motility before loss of viability further complicated interpretation of the distribution of viable spermatozoa.

Still prior to development of a good Staflo microscopic system, this loss of motility also prevented understanding the possible interplay between intrinsic motility and electric field, in the flow-chamber. As before, the very poor results of artificial insemination suggest that the loss of motility was irreversible. Thus, the Hanks-glycine medium used in these experiments was highly unsatisfactory for biological work. As also noted in Chapter IV, the presence of diluted seminal plasma appeared to enhance the relative preservation of motility in the respective fractions.

The differences among the 5, 6.25 and 7.5 V/cm groups of experiments are consistent with the known relationships between the intrinsic motility and electrophoretic mobility of spermatozoa. Even at temperatures of 5-6°C, there was a significant decrease in the net electrophoretic migration because of the oppositely directed intrinsic motility vector. The most highly motile spermatozoa showed very little net migration in the electric field, and, either through "exhaustion" or because of subsequent dilution, lost their motility before or shortly after the runs were completed. Relative enrichment of the lower collection fractions with regard to motile and viable spermatozoa was demonstrated in the 6.25 V/cm experiments at 3.5°C and 5°C.

The 1 x 12 flow-cell experiments further demonstrated the need for accurate temperature control which later became possible upon construction of a cold room. With temperature control, the glycine-phosphate and several greater-increment density gradients, good reproducibility was achieved in predominantly electrophoresis experiments.

The 3 x 16 flow-cell and ancillary equipment were tested in a series of upward- and downward-electrophoresis experiments with both rabbit and fowl spermatozoa. The horizontal and vertical distributions of spermatozoa

in the flow-chamber approximated those predicted for "ideal" migration patterns by Tippetts[263]. The glycine-phosphate buffer used in the 3 x 16 flow-cell experiments, in addition to being far superior to the Hanks-glycine medium for the preservation of spermatozoa, also demonstrated excellent pH stability (less than 0.1 pH unit change during an experiment). This was without the "high conductivity" barrier used in subsequent experiments (Chapters VI and VIII).

The Staflo microscope and slit lamp technique were both used to measure migration distances in the flow-chamber. The slit lamp was more practical for observing aberrations of the gross migration profile, whereas the microscopic system gave the additional capability of observing the orientation and degree of motility, and cell-cell interactions such as microscopic "cluster sedimentation". Several experiments in this series illustrated the need for such observations if proper interpretation of the net migration was to be made.

All rabbit and fowl spermatozoa had a net negative charge from pH 6-7 in all buffers used and, at temperatures of 2-7°C, had a tail-anode orientation in the electric field regardless of the direction of electrophoretic migration. With both upward and downward migration, the total spermatozoa distribution suggested a unimodal distribution of electrophoretic mobilities, the averages of which are summarized in Table X. The values have been normalized as described in Chapter III to those expected in the glycine-phosphate buffer with 1% sucrose at 3°C in order to make some subsequent comparisons.

The electrophoretic mobility of fowl spermatozoa in these experiments was seen to be approximately twice that of rabbit spermatozoa. Additional electrophoretic determinations are given in Chapter VI and VIII for fowl and rabbit spermatozoa, respectively.

VI.   STAFLO ELECTROPHORESIS: FOWL SPERMATOZOA

Contents

A. <u>Preservation of Motility and Fertilizing Capacity Under Conditions of</u>
    <u>Low Temperature and Low Spermatozoa Concentrations</u>

1. <u>Introduction</u>

It is well recognized that knowledge of the biophysical properties

of many types of cells has contributed greatly to an understanding of their

role in biological systems. In this regard, the properties of spermatozoa

should be of great interest in the biology of reproduction. In the study

and practice of mammalian reproduction, considerable flexibility is pro-

vided in work with a number of species by the ability to store spermatozoa

for weeks or longer at low temperatures, with maintenance of their vital

functions, prior to artificial insemination. In contrast, for fowl sperm-

atozoa, such success has not been achieved and the common practice is to

inseminate concentrated semen spermatozoa immediately after collection. The

inability of fowl spermatozoa to survive low temperature storage conditions

also severely limits the feasibility of conducting many biophysical studies

on these cells. This section is concerned with the preservation of the motility and fertilizing capacity of fowl spermatozoa not only at low temperatures, but in low concentrations and in low ionic strength media.

A convenient means of reversibly controlling motility is through the control of temperature. For complete immobilization of spermatozoa, depending upon the suspension medium, temperatures near 0°C are required[35]. Varying specified degrees of motility can be achieved by selection of suitable temperatures above 0°C.

Even though the biological qualities of spermatozoa are most easily preserved at high sperm concentrations, for certain purposes, it is desirable or essential to work at low concentrations and still maintain these properties. For example, in both analytical and preparative electrophoresis, the requirements for observation of individual spermatozoa and for hydrodynamic stability demand spermatozoa concentrations ranging from about 1/300 - 1/30 of those in undiluted ejaculates[156-158]. Many electrophoresis experiments further require media with ionic strengths much less than those of physiological solutions. The inability to preserve motility and fertilizing capacity under such extreme conditions severely limits the possible kinds of experimental manipulations of spermatozoa.

Many investigators interested primarily in artificial insemination have studied the effects of cooling or dilution of fowl spermatozoa but not combinations of the two factors as noted above. With regard to temperature, Van Wambeke observed almost no loss of fertilizing capacity of fowl spermatozoa diluted with equal volumes of either of two diluents containing skimmed milk and stored at 2-5°C for 24 hours[231]. Even though the semen was diluted 1:2, the cell concentration is very high in the sense used in this paper. Lorenz, in his review, mentions work by others at room

temperature which showed the following: (1) 1:3 dilution with seminal plasma gave a slight decrease in fertility but no additional loss with dilutions of up to 1:63, provided that the same number of spermatozoa was inseminated; (2) dilutions of up to 1:50 in an artificial medium resulted in only a slight loss of fertility for inseminations of at least 10^8 spermatozoa; poor results were obtained with greater dilutions or with insemination of fewer spermatozoa and (3) 1:1250 dilution in saline or in Tyrode's or Baker's solutions produced immediate irreversible immobilization[140].

Thus, from the above, it is seen that the fertilizing capacity of fowl spermatozoa has been maintained for brief periods in media of high ionic strength during storage under conditions of (1) low temperature-high concentration, or (2) room temperature-low concentration. In this section, we report a significant extension of the limiting experimental conditions of this kind, namely, preservation of motility and fertilizing capacity under the combined conditions of low temperature and low sperm concentration, in 2 low ionic strength media suitable for electrophoresis experiments. In addition, 2 high ionic strength media were evaluated for storage of spermatozoa at low temperature and high concentrations.

2. <u>Materials and Methods</u>.

a. <u>Characteristics of Ejaculates</u>. The characteristics of the ejaculates from the White Leghorn roosters used in these experiments were as follows: volume, 0.4 - 1.0 ml; spermatozoa concentration, 2.5 - 3.0 x 10^9/ml; motility, 80%-90%; fertilizing capacity, 85%-95% with insemination of 0.1 ml of normal, undiluted semen at 4-5 day intervals.

b. <u>Evaluation of Spermatozoa</u>. The customary evaluation scale was used for high concentration populations of spermatozoa. It ranges from Grade I (extremely poor motility) to Grade VI (a "vigorous swarming pattern"). In

addition, with low concentrations it was possible to estimate the percentage of individually motile spermatozoa and to utilize a scale of degrees of motility as follows: (1) immotile, (2) feeble tail-wagging, (3) vigorous tail-wagging, (4) feeble forward motion and (5) vigorous forward motion.

c. __Cooling Spermatozoa__. Initial cooling of spermatozoa in all experiments was accomplished by adding an equal volume of dilution medium to pooled ejaculates in a 15 ml glass centrifuge tube which then was capped and placed in the center of a 2 lb coffee can full of sawdust. The coffee can was covered and immersed to the brim in ice water. Cooling curves determined with a temperature recorder (Varian Associates) showed a temperature decrease from 29°C to 2.5°C in 120 minutes, with a maximum cooling rate of 0.30°C per minute.

d. __Media__.

(1) __Egg yolk medium__. The egg yolk-glycine-citrate medium is a modification of that used by Bhattacharya[35]: 40% by volume whole egg yolk and 60% glycine-citrate solution consisting of 20 g sodium citrate dihydrate, 20 g glycine, 5 g fructose, 0.25 g citric acid, 1.5×10^6 units penicillin G, diluted to 1000 ml with distilled water; a "high" ionic strength medium.

(2) __Vitalizing medium__. The glucose vitalizing medium is a modification of the Krebs-Henseleit-Ringer solution used by Bhattacharya[35]: 0.9 g NaCl, 0.05 g KCl, 0.05 g $Na_2HPO_4 \cdot 12H_2O$, 0.04 g $MgSO_4$, 0.03 g $NaHCO_3$, 0.5 g glucose, 10^5 units penicillin G, diluted to 100 ml with distilled water. pH adjusted to 7.0 with HCl; a "high" ionic strength medium.

(3) __Glycine-citrate buffer__. A "low" ionic strength solution consisting of 2.0 g sodium citrate dihydrate, 9.25 g glycine, 3.0 g fructose, 0.25 ml of 10% citric acid solution, 48.6 g sucrose, 10^6 units of penicillin G, diluted to 1,000 ml with distilled water; pH 6.94.

(4) __Glycine-phosphate buffer__. A "low" ionic strength solution consisting

of 2.2 g $Na_2HPO_4 \cdot 12H_2O$, 0.20 g NaCl, 0.20 g $NaHCO_3$, 0.016 g KH_2PO_4, 9.25 g glycine, 23 g fructose, 10^6 units penicillin G, diluted to 1,000 ml with distilled water. pH adjusted to 7.1 with HCl.

As mentioned in the text, dextran (MW 73,000; Sigma Chemical Company) or sucrose was added to the glycine-citrate and glycine-phosphate buffers in some instances to simulate density gradient conditions for electrophoresis experiments.

3. Results.

a. Comparison of the two "high" ionic strength media. Egg yolk medium and vitalizing medium, both of which have been found useful in work with rabbit spermatozoa[35] were evaluated for use in the initial dilution and cooling of fowl spermatozoa and for the temporary storage of these spermatozoa reconcentrated after dilution in low ionic strength media. Semen was obtained by abdominal massage[266]. It was pooled and then diluted with equal volumes of either egg yolk medium or vitalizing medium and placed in separate 15 ml glass centrifuge tubes. Both samples were cooled continuously from room temperature to 4°C in 2 hours and were held at that temperature for 2 additional hours. The tubes then were removed from the cooling container and allowed to warm naturally to room temperature. After warming there were no apparent differences in morphology or in the high degree of motility of the spermatozoa in the 2 samples. Two groups of 2 chickens each were inseminated with 0.1 ml of the respective samples. The procedure was repeated twice with fresh spermatozoa at 4-day intervals and eggs were collected from the day after the first insemination until 4 days after the third insemination. The results are presented in Table XI. Only 9.1% of the eggs were fertilized by spermatozoa stored in the egg yolk medium compared to 80% fertility in the vitalizing medium.

Table XI. Preservation of Fertilizing Capacity of Fowl Spermatozoa
Diluted 1:2 and Stored in "High" Ionic Strength Media at 4°C.

	Egg Yolk Medium	Vitalizing Medium
Total number of eggs	33	40
Number of fertile eggs	3	32
Percentage of fertile eggs	9.1	80

b. Comparison of the two "low" ionic strength media.

(1) Motility. The preservation of motility at 3°C in "low" ionic strength

glycine-citrate and glycine-phosphate buffers was evaluated in solutions of

each adjusted with sucrose to 310, 368 and 427 mosm. Pooled semen was di-

luted 1:2 with vitalizing medium and cooled from room temperature to 3°C

in 2 hours. Aliquots were then diluted in each of the 6 solutions, to con-

centrations of 80 x 10^6 spermatozoa/ml. Additional dilutions were made on

each of the suspensions resulting in concentrations of 40, 27, 20, 13 and

8 x 10^6 spermatozoa/ml, giving a total of 36 samples.

After 1 hour at 3°C, aliquots of selected 310, 368 and 427 mosm solu-

tions of both buffers were allowed to warm to room temperature. Spermatozoa

in the 310 and 368 mosm solutions showed no change from their initial motility

(80%-90% vigorous forward motion). On the other hand, those in all of the

427 mosm solutions exhibited a marked decrease in motility with approximately

10% having feeble forward motion and 15%-20% feeble tail-wagging. Inter-

mittent observations made over the next 5 hours established a continuous

trend of loss of sperm quality in some solutions, and a relative constancy

in others. These were culminated by the final observations at room temperature

following storage for 6 hours at 3°C. In the 310 mosm solutions of both

buffers, and at all 6 concentrations, approximately 70% of the spermatozoa

had vigorous forward motion. In all 368 mosm solutions of both buffers,

approximately 50% of the spermatozoa had vigorous forward motion and 20%

exhibited vigorous tail-wagging. Moderately severe tail-tail agglutination

occurred in all 368 mosm solutions whereas agglutination was rare in the

310 mosm solutions. For the 427 mosm solutions of both buffers, less than

5% of the sperm had either tail-wagging or forward motion. In summary,

no qualitative differences were found between any of the corresponding 310,

368 or 427 mosm solutions of "low" ionic strength glycine-citrate and

glycine-phosphate buffers. Furthermore, both 310 mosm solutions gave

excellent preservation of motility; i.e. less than a 15% decrement from

the initial values was observed, in concentrations ranging from 8-80 x 10^6/ml

after 6 hours storage at 3°C.

(2) _Fertilizing capacity_. The glycine-citrate and the glycine-phosphate

buffers were also compared to each other and to vitalizing medium with

regard to preservation of fertilizing capacity after dilution and brief

storage. Pooled semen was diluted 1:2 with vitalizing medium and cooled

to 3°C in 2 hours. One-third of the sample was diluted in vitalizing medium

to approximately 250 x 10^6 spermatozoa/ml and stored at 3°C for 2 1/2 hours.

The remainder of the sample was divided between the two 40 ml glass centri-

fuge tubes. One suspension was diluted with glycine-citrate and the other

with glycine-phosphate buffer to spermatozoa concentrations of approximately

350 x 10^6/ml. After 1 hour, these samples were further diluted to 80-90

x 10^6/ml and held at 3°C for an additional 1 1/2 hours. This dilution-

storage protocol was repeated in 4 and 8 days. For each of the 3 trials,

the same 3 groups of 6 hens each were inseminated. Eggs were collected

from the day after the first insemination until 4 days after the third

insemination. In the first trial, reconcentration of the diluted spermatozoa was not attempted because the 2% dextran, which was used to simulate density gradient conditions, interfered with the centrifugation process. Each hen was inseminated with approximately 1.0 ml of suspension containing a total of 86-100 x 10^6 spermatozoa as indicated in Table XII A. In trials 2 and 3, where sucrose was used to simulate the density gradient conditions, reconcentration of the spermatozoa in all 3 suspensions was accomplished by centrifugation (50 minutes at 400 g in trial 2, 30 minutes at 1,500 g in trial 3). The volumes and total number of spermatozoa inseminated in trials 2 and 3 are shown in Table XII B.

The results of trial 1 are shown in Table XII A. It is seen that a much higher percentage of eggs was fertilized by spermatozoa diluted and stored in the glycine-citrate buffer than in either of the 2 other media. The differences between the fertilization rate of 56.3% with glycine-citrate buffer and the rates of 15.8% (glycine-phosphate) and 27.2% (vitalizing medium) are significant at the $P < 0.01$ and $P = 0.07$ levels, respectively, in a one-tail test[90].

The pooled results of trials 2 and 3 are shown in Table XII B. The largest difference among the fertilization rates, i.e. that between the 41.9% in vitalizing medium and 26.5% in glycine-citrate buffer, is signi-. ficant only at the $P = 0.10$ level. The spermatozoa used in trials 2 and 3 did exhibit increased evidence of certain morphological changes following centrifugation, the most common of which was severe bending of the spermatozoa to give apposition of head and proximal tail segments.

The average results of all 3 trials are given in Table XII C. There is no difference between the fertilization rates with glycine-citrate buffer and vitalizing medium ($P \gg 0.10$). The fertilization rates with

Table XII. Preservation of Fertilizing Capacity of Fowl Spermatozoa Diluted 1:30
and Stored in "High" and "Low" Ionic Strength Media at 3°C.

	Low Ionic Strength		High Ionic Strength Vitalizing Medium[a]
	Glycine-Citrate Buffer[a]	Glycine-Phosphate Buffer[a]	
A. Trial 1: (no recentrifugation)			
Inseminate:			
Volume (ml)	1.0	1.0	1.0
Total Spermatozoa	86×10^6	95×10^6	100×10^6
Total Number of Eggs	16	19	11
Number of Fertile Eggs	9	3	3
Percentage of Fertile Eggs	56.3	15.8	27.2
B. Trials 2 and 3: (recentrifugation)			
Inseminate:			
Volume (ml)	0.2	0.2	0.1
Total Spermatozoa	220×10^6	210×10^6	150×10^6
Total Number of Eggs	34	30	31
Number of Fertile Eggs	9	9	13
Percentage of Fertile Eggs	26.5	30	41.9
C. Average of Trials 1, 2 and 3			
Total Number of Eggs	50	49	42
Number of Fertile Eggs	18	12	16
Percentage of Fertile Eggs	36	24.5	38.1

a. 6 hens in each group

the glycine-citrate and glycine-phosphate buffers differ only at the

$P = 0.12$ level while the latter and the vitalizing medium differ at the

$P = 0.08$ level. Thus, any advantage of using the glycine-citrate buffer

must be considered slight.

c. Comparison of best "high" and "low" ionic strength media at 1:30

   dilutions. The effects of moderate dilution in vitalizing medium

and in glycine-citrate buffer on the fertilizing capacity of fowl sperm-

atozoa were compared, using a 1:2 dilution control. Pooled semen was di-

luted 1:2 in vitalizing medium and cooled from room temperature to 3°C in

2 hours. One-third of the sample (the control) was stored at this temp-

erature without further dilution. The remainder was divided equally be-

tween two 40 ml glass centrifuge tubes and diluted 1:30 with either

vitalizing medium or with glycine-citrate buffer. After 1 hour, the

diluted spermatozoa were reconcentrated by centrifugation for 50 minutes

at 250 g. Recovery was approximately 85%-90%. After removal of the

supernatant, both samples of spermatozoa were resuspended in vitalizing

medium. This dilution-storage protocol was repeated 3 days later. On

each occasion, the same 3 groups of 6 chickens each were inseminated with

0.20 ml of suspension containing approximately 200×10^6 spermatozoa. Eggs

were collected from the day after the first insemination until 7 days after

the second insemination. The results are shown in Table XIII. There are

no significant differences among the 3 fertilization rates.

4. Discussion.

a. Effectiveness of various media. The poor preservation of fertilizing

capacity by media containing egg yolk, despite maintenance of motility,

has been reported by others[140]. In addition to its superiority in preserv-

ing fertilizing capacity, vitalizing medium is a clear, colorless solution

of low viscosity. In contrast, egg yolk medium exhibits decreased light

Table XIII. Effects of Dilution and Media on the Preservation of the Fertilizing Capacity of Fowl Spermatozoa Stored at 3°C.

	Ionic Strength		
	"Low"	"High"	
	Glycine-Citrate (1:30 dilution)	Vitalizing Medium (1:30 dilution)	Vitalizing Medium (1:2 dilution)
Total number of eggs	34	46	38
Number of Fertile Eggs	20[a]	23	24
Percentage of fertile eggs	58.8	50	63.2

a.3 of 20 eggs contained dead embryos whereas all other fertile eggs contained viable embryos on the 17th day of incubation.

transmission, a high viscosity and a potential for coating equipment with a protein film – all undesirable properties in certain experimental systems.

For the preservation of motility, no difference was found between the "low" ionic strength glycine-citrate and glycine-phosphate buffers. For the preservation of fertilizing capacity, the data in Table XII C suggest a possible slight advantage to using the glycine-citrate buffer. Consequently, that medium was selected for subsequent dilution studies and for electrophoresis experiments to be reported in Section B.

In the study summarized in Table XIII, the fertilizing capacity of spermatozoa diluted 1:30 in "low" ionic strength glycine-citrate buffer, stored for 1 1/2 hours at 3°C and reconcentrated by centrifugation is comparable to that obtained for spermatozoa diluted 1:2 in vitalizing medium and stored under the same time-temperature conditions without centrifugation. This suggests that fowl spermatozoa can withstand the stresses expected in Staflo electrophoresis experiments: low ionic strength, low temperature, moderate dilution and reconcentration by centrifugation.

Comparison of the data in Table XIII for the 1:30 dilution (with centrifugation) and the 1:2 dilution (no centrifugation) in vitalizing medium reveals no difference (P > 0.10). Thus, if use of a "high" ionic strength medium is indicated, vitalizing medium could be used for studies with fowl spermatozoa under conditions of moderate dilution.

b. Factors Affecting Fertilization Rates.

(1) Artificial insemination. The fertilization rate of 80% achieved with spermatozoa diluted 1:2 in vitalizing medium (Table XI) hardly differs from the optimum range of 85%-95% expected with our flock. If there is a slight difference, it might be due entirely to the cooling to 4°C (2 hours) and storage at 4°C (2 hours).

The difference between the fertilization rates of 80% (Table XI) and 63.2% (Table XIII), with 1:2 dilutions in vitalizing medium and storage under the same conditions, is attributed primarily to using young hens of unknown fertility in the latter study. Thus, it is expected that a fertilization rate approaching 80% would have been obtained if hens of proven fertility had been available. Likewise, a comparable improvement could be anticipated in the 58.8% fertilization rate for spermatozoa diluted 1:30 in the "low" ionic strength glycine-citrate buffer (Table XIII).

(2) Osmolarity. For the preservation of motility, the 310 mosm solutions of both the glycine-citrate and glycine-phosphate buffers were far superior to the 368 and 427 mosm solutions, with storage at 3°C for 6 hours in concentrations from 8×10^6 - 80×10^6 spermatozoa/ml. In other studies, using 310 mosm solutions of both buffers, the fertilizing capacity was preserved for 2 hours at 3°C at 1:30 dilutions. Even though the preservation of this property was not evaluated in the 368 mosm solutions, the more rapid loss of motility therein suggests that a corresponding loss of fertility would also occur. Other investigators, using different media, have reported different optimum osmolarities for the preservation of motility and fertilizing capacity - 300 and 350 mosm, respectively[140]. Our observations favor use of the lower (310) mosm solutions for sustaining both biological properties.

The marked decrease in motility, after 1 hour at 3°C, for all spermatozoa concentrations in the 427 mosm glycine-citrate and glycine-phosphate buffers is in contrast with the excellent preservation of motility for 4 hours in the 415 mosm egg yolk medium. Thus, egg yolk has an important protective effect on motility in overcoming otherwise adverse effects of high osmolarity. Other observations in this study indicated excellent preservation of fowl sperm motility in egg yolk medium for more than 24 hours. In

spite of this, the observed loss of fertilizing capacity (Table XI) suggests that irreversible damage was done to the spermatozoa during the 4-hour exposure to egg yolk medium. (The probable dilution and/or absorption of the medium in the female genital tract should have largely eliminated the influence of the medium shortly after artificial insemination). It is recognized that these IN VITRO observation times are short considering that good fertility is maintained in chickens for 5-6 days after mating and that some fertile eggs may be obtained as late as 35 days thereafter[220]. Never-theless, these results point to a probable differential vulnerability of fowl spermatozoa with respect to the 2 key biological characteristics: motility and fertilizing capacity.

(3) Dilution and Reconcentration. Because the usual practice with fowl is to inseminate 0.05-0.10 ml of highly concentrated spermatozoa (undiluted or 1:2 dilution), immediately after collection, dilute suspensions of spermatozoa in this study were generally reconcentrated by centrifugation prior to insemination. An important question is whether or not the mor-phological changes seen following the centrifugation process negate the advantages of inseminating small volumes. Comparison of the fertilization rates in Table XII for dilute (56.3%) and reconcentrated (26.5%) spermatozoa suggests that, at least for the glycine-citrate buffer, centrifugation is detrimental (significant at the $P = 0.02$ level). Although the reverse appears to be true for the spermatozoa in vitalizing medium and in glycine-phosphate buffer, the differences are not significant ($P > 0.10$).

The results of this part of the study indicate that successful insem-ination of low concentrations of spermatozoa is possible in glycine-citrate buffer. Furthermore, if centrifugation is required to achieve a high concentration sample, insemination with a moderately dilute sample actually

appears to be preferred provided that at least 80×10^6 spermatozoa can be inseminated in a volume of 1 ml or less.

c. <u>Additional Studies</u>. Because the 415 mosm "high" ionic strength egg yolk medium preserved the motility of the fowl spermatozoa much better than did the 427 mosm "low" ionic strength glycine-citrate and glycine-phosphate buffers, the preservation of both motility and fertilizing capacity in an egg yolk medium adjusted to 310 mosm should also be evaluated. This would shed light on the question of whether osmolarity or some other property was responsible for the loss of fertilizing capacity in media containing egg yolk.

The excellent preservation of motility in the 310 mosm "low" ionic strength glycine-citrate and glycine-phosphate buffers at concentrations as low as 8×10^6 spermatozoa/ml shows that meaningful analytical electrophoresis studies can be conducted. The analytical information is of interest on 2 accounts: (1) in providing physical characterizations of spermatozoa populations and (2) in revealing optimum conditions for preparative electrophoresis separations. It is expected that information obtained in analytical studies can be exploited in preparative experiments.

It was seen that the "low" ionic strength glycine-citrate buffer excellently preserved both motility and fertilizing capacity. In view of the fertilization results achieved with different media by others at room temperature with dilutions of 1:50 and 1:63[140], successful insemination may be possible after even greater dilutions at low temperature in glycine-citrate buffer. Furthermore, one can speculate that this buffer might be an even better diluent than vitalizing medium for storage of spermatozoa at high concentrations. For this reason, it would be useful to extend the present study to a comparison of the 2 media at 1:2 dilutions with longer

storage times at 3°C. Effective extension of successful spermatozoa

storage, as well as insemination at lower concentrations, could be of

considerable practical benefit for work in poultry husbandry and genetics.

B. Electrophoresis Experiments

1. Introduction

In contrast to the abundance of information concerning the electro-

phoretic properties of mammalian spermatozoa, there have been few such

reports on fowl spermatozoa. Undoubtedly, major reasons for the greater

interest in mammalian spermatozoa are the economic and sociological con-

sequences of finding a way to separate genetically differing, e.g. X- and

Y-chromosome bearing, spermatozoa. Despite the work of Schröder[206] and

of Gordon[91], it is uncertain that apparent differences in the electro-

phoretic properties of rabbit spermatozoa can be attributed to different

chromosomal composition[18,174,233]. Because all fowl spermatozoa contain

X-chromosomes, the sex control incentive is lacking in the study of this

species. However, in addition to basic knowledge about the properties of

fowl spermatozoa, electrophoretic studies are motivated by the availability

of cocks with a translocation heterozygote producing spermatozoa with

differing chromosomal composition and fertilizing capacity[120]. It would

also be of interest to compare the electrophoretic properties of sperm-

atozoa from cocks of lines of poor fertility with those from normal cocks.

The stable-flow free-boundary (Staflo) method has been used to charac-

terize cell populations such as rat bone marrow[159] and rabbit spermatozoa

(Chapters IV, V and VIII). The flexibility of this method provides an

opportunity for either analytical or preparative studies using sedimentation

or electrophoresis or a combination of the two. In addition, one can achieve

a fractionation of spermatozoa populations using differing degrees of

intrinsic motility at specified controlled temperatures.

In Section A, it was shown that fowl spermatozoa, which were subjected to cooling, dilution and storage at 3°C for 1 1/2 hours in low ionic strength glycine-citrate medium at concentrations expected in Staflo electrophoresis experiments and then reconcentrated by centrifugation, gave a fertilization rate of 58.8%, as compared to a control value of 63.2%. This section reports a series of experiments which 1) confirm the conclusion from Section A that the fertilizing capacity of spermatozoa could be maintained during Staflo electrophoresis experiments; 2) characterize the sperm- atozoa populations with regard to average and extreme values of electrophor- etic mobility; and 3) partially fractionate and relatively enrich the spermatozoa populations by utilizing a combination of electrophoresis and intrinsic motility.

2. Materials and Methods

a. Sample Preparation. Ejaculates were collected from mature White Leg- horn cocks by abdominal massage[266] and pooled until 2.0 ml or more of undiluted semen was obtained. A 0.5 ml aliquot of pooled semen was diluted 1:2 with modified Krebs solution for a control specimen and the remainder was diluted 1:2 with glycine-citrate buffer. A 0.10 ml aliquot was diluted 1:100 in a solution of 1% formalin in normal saline for counting in a hemo- cytometer. Both samples were cooled from room temperature to 2°C in 45 minutes. The glycine-citrate sample then was diluted to a spermatozoa concentration of approximately 250-300 x 10^6/ml and subjected to Staflo electrophoresis.

b. Staflo Apparatus. The essential features of the Staflo apparatus employed in these experiments are a 3 x 16 channel flow-cell (designated No. XI), duplicate 48-channel collection systems and a 16-channel, multi- syringe pumping system. Sixteen 50 ml plastic syringes on the pump rack

contain the density gradient solutions which are delivered by polyethylene tubing to the respective inlets on the flow-cell. A variable speed drive allows a wide range of accurately controlled solution flow rates. A 20 ml syringe is often used for the spermatozoa sample suspension in order to achieve an initial sample stream thickness approximately 1/2 that of other fluid layers with resulting greater resolution of the migrating species ("thin sample technique"[229]). The spermatozoa suspension is stirred magnetically to maintain a uniform concentration throughout the experiment.

The inlets to the flow-chamber are arranged vertically as shown in Figure 48, thereby forming flowing layers, each 0.125 cm high, 0.80 cm wide and 30 cm long. The multiple, horizontally flowing layers are stabilized by hydrodynamic feedback principles, rather than by use of any solid supporting medium[156,159]. The fluid in each of the 16 layers exits the flow-cell through 3 horizontal channels, thereby allowing separation of the faster moving fluid in the central region from the slower moving fluid near each sidewall. In this study, only the collection fractions from the central channel of the flow-cell were evaluated.

In these experiments, only 4 of the 6 electrode segments were activated giving an electric field across the downstream 2/3's (20 cm) of the flow-cell as shown schematically in Figures 48 and 49.

c. __Medium and Density Gradient.__ Using the low ionic strength glycine-citrate buffer described in Section A, a sucrose density gradient was established by weight/volume percent in the flowing layers as follows: No. 4, 1.0%; No. 5, 1.5%; No. 6, 2.0%; No. 7, 2.5%; No. 8, 3.0%; No. 9, 4.5%; No. 10, 4.8%; No. 11, 5.1%; No. 12, 5.4%; and No. 13, 5.7%. Layers 1-3 contained a higher conductivity medium resulting from a glycine-citrate content approximately 3 times that of layers 4-12. Layers 14-16 contained the

STAFLO FLOW-CELL; UPWARD ELECTROPHORESIS OF FOWL SPERM
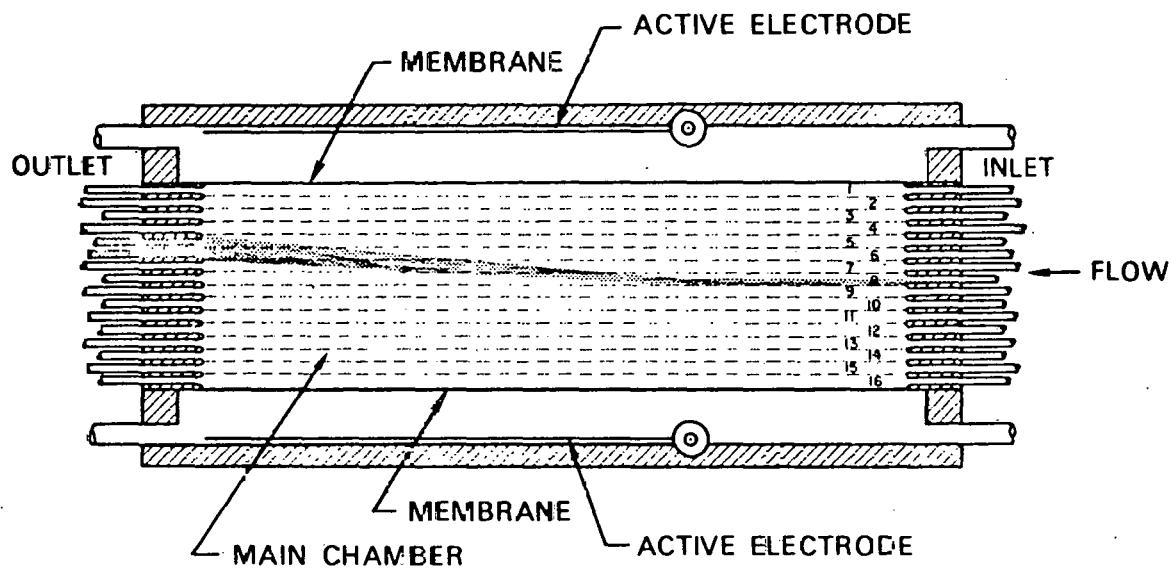
DBL 688-5417

Fig. 48. Schematic diagram of Staflo flow-cell illustrating fowl spermatozoa upward-electrophoresis migration pattern.

THREE-DIMENSIONAL STAFLO MIGRATION PATTERN
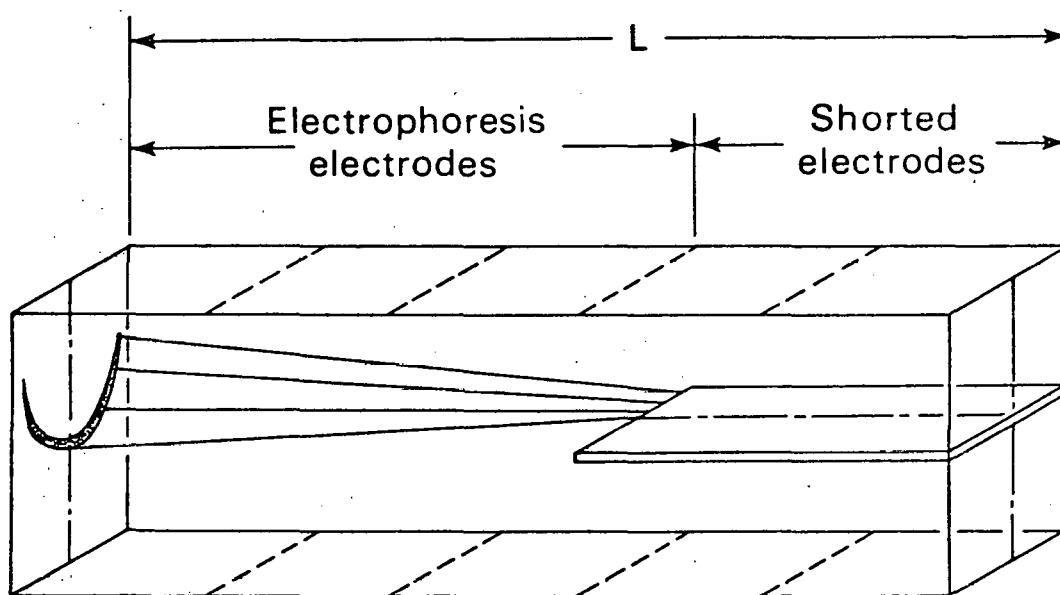
DBL 688-5381

Fig. 49. Schematic diagram showing ideal three-dimensional Staflo upward-electrophoresis migration pattern of fowl spermatozoa.

same medium with 6% sucrose. The resulting top and bottom "conductivity

barriers" buffer the intermediate layers against changes in pH during the

experiment and confine the migrating species to the intermediate layers

of the flow-cell[159]. Initial discontinuities between layers are smoothed

by gradient-stabilizing diffusion of sucrose during the first 1/3 (10 cm)

of the flow-chamber prior to reaching the region of the "programmed"

electric field[229]. The spermatozoa suspension was introduced into layer

8 and was initially supported by the "density shelf" provided by the sucrose

increment from layer 8 to 9.

d. <u>Temperature Control</u>. All experiments were conducted in a refrigerated

room with good temperature regulation. Temperature measurements inside

the flow-chamber during electrophoresis have shown the average temperature

rise to be less than 0.5°C under the operating conditions used in these

experiments.

e. <u>Observation and Evaluation of Spermatozoa</u>. The gross electrophoresis

pattern was observed by eye, using the "slit lamp technique"[229]. Individual

spermatozoa and the microscopic steady-state pattern were observed with a

special microscopic optical system developed for use with the Staflo appar-

atus (Chapter III). Immediately after each run, the spermatozoa concentra-

tion in each collection container was determined with a hemocytometer. The

spermatozoa in each fraction were reconcentrated by centrifugation in the

cold room and subsequently evaluated at room temperature for motility and

viability (nigrosin-eosin staining[109]). The quality of motility was judged

on a scale of I-VI ranging from essentially no motility (I) to a vigorously

swarming pattern (VI). The nigrosin-eosin stained spermatozoa were cate-

gorized as unstained (viable) or stained (non-viable). The spermatozoa

from each fraction and from the control sample were inseminated into sep-

arate groups of White Leghorn hens between 1300 and 1500 hours on the

day of the experiment.

3. Results

Two groups of electrophoresis experiments are reported. The first group was conducted at 4°C or lower to minimize the effect of intrinsic motility on migration. The second group of experiments was conducted at 5.5°C in a deliberate attempt to achieve enrichment of one collection fraction with regard to motile spermatozoa. Figure 48 illustrates the migration pattern (overall side-view appearance of a continuously flowing sample stream in the flow-chamber) observed in both sets of experiments. The paraboloid migration profile (cross-section of the migration pattern) observed with the "slit lamp technique" is illustrated in Figure 49. Vertical migration distances measured in the longitudinal centerline by this technique were used to calculate the electrophoretic mobility values.

a. Electrophoresis Experiments. General observations during the series of experiments at temperatures of 4°C or lower indicated that 1) the migration patterns determined by the "slit lamp technique" were essentially the same at spermatozoa concentrations of 25 x 10^6 and 250 x 10^6/ml; 2) in all experiments, the height of the migration profile at the outlet in the centerline of the flow-chamber was less than 2.0 mm, thereby giving a 2-layer fractionation of the sample; 3) at pH 7.0 and with the top electrode positive, all spermatozoa were oriented vertically with heads down and tails upward; and 4) at a flow-chamber temperature of 4°C, a high percentage of individual spermatozoa showed a moderate degree of tail-wagging.

Despite an attempt to effect an even division of the sample between the 2 layers at the outlet, the upper collection fraction generally had a lower spermatozoa concentration than did the bottom one. In all experiments, the percentage and degree of motility in the bottom fraction were consistently

greater than in the upper one (though less than in the control sample).
In one experiment, the upper fraction, containing only 10% of the sperm-
atozoa, at a concentration of 10 x 10^6/ml had only 1-2% motile spermatozoa
as compared to about 70% motility in the bottom fraction. When comparable
numbers of spermatozoa were collected in the 2 fractions, for experiments
at 4°C or lower, there were no significant differences in viability
(staining) among the 2 fractions and the control spermatozoa. Even though
the spermatozoa fractions in several experiments had Grade II or III
motility, insemination of 150-200 x 10^6 spermatozoa in 0.15 ml failed to
give any fertile eggs. Control inseminations with Grade III or IV sperm-
atozoa did give fertile eggs. Possible reasons for the loss of fertilizing
capacity of the spermatozoa in the collection fractions in this first group
of experiments are considered in the Discussion.

The average and ranges of apparent electrophoretic mobilities for
one pair of electrophoresis runs at 4°C with the same Staflo operating
conditions but differing spermatozoa concentrations are shown in Table
XIV.

Table XIV. Apparent Electrophoretic Mobilities of Fowl
Spermatozoa at 4°C.

| Spermatozoa Concentration | Apparent Electrophoretic Mobility [Units of 10^{-4}(cm/sec)/(V/cm)] | |
	Average	Range
25 x 10^6/ml	1.56	1.50 - 1.61
250 x 10^6/ml	1.69	1.50 - 1.79

b. **Electrophoresis-Enrichment Experiments.** Two enrichment experiments
were conducted using upward electrophoresis at 5.5°C. Figure 50 is a photo-
graph of the steady-state migration pattern during one of these experiments.
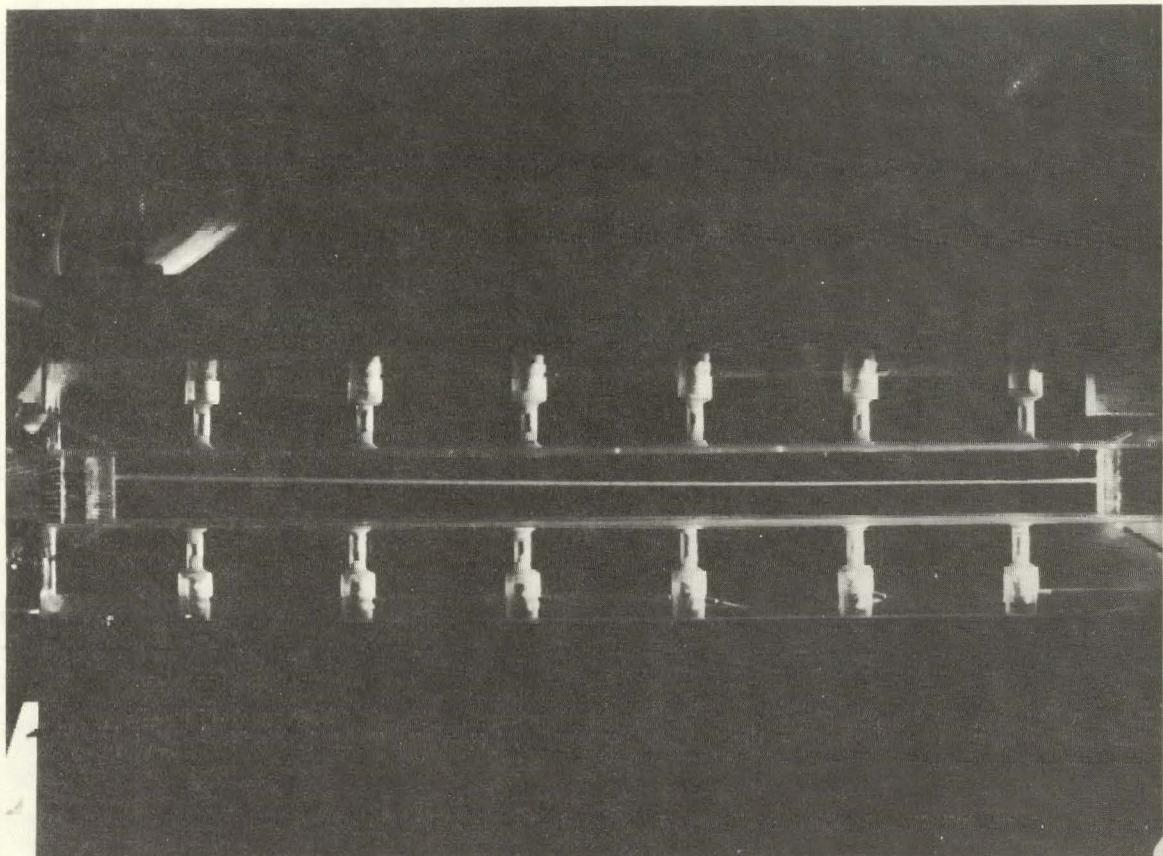
XBB 7011-5006

Fig. 50. Photograph of upward-electrophoresis migration pattern with
fowl spermatozoa in a 3 x 16 Staflo apparatus enrichment experi-
ment at 5.5°C.

Again, because the pattern spread was small, only 2 fractions were obtained (containers 5 and 6). The spermatozoa in the fractions were reconcentrated by centrifugation for 5 minutes at 1,400 g at 5°C prior to evaluation at room temperature, and artificial insemination. The same 3 groups of 6 hens each were inseminated on each occasion with the respective collection fractions or control spermatozoa. Eggs were collected from the day after the first insemination until 4 days after the second insemination (a total of 8 days). The fractionation results with respect to spermatozoa distribution, motility, viability, fertility and hatchability are summarized in Table XV.

In Table XV, it is seen that fraction 6 has both a higher concentration and a better quality of spermatozoa than fraction 5, judged by motility and viability. In addition, the percentages of fertility and hatchability for fraction 6 are significantly higher than those for fraction 5 (P $<$ 0.01 in a one-tail test[90]). There was no difference between fraction 6 and the control with regard to motility, viability, fertility or hatchability. Following one of these experiments, which used a concentration of 250 x 10^6 spermatozoa/ml, a comparison run was made using 1/10 the initial concentration. Although the pattern spread was slightly greater for the higher concentration, the average migration distances were the same. The mobility, calculated from the average migration distance was slightly greater ($\approx$2 x 10^{-4} cm/sec/V/cm at 5.5°C) than in Table XIV. However, the measurements in Table XIV are considered more reliable because the migration distances involved were greater.

4. <u>Discussion</u>

a. <u>Technical Considerations</u>. In view of the report by Proudfoot and Stewart[189] that the fertilizing capacity of fowl spermatozoa did not appear

Table XV. Average Enrichment-Fractionation and Artificial Insemination
Results of Two Electrophoresis Experiments with Fowl Spermatozoa at 5.5°C.

	Fraction 5	Fraction 6	Control
Sperm Conc. (units of 10^6/ml)	87	140	1500
Percent of Sperm Collected:			
Average	38.3	61.7	-
Range	36.2-39.8	60.2-63.8	-
Motility (grade)	II	IV	IV
Viability (unstained) (%)	77	90.5	91.8
Artificial Insemination:			
Volume (ml)	0.15	0.15	0.10
No. of Sperm (units of 10^6)	145	170	150
Evaluation of Eggs:			
Total Number	39	46	46
Number Fertile	5	26	25
Percent Fertile	12.8	56.6	54.4
Number Hatchable	3	19	21
Percent Hatchable	7.7	41.3	45.7

to be affected by a rapid temperature fall during collection, cooling from room temperature to 3°C was accomplished in 45 minutes in these experiments as compared to 2 hours in Section A. The fertilization rates in Table XV are comparable to those in the dilution experiments in Section A and confirm the report that rapid cooling is not detrimental.

Another consideration to bear in mind in comparing the results of Sections A and B is the 25 - 45 minute shorter centrifugation time used in the present experiments. This reduction was possible in part because the collection fractions contained only 8 - 10 ml compared to the 15-20 ml volumes used in Section A.

It was noted that for several of the earlier "electrophoresis experiments" no fertile eggs were obtained following insemination of adequate numbers of reconcentrated spermatozoa of Grade II or III motility. Control inseminations, however, gave fertile eggs. Furthermore, in the subsequent "electrophoresis enrichment experiments", a fertilization rate of 56.6% was achieved from Staflo collection fraction 6 as compared with the 54.4% control value. It is possible that the loss of fertilizing capacity (but not motility) in the early experiments was due to trace contamination of the apparatus either by Roccal (benzalkonium chloride) used in cleaning or by a toxic substance associated with new polyethylene tubing.

b. Electrophoretic Mobility Measurements. Even though there was good agreement between the average values of electrophoretic mobility for the similar pairs of high and low concentration runs, the range of mobilities was somewhat greater for both high concentration experiments. The greater spread in the steady-state migration pattern of the high concentration runs may reflect a small degree of density gradient instability. (Alternatively, this may be a consequence of the relative difficulty in defining

the limits of the low concentration migration pattern). Only having a slight instability at a concentration as high as 250 x 10^6 spermatozoa/ml is remarkable in view of the threshold for instability of 25 x 10^6/ml for rabbit spermatozoa in other density gradients (Chapters IV, V and VIII). The increased stability in the fowl spermatozoa experiments may be attributed to 1) a larger increment in the density gradient between layers (0.5% sucrose/layer vs. 0.10 - 0.15% sucrose/layer for rabbit spermatozoa experiments) and 2) a lower sedimentation rate for fowl spermatozoa[35].

In addition to the apparent electrophoretic mobility of 1.69 x 10^{-4} cm/sec/V/cm for the 250 x 10^6/ml spermatozoa sample shown in Table XIV, an average value of 1.52 x 10^{-4} cm/sec/V/cm was obtained in another experiment under similar conditions, at 4°C. When these values are normalized to conditions of a 1% sucrose density gradient at 3°C, they become 1.75 x 10^{-4} and 1.57 x 10^{-4} cm/sec/V/cm, respectively. These latter values compare favorably with the 1.68 x 10^{-4} cm/sec/V/cm observed in a downward electrophoresis experiment using the glycine-phosphate buffer described in Section A, corrected to 1% sucrose at 3°C (Chapter V). They also agree well with the value of 1.55 x 10^{-4} cm/sec/V/cm reported by Bey, also in a low ionic strength medium[251].

It is difficult to evaluate precisely the relative electrophoretic mobilities of live and dead spermatozoa because even at 4°C, a mild degree of tail-wagging was observed in the flow-chamber. It was noted that in such low temperature experiments, where substantial numbers of spermatozoa were found in both collection fractions, there was no difference between them in staining properties. In contrast, in the enrichment experiments at 5.5°C, a significant difference in viability was observed between fractions 5 and 6 as shown in Table XV. This difference probably results from the

greater degree of intrinsic motility of (viable) spermatozoa at 5.5°C.

The electrophoretic mobility of immotile (non-viable) spermatozoa at 4°C can be estimated from the experiment in which the upper collection fraction contained 99% immotile spermatozoa, at a concentration of 10 x 10^6/ml. Because it was shown in Section A that concentrations as low as 8 x 10^6/ml could be tolerated for up to 6 hours at 3°C, it is probable that the spermatozoa were immotile at the beginning of the experiment. The average electrophoretic mobility of these immotile spermatozoa was 1.67 x 10^{-4}cm/sec/V/cm compared to average and minimum apparent mobility values of 1.52 x 10^{-4} and 1.36 x 10^{-4}cm/sec/V/cm for the whole sample. Theoretically, at least, the minimum value represents the slowest electro-phoresing-fastest swimming spermatozoa. In order to define the true con-tribution of motility to the net migration, experiments should be conducted at temperatures ranging from less than 1°C to about 15°C, and with lower spermatozoa concentrations to insure that the migration is not influenced by any kind of density gradient instability.

c. <u>Relative Enrichment.</u> Because of the superior quality of the initial spermatozoa suspensions (Table XV), absolute enrichment of a collection fraction, compared to the control sample, was not achieved. However, with an initial sample of about 50% motile spermatozoa rather than 90%, especially if a slightly higher temperature and/or greater migration distance is used, absolute enrichment would be expected. Such enrichment has been obtained in experiments with rabbit spermatozoa (Chapter IV). It is significant that despite the additional stresses of dilution, electrophoresis and cen-trifugation, the spermatozoa in collection fraction 6 were as good as the control sample by all of the criteria in Table XV.

Because the minimum spermatozoa concentration encountered in the en-

richment experiments was comparable to those at which motility and

fertilizing capacity were shown to be maintained in Section A, all of the

differences between fractions 5 and 6 in Table XV are most likely a con-

sequence of intrinsic motility. Thus, at 5.5°C in the glycine-citrate

medium, the downward oriented intrinsic motility vector is believed to

have been of sufficient magnitude to give the depletion in layer 5 and

the relative enrichment in layer 6 with regard to all of the criteria

enumerated in Table XV.

The fertilization rate of 56.6% obtained with spermatozoa from

collection fraction 6 in the enrichment experiments is essentially the

same as the 58.8% from spermatozoa diluted 1:30 in glycine-citrate buffer,

stored at 3°C for 1 1/2 hours and reconcentrated by centrifugation prior to

insemination (Section A, Table XIII). The difference between the respective

control fertilization rates of 54.4% and the 63.2% in the 2 studies was

also not significant (P$\gg$0.10). Therefore, it can be said that Staflo

electrophoresis has not adversely affected the motility and fertilizing

capacity of the spermatozoa.

The combined results of Sections A and B have shown that analytical

experiments can be conducted at 3°C using spermatozoa concentrations as low

as 8 x 10^6/ml, and that the results can be exploited in Staflo electro-

phoresis or electrophoresis-enrichment experiments. A natural extension

of the present fractionation studies is the evaluation of the fertilizing

ability of sub-populations of spermatozoa characterized by differing degrees

of intrinsic motility, obtained by differential fractionation in Staflo-

electrophoresis-motility or sedimentation-motility experiments. With

regard to fowl spermatozoa, this extension would be of particular interest

in studying the "translocation heterozygote" spermatozoa. For mammalian

spermatozoa, there is the additional possibility of obtaining information on the relationship of motility to the sex ratio of offspring.

C. Summary and Conclusions

Although both egg yolk medium and vitalizing medium preserved the motility of fowl spermatozoa during storage for 2 hours at 4°C, vitalizing medium was far superior in preserving the fertilizing capacity (80% vs. 9.1% for egg yolk medium).

310 mosm solutions of low ionic strength glycine-citrate and glycine-phosphate buffers were far superior to 368 and 427 mosm solutions in the preservation of motility during storage at 3°C for spermatozoa concentrations of 8-80 x 10^6/ml. After storage for 6 hours in the 310 mosm solutions, there was only a 15% decrement from initial motility values at all concentrations. The percentages of motility were much lower in the 368 and 427 mosm solutions.

In regard to the preservation of fertilizing capacity there was no difference between the glycine-citrate and glycine-phosphate buffers after storage at 1:30 dilutions for 1 1/2 hours at 3°C. In a second study, the preservation of fertilizing capacity of spermatozoa after storage at 1:30 dilutions in glycine-citrate medium for 1 1/2 hours at 3°C, followed by reconcentration by centrifugation, was the same as that of spermatozoa diluted 1:2 in vitalizing medium and stored under the same time-temperature conditions without further handling. This suggested that fowl spermatozoa could be subjected to the stresses of low temperature, low concentration and centrifugation expected in Staflo electrophoresis experiments, with low ionic strength media, and still maintain their vital functions. This was confirmed in the enrichment-electrophoresis experiments where a fertilization rate of 56.6% was obtained with spermatozoa in the lower collection

fraction, compared to 54.4% for the control.

The electrophoresis experiments showed the same average electrophoretic mobilities for fowl spermatozoa at concentrations of 25×10^6 and 250×10^6/ml and showed comparable mobilities for upward and downward electrophoresis. The higher concentration gave an apparently slightly greater spread of the migration pattern with associated greater range of electrophoretic mobilities. The average apparent electrophoretic mobility for fowl spermatozoa in these experiments is about 1.6×10^{-4} (cm/sec)/(V/cm). This value compares favorably with those obtained in Chapter V (Table X).

At a flow-chamber temperature of 5.5°C, relative enrichment in the lower of 2 collection fractions, with an upward-electrophoresis migration pattern, was obtained with regard to motility, viability, fertilizing capacity and hatchability. The enrichment is attributed to the intrinsic motility of the spermatozoa at that temperature. Because of the high quality of the initial spermatozoa sample, the lower fraction did not differ from the control, according to these criteria.

These studies showed that the motility of fowl spermatozoa could be well preserved after storage for 6 hours at 3°C in concentrations as low as 8×10^6/ml in low ionic strength media - conditions desirable for analytical electrophoresis studies. Furthermore, the excellent fertilization results with "Staflo spermatozoa" in the enrichment experiments show that information learned in analytical studies can be exploited in preparative electrophoresis experiments using the Staflo apparatus.

VII. EQUILIBRIUM DENSITY GRADIENT CENTRIFUGATION:
RABBIT AND FOWL SPERMATOZOA

Contents

A. Introduction

The review of the literature on the centrifugation of spermatozoa

in Chapter II, A, 4, summarizes the work by many investigators to deter-

mine the apparent buoyant density of bull and rabbit spermatozoa. There

were marked differences in the reported specific gravities determined in

different density gradients. In order to better understand the behavior

of rabbit and fowl spermatozoa in Staflo sedimentation and sedimentation-

electrophoresis experiments, the work in this chapter was done to deter-

mine the magnitude and range of specific gravities of both rabbit and

fowl spermatozoa. At the time this work was done (summer, 1966), the reports by Benedict, Schumaker and Davies[32], and by Lavon, Volcani, Amir and Danon[132,133] had not been published.

This chapter presents results of equilibrium density gradient centrifugation experiments with rabbit and fowl spermatozoa using both dextran and sodium iothalamate density gradients. The studies showed both similarities in the specific gravity of fowl and viable rabbit spermatozoa and marked differences in the properties of the spermatozoa of the 2 species when under the influence of osmotic stress.

B. Materials and Methods

1. Media and Density Gradients

Bovine serum albumin (BSA), dextran (Sigma Chemical Company, MW 15-20,000; 73,000 and 215,000), Ficoll and several radiographic contrast media were evaluated for use in equilibrium density gradient centrifugation. In general, sufficiently dense solutions of BSA, Ficoll and dextran could not be prepared without encountering limiting degrees of viscosity. The work reported below was accomplished with density gradients prepared with dextran or sodium iothalamate (Angio-Conray or Conray-400) in 0.3% or 0.9% NaCl. Because the motility of both fowl and rabbit spermatozoa was not maintained well in 0.3% saline-sodium iothalamate solutions, 0.9% saline solutions were used for most experiments. The effects of radiographic contrast media on the motility of fowl spermatozoa and on the motility and fertilizing capacity of rabbit spermatozoa are reported in Appendix D.

2. Centrifugation

Linear density gradients were preformed at room temperature with a gradient machine using dextran or sodium iothalamate in normal saline.

The centrifuge tubes then were cooled to approximately 3°C in a refriger-
ated room. Spermatozoa, which had been collected and cooled slowly to
3°C as described in Chapter III, were suspended in a gradient material
solution slightly less dense than the top of the linear density gradient.
The suspension was layered on the density gradient and the boundary was
stirred carefully to eliminate the interface. Centrifugation was accom-
plished at 4°C with the Beckman Model L Ultra-centrifuge using the SW39
swinging-bucket rotor. After centrifugation, the tubes were examined in
the cold room where the migration bands were measured, photographed and
fractionated by pipetting. After removal from the centrifuge tubes,
spermatozoa in the separate bands were resuspended in approximately 25 ml
of a 0.5% glucose-normal saline solution and were centrifuged for 15
minutes at 3,300 rpm in a clinical centrifuge. The pellet of spermatozoa
was resuspended in egg yolk medium (see Chapter VI) and evaluated for
motility and vital staining characteristics. The total time of exposure
of the spermatozoa to the sodium iothalamate, when it was used, ranged
from 25-35 minutes, approximately 20 minutes longer than the centrifugation
time.

3. Evaluation of Spermatozoa

Spermatozoa were evaluated by phase contrast microscopy and by
nigrosin-eosin staining as described in Appendix B. No artificial insem-
ination studies were done in the experiments described in this chapter.

C. Results

1. Dextran Gradient Experiments

a. Fowl Spermatozoa. In preliminary experiments using a linear density
gradient of 15-20,000 MW dextran and a force of 1,500 g in an International
Centrifuge, a centrifugation time of nearly 60 minutes was required for

fowl spermatozoa to reach equilibrium. Fowl red blood cells, on the other

hand, reached equilibrium in 15 minutes or less. The apparent specific

gravities for spermatozoa and red blood cells were 1.15-1.17 and 1.14,

respectively.

b. <u>Rabbit Spermatozoa</u>. Centrifugation of 2 samples of rabbit spermatozoa

in linear density gradients of 15-20,000 MW dextran in a phosphate buffered

medium gave low-density bands corresponding to a specific gravity range

of 1.16-1.21. Spermatozoa also were found at the bottom of the centrifuge

tube which corresponded to a specific gravity of 1.23. Because some

spermatozoa appeared to have a specific gravity greater than the maximum

specific gravity of the solution (1.23) and the viscosity of this dextran

solution was very high, sodium iothalamate was used in subsequent studies.

2. <u>Sodium Iothalamate Gradient Experiments</u>

a. <u>Fowl Spermatozoa</u>. The results of the centrifugation of fractions

of individual ejaculates from White Leghorn cocks are presented in Figure

51 and Table XVI. Figure 51A shows that, with centrifugation times of 5,

9, and 15 minutes at 5,800, 6,600 and 6,700 g, respectively, the apparent

specific gravity of spermatozoa from the same ejaculate increased pro-

gressively to a maximum range of 1.19-1.21 at the 15 minute centrifugation

time. The last 4 ranges of apparent specific gravities shown in Figure

51 A agree within the accuracy of measurements in these experiments. Figure

51 B shows the results of 3 centrifugation runs with the same fowl sperm-

atozoa sample using a density gradient prepared with sodium iothalamate in

0.3% saline. The 0.3% saline was used to reduce the osmolarity of the

linear density gradient. There appears to be a slight increase in the

apparent specific gravity with an increase in centrifugation time from

6 1/4 to 10 3/4 minutes but no further increase with time. The maximum

range of specific gravities is 1.19-1.20, slightly less than in the gradient

RANGES OF APPARENT SPECIFIC GRAVITIES OF FOWL SPERMATOZOA

MEDIUM	CENTRIFUGATION		SPECIFIC GRAVITY				
	(min)	g (avg)	1.14	1.16	1.18	1.20	1.22
A. Normal saline + sodium iothalamate	5	5,800					
	9	6,600					
	15	6,700					
	10*	8,000					
	11	10,000					
	16	10,000					
B. 1/3 normal saline + sodium iothalamate	6¼	8,900					
	10¾	9,100					
	15¾	9,100					

*2 tubes

Fig. 51. Ranges of apparent specific gravities of fowl spermatozoa in sodium iothalamate density gradients in A) normal saline and B) one-third normal saline background solutions.

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Table XVI. Ranges of Apparent Specific Gravities of Fowl Spermatozoa
in Sodium Iothalamate Density Gradients.

Centrifugation		Specific Gravity	
Time (min)	g (av.)	Range	Average
A. Normal Saline			
5	5,800	1.158-1.189	1.174
9	6,600	1.176-1.197	1.187
15	6,700	1.192-1.210	1.201
10 (2)	8,000	1.201-1.212	1.207
11	10,000	1.195-1.205	1.200
16	10,000	1.195-1.205	1.200
B. 1/3 Normal Saline			
6 1/4	8,900	1.187-1.196	1.192
10 3/4	9,100	1.192-1.200	1.196
15 3/4	9,100	1.192-1.201	1.197

using 0.9% saline.

Fowl spermatozoa gave a continuous band in the density gradient, generally 2-4 mm wide. The narrowest bands were obtained with the highest quality ejaculates and, after centrifugation and washing, most spermatozoa still were intact and had a high degree of motility. With poor quality ejaculates, a differential distribution of spermatozoa and component parts thereof was observed. Above the top of the discrete band, there were sperm tails. In the top of the band, spermatozoa were intact and had excellent motility. In the bottom of the band, spermatozoa showed signs of deteriorating and had either a poor degree of motility or were immotile. At the bottom margin of the band and slightly below, all spermatozoa were deteriorating or were represented by tails with remnants of heads. Deterioration was manifested first by swelling of the sperm head to 2-3 times its normal width, later by the formation of bead-like masses of nuclear material and finally by scattering of the bead-like material, leaving the tail with a knob projecting toward the former head region. Because of the fragility of the fowl spermatozoa heads, no intact heads were ever observed separated from tails. Swelling and disintegration of fowl spermatozoa heads was also observed after storage for 24 hours or more in egg yolk and other media, suggesting that this deterioration is a general phenomenon. Distortion of this differential distribution was observed in the presence of clumping for it appeared that viable spermatozoa were carried to the lower margin of the band by clumps. No clumps were ever observed in the upper part of the band.

In fresh ejaculates of poor quality, several percent of the spermatozoa were bent in the vicinity of the neck piece, giving a hairpin shape. In centrifuged samples, the frequency of this abnormality was

greatly increased. Despite this damage, many such individual spermatozoa often had a high degree of motility.

b. Rabbit Spermatozoa. The results of rabbit spermatozoa experiments are summarized in Figure 52 and Table XVII. Three or more discrete bands were observed in each experiment. Spermatozoa from the same ejaculate were used in the first 3 experiments in Figure 52 (and Table XVII). It is seen that the apparent specific gravity of the upper band increases with increasing centrifugation time until a range of 1.195-1.210 is reached. This range was observed even with centrifugation for 90 minutes at 27,000 g. The middle band appears to plateau at a specific gravity range of 1.26-1.29. There is a suggestion that the apparent specific gravity of the lower band continues to increase over the range 1.31-1.37, but the formation of sub-bands in that range complicates interpretation thereof.

In addition to the differences in specific gravity, there was a marked difference in the quality of the spermatozoa in the 3 bands. Table XVIII A summarizes the results of one experiment in which the upper and lower bands were at the top and bottom of the density gradient. There is a dramatic decrease in the quality of the spermatozoa, judged by vital staining, morphology and motility, from top to bottom. By all criteria, the upper band was enriched with regard to the initial sample. This differential distribution is confirmed by the phase contrast observations of morphology and motility for a second experiment, as shown in Table XVIII B. Again, there is a marked difference between the upper and lower bands and absolute enrichment of the upper band.

D. Discussion

The results of the experiments reported above are considered in terms

RANGES OF APPARENT SPECIFIC GRAVITIES
OF RABBIT SPERMATOZOA

CENTRIFUGATION		SPECIFIC GRAVITY				
(min)	g (avg)	1.15	1.20	1.25	1.30	1.35
		UPPER BAND		MIDDLE BAND	LOWER BAND	
5	5,800	—		———	—	
9	6,600	—		——	—	
15	6,700		—	——	— —	
11	40,000	—		—	— ——	
90	27,000	—		—— ·····	——	

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Fig. 52. Ranges of apparent specific gravities of rabbit spermatozoa in a sodium iothalamate density gradient.

Table XVII. Ranges of Apparent Specific Gravities of Rabbit Spermatozoa in a
Sodium Iothalamate Density Gradient in 0.9% Saline.

| Centrifugation | | Specific Gravity | | | | | |
| Time (minutes) | g (av.) | Upper Band | | Middle Band | | Lower Band | |
		Range	Average	Range	Average	Range	Average
5	5,800	1.176-1.189	1.183	1.258-1.294	1.276	1.322-1.330	1.326
9	6,600	1.183-1.197	1.190	1.262-1.285	1.274	1.323-1.337	1.330
15	6,700	1.204-1.211	1.208	1.267-1.293	1.280	1.312-1.322	1.317
						1.335-1.350	1.343
11	40,000	1.195-1.206	1.201	1.283-1.290	1.287	1.320-1.330	1.325
						1.342-1.366[a]	1.354
90	27,000	1.195-1.208	1.202	1.270-1.286	1.278	1.332-1.358[a]	1.345
				1.290-1.310[b]	1.300		

a. A very small number of spermatozoa were slightly lower in the gradient.

b. Second part of band was thinly scattered.

Table XVIII. Viability and Morphology of Rabbit Spermatozoa
in a Linear Density Gradient.[a]

| Sample | Percent of Sperm | Nigrosin-Eosin Staining | | | Phase Contrast | | | |
		U (%)	MNS (%)	S (%)	C (%)	CL (%)	FM (%)	TW (%)
A. Specimen #1								
Upper band	44.0	94.5	4.5	1.0	94	6	11.6	29.5
Int-band 1)	3.4	65.5	18.2	16.3	68.2	31.8	0	1
Int-band 2)	2.5	22	16	62	53.2	46.8	0	0
Lower band	50.1	6.0	13.5	81.5	15.5	84.5	0	0
Initial Sample	100	37	12	51	65.2	34.8	7.5	17.5
B. Specimen #2								
Upper band	51.2				99	1	0	90
Int-band	18.2				31	69	0	8
Lower band	30.6				7	93	0	3
Initial Sample	100				62	38	5-10	50-60

a. See Glossary for explanation of abbreviations

of (1) technical aspects of equilibrium density gradient centrifugation,

(2) a comparison of fowl and rabbit spermatozoa centrifugation results,

(3) a comparison with Staflo sedimentation results and (4) a comparison

with the work of other investigators.

1. Technical Considerations

As shown in Figures 51 and 52, when sufficient time and centrifugal

force are used to assure that equilibrium is reached, reproducible results

are obtained with both rabbit and fowl spermatozoa. There were no signi-

ficant variations in results using spermatozoa from different male rabbits

or from different cocks.

The mechanical preparation of the linear density gradient was uncom-

plicated with the low viscosity contrast media, but difficult with high

concentrations of dextran because of the increased resistance to flow

through the small bore tubing of the gradient former. The high viscosity

of dextran density gradients also significantly increased the time required

for fowl spermatozoa to reach equilibrium. This is due primarily to the

extreme axial ratio (f/f_0^{204}) from the length of approximately 150 microns

and thickness of 0.5 microns. The high viscosity also made it difficult to

remove spermatozoa from the centrifuge tubes. In addition to these problems

due to viscosity, the dextran density gradient did not give a sufficiently

high specific gravity to study the most dense rabbit spermatozoa. Therefore,

sodium iothalamate was used for the equilibrium centrifugation of both rabbit

and fowl spermatozoa because solutions of high specific gravity and low vis-

cosity could be prepared readily.

2. Comparison of Fowl and Rabbit Experiments

The most striking difference between the results of equilibrium cen-

trifugation of fowl and rabbit spermatozoa in the sodium iothalamate

density gradient is the formation of a single band with the former compared

to 3 or more bands with the latter. As shown in Tables XVI and XVII, the apparent specific gravity of fowl spermatozoa ranges from 1.192-1.212 which is approximately the same as the range of 1.195-1.211 for the low-density band of rabbit spermatozoa. Thus, the similarity applies only to the highest quality rabbit spermatozoa.

Even though there was only 1 band of fowl spermatozoa in the centrifuge tube, the difference in the quality between the top and the bottom of that band suggested a differential distribution of viable and non-viable cells. The observation of deteriorating fowl spermatozoa at the high density border of the band suggests that either (1) disintegration occurs before further migration takes place or (2) swelling decreases the specific gravity and arrests migration. The finding of tails slightly below the band, as well as above it, suggests that non-viable spermatozoa incorporated some sodium iothalamate, migrated to the lower margin of the band, remained intact (although swollen) during centrifugation and then disintegrated during the pipetting and washing of the fractions of the migration band. These observations show that fowl spermatozoa are quite fragile compared to rabbit spermatozoa, which maintain their physical integrity, except for loss of the acrosomal cap, even after loss of viability.

In all experiments using the sodium iothalamate density gradients, rabbit spermatozoa segregated into at least 3 major bands characterized by different ranges of specific gravities. The marked differences in the quality of the spermatozoa in the 3 bands, as shown in Table XVIII, suggests that their formation is dependent primarily upon the relative viability of the spermatozoa. Inasmuch as the spermatozoa were immobilized both by the low temperature and by the high concentration of sodium iothalamate, there was no influence of motility on the distribution. It is easy to postulate a low-density group consisting of viable spermatozoa

which are affected uniformly to a slight degree, or not at all, by the sodium iothalamate, and a high-density group consisting of non-viable spermatozoa which experience a marked increase in apparent specific gravity due to the hyperosmolar environment.

Mechanisms by which hyperosmolar solutions of low molecular weight substances used in density gradients might modify the apparent specific gravity of spermatozoa include the following: (1) simple dehydration, (2) attachment of the molecules to the membranes of both viable and non-viable cells and (3) selective penetration of the cells depending upon their relative viability. In regard to dehydration, the estimated difference of about 100 mosm between the 0.3% and 0.9% saline-iothalamate solutions gives a difference in the average specific gravity of only 0.004 in Table XVI. This is much less proportionately (2%) than the 15-20% change in the osmolarity and suggests a non-linear relationship of the 2 factors. There is a suggestion of dehydration also because of the difference in the apparent specific gravity range of 1.190-1.210 in the sodium iothalamate gradient compared to the range of 1.15-1.17 observed in the dextran density gradient.

The formation of a discrete intermediate band of rabbit spermatozoa suggests the loss of 1 or more selective mechanisms functioning in the viable cells to exclude iothalamate, but not a state of complete penetrance as in the spermatozoa in the lower band. This phenomenon may be related to the observation in nigrosin-eosin stained preparations of morphologically normal but stained spermatozoa in which the post-nuclear cap region is stained, while the remainder of the head is unstained. It is possible that attachment of or penetration by the iothalamate molecules in that region of the spermatozoon could give a population of cells with a relatively

discrete intermediate range of specific gravities. The analogy with vital staining is not complete for viable rabbit spermatozoa take up eosin at 4°C, whereas, if they do take up some iothalamate, they are more selective than non-viable cells. Benedict and co-workers report no difference in the apparent specific gravity at 5°C and 37°C, suggesting that the influence of certain low molecular weight substances on the specific gravity of rabbit spermatozoa is not temperature dependent[32].

3. Correlation with Staflo-Sedimentation Studies

In Staflo-sedimentation experiments reported in Chapter VIII, a range of sedimentation velocities from 0-1 x 10^{-4} cm/sec was observed for rabbit spermatozoa in a sucrose density gradient. The distribution of spermatozoa observed in those experiments suggested a uniform distribution of sedimentation velocities among the spermatozoa population. This is in contrast to the suggestion of a trimodal distribution of sedimentation velocities in these equilibrium centrifugation experiments. Because of the short migration distances in the Staflo-sedimentation experiments, overlap among several different-density groups of spermatozoa could have occurred, giving the appearance of a uniform distribution. Nevertheless, the formation of 3 discrete bands in the linear density gradient is attributed to a differential vulnerability of viable and non-viable cells to penetration by sodium iothalamate rather than to distinct groups of spermatozoa with different specific gravities.

Bhattacharya reported partial bimodal distributions of rabbit and bull spermatozoa and a unimodal distribution of fowl spermatozoa in sedimentation experiments[35]. Bedford and Bibeau observed a suggestion of a bimodal distribution of rabbit spermatozoa using a similar apparatus, but most distributions could be interpreted as unimodal or uniform[29]. Schilling

observed a unimodal distribution of bull spermatozoa using a similar device but different medium from that used by Bhattacharya[205]. The disagreement among many investigators using similar equipment shows that there is still some question as to the sedimentation properties of both rabbit and bull spermatozoa. The results of these centrifugation experiments illustrate a need for considering the effects of the medium on the sedimentation as well as centrifugation properties of rabbit and other spermatozoa.

4. Comparison with Work by Other Investigators

Table XIX summarizes the wide range of specific gravities of rabbit and bull spermatozoa reported by other investigators and also includes my results. As shown in Table XIX, Benedict, Schumaker and Davies obtained 3 bands of rabbit spermatozoa in a linear density gradient prepared with umbradilic acid[32]. They observed a wider low-density band which overlapped the one I obtained. The intermediate bands were very similar and I observed a greater specific gravity for the higher density spermatozoa. I did not observe a splitting of the low-density band of rabbit spermatozoa, as reported by Benedict and co-workers, in any of my experiments.

The range of 1.09-1.18 for the specific gravity of rabbit spermatozoa reported by Beatty using high molecular weight dialyzed colloidal silica or dextran solutions[20], is significantly less than the range observed by Benedict, et al[32] and me with low molecular weight substances. As noted earlier, using a density gradient prepared with 15-20,000 MW dextran, I observed a specific gravity range of 1.16-1.21 for the low-density band of rabbit spermatozoa. Inasmuch as many spermatozoa formed a pellet at the bottom of the centrifuge tube, it appears that some spermatozoa had a specific gravity greater than 1.23. Unfortunately, the viability of the spermatozoa in the bottom of the centrifuge tube was not evaluated to see

Table XIX. Comparison of Specific Gravity Measurements on Rabbit and Bull

Spermatozoa in Different Density Gradients.

Sperm Source	Specific Gravity Range	Mean	Sub-Groups	Main Constituents of Density Gradient	Reference
Bull	1.24-1.35	1.2867	First ejaculate	Methyl glucamine salt of umbradil	Lindahl and
		1.2897	Second ejaculate	(2,5-diiodine-4-pyridone-N-acetic acid)	Kihlstrom[138]
		1.2668	Third ejaculate		
Bull			Heads 1.276; tails & midpieces, 1.035-1.056	"	Kihlstrom[124]
Bull	1.10-1.125	-	Epididymal sperm	Ficoll (synthetic polysaccharide)	Lindahl and Thunqvist[139]
Bull	1.150-1.226	1.187	Low density band	Methyl glucamine salt of 3,5-di-iodo-4-	Benedict, Schumaker
	1.255-1.318	1.283	High density sub-band	pyridone-N-acetic acid (umbradilic acid)	and Davies[32]
	1.286-1.324	1.303	High density sub-band	"	"
Rabbit	1.150-1.210	1.185	Low density band	"	"
	1.273-1.306	1.283	High density band	"	"
	1.280-1.313	1.294	High density band	"	"
Bull	1.0376-1.0927	-		Di-N-butyl phthalate, di-ethyl-phthalate, di-iso-butyl-phthalate	Lavon, Volcani, Amir and Danon[132]
Rabbit	1.09-1.18	1.132		Dialysed colloidal silica, dextran	Beatty[20]
Rabbit	1.195-1.211	1.203	Low density band	Sodium Iothalamate	Pistenma - this report
	1.267-1.310	1.288	Intermediate density band	"	"
	1.312-1.330	1.321	Bottom band #1	"	"
	1.332-1.366	1.351	Bottom band #2	"	"
Rabbit	1.155-1.215	-	Upper band	Dextran (20,000 MW, Sigma)	"
	> 1.23	-	Bottom band (s)	"	"

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if they were non-viable as expected from the sodium iothalamate density
gradient experiments. The dextran gradient values of the apparent specific
gravity of rabbit spermatozoa are higher than those reported by Beatty
yet lower than those obtained with the sodium iothalamate density gradients.
These differences illustrate the need for further study of the effect of
osmotic pressure on the apparent specific gravity of spermatozoa and other
cells.

Similar differences in Table XIX are found in the reported apparent
specific gravities of bull spermatozoa which vary from a range of 1.0376-
1.0927, reported by Lavon and co-workers using a density gradient of di-
n-butyl phthalate and di-ethyl-phthalate[133] to a range of 1.24-1.35 re-
ported by Lindahl and Kihlström using MGU[138].

The differences in quality of the spermatozoa in the several bands
observed in my experiments, examples of which are given in Table XVIII,
appear to be greater than that observed by Benedict and co-workers[32].
Spermatozoa from the high-density band in my experiments had almost no
motility and very low viability, suggesting that the fertilizing capacity
was negligible. My impression is that any viable and motile spermatozoa
in the high-density band reached that level in the centrifuge tube by
droplet sedimentation or by being in a clump with "dead" spermatozoa.
Thus, I am surprised that Benedict and co-workers were able to get offspring
from the insemination of spermatozoa from the high-density bands even though
the fertility rate was only 4-6%.

E. Summary and Conclusions

For physiological reasons, it is desirable to use dextran density
gradients rather than hyperosmolar solutions of radiographic contrast media
or similar substances. However, in addition to severe technical problems

with high concentrations of dextran, a 65.8% (weight/volume) solution of 15-20,000 MW dextran (specific gravity 1.23 at 4°C) was not sufficiently dense for isopycnic centrifugation of all of the rabbit spermatozoa. In this linear density gradient, the lightest band of rabbit spermatozoa had a specific gravity range of 1.16-1.21 while that for fowl spermatozoa ranged from 1.15-1.17.

In a linear density gradient of sodium iothalamate in saline, centrifugation for approximately 10 minutes at 9,000 g was required to reach equilibrium with both fowl and viable rabbit spermatozoa. Fowl spermatozoa gave a single band in the density gradient with an apparent specific gravity range of 1.19-1.21. Under the same conditions, rabbit spermatozoa always gave 3 or more bands, the lightest of which had this same apparent specific gravity range of 1.19-1.21. The two other bands formed by rabbit spermatozoa generally were in the ranges of 1.26-1.29 and 1.32-1.36. There was a suggestion that the apparent specific gravity of non-viable rabbit spermatozoa increased with increasing centrifugal force and centrifugation time in this density gradient.

The discrepancy between results using dextran and sodium iothalamate density gradients are similar to those summarized in Table XIX. Possible mechanisms were examined for the apparent high specific gravity in the sodium iothalamate density gradient.

There was a marked differential distribution of viable and non-viable rabbit spermatozoa among the 3 bands with unequivocal absolute enrichment of the upper band in regard to viable and motile spermatozoa. All sperm-

atozoa in the lowest band were essentially non-viable. Thus, with
equilibrium density gradient centrifugation in a sodium iothalamate gradi-
ent one can obtain a population of high quality rabbit spermatozoa from
a lesser quality starting sample. The homogeneity of the most dense (non-
viable) band of rabbit spermatozoa can be modified by clumps which tend
to carry viable cells into the higher density regions of the gradient,
along with the non-viable ones.

A differential distribution of fowl spermatozoa within the single,
continuous band was also found. The highest quality spermatozoa were on
top and deteriorating cells were on the bottom. The disintegration of non-
viable fowl spermatozoa is in marked contrast to the morphological integrity
of non-viable rabbit spermatozoa except for the latter's loss of the acro-
somal cap. Thus, the discreteness of the band of fowl spermatozoa in the
density gradient is an indication of the quality of the sample.

It was shown (Appendix D) that for rabbit spermatozoa, neither the
motility nor fertilizing capacity was affected by exposure to low concen-
trations of sodium iothalamate at 2-4°C for many hours. Fowl spermatozoa
experienced only a mild loss of motility after exposure for about 30
minutes to high concentrations of several contrast media. The preservation
of vital functions in these media suggests that they might be useful for
the separation of viable and non-viable cells with other cell systems,
as well.

VIII.   MULTI-CHARACTERIZATION STAFLO STUDIES: RABBIT SPERMATOZOA

Contents

A. Introduction

A review of work by others on the sedimentation and electrophoretic
properties of spermatozoa is presented in Chapter II. The results of early
Staflo-sedimentation and -electrophoresis studies on rabbit spermatozoa
are reported in Chapters IV and V, respectively. These early studies
suggested a poorly defined heterogeneity in the sedimentation and electro-
phoretic properties approximately resembling a normal distribution. In-
teresting observations included a near-vertical orientation of the sperm-
atozoa with the head down and tail up in sedimentation experiments, and a
vertical orientation in sedimentation-electrophoresis experiments with the
tail pointing toward the positive electrode, irrespective of the direction
of migration. In addition, a significant effect of motility on the fraction-
ation of the spermatozoa in the Staflo apparatus was observed at temperatures

of 4°C and above where some spermatozoa had sufficient intrinsic motility to overcome the force of the electric field. Some of the influences of the pH and ionic strength on the electrophoretic properties of spermatozoa, as reported by Bangham[18], were also observed in these early studies.

The purpose of the experiments reported in this chapter was to explore the relationships among the sedimentation rate, electrophoretic mobility and intrinsic motility of rabbit spermatozoa utilizing the flexibility of the Staflo method. A series of sedimentation and sedimentation-electrophoresis experiments, the possible types of which were discussed in Chapter III, was conducted, using the same medium and density gradient. The "low" ionic strength glycine-phosphate buffer evaluated in Chapters V and VI was selected because of its good pH stability in electrophoresis experiments and its excellent preservation of motility. A sucrose density gradient was selected to minimize discontinuities between layers and to enhance the sedimentation of spermatozoa.

B. Materials and Methods

1. Sample Preparation

Spermatozoa samples were obtained from mature Dutch Belt, California or New Zealand White rabbits using the artificial vagina described in Appendix C. Each ejaculate was diluted immediately by adding 1.0 ml of the glycine-phosphate buffer at 37°C to the collection vial. The sample then was allowed to cool to room temperature in an insulating nest of glass wool. A 0.05 ml aliquot of this sample was then diluted to 1.0 ml in egg yolk medium and was used for the initial evaluation of forward motility, for the initial estimate of the spermatozoa concentration in the sample, and for control inseminations. Further cooling to the experimental temperature was accomplished as described in Chapter III.

After the spermatozoa had been cooled to the temperature of the experiment, the sample was diluted to a concentration of approximately 20×10^6/ml. Even though the density gradient used in these experiments supported a suspension of up to 30×10^6/ml, lower concentrations were used to avoid "cluster sedimentation" discussed in Chapter IV. Generally, a single ejaculate provided sufficient spermatozoa for an experiment. If it was necessary to pool ejaculates from 2 or more rabbits, only spermatozoa from rabbits of the same breed were used.

A 1.0 ml aliquot of the 20×10^6/ml suspension, called the initial sample (IS), was set aside for immediate evaluation and for later comparison with the fractionated spermatozoa. The remainder of the initial sample was introduced into layer 8 in all experiments over the small density shelf between layers 8 and 9, and was subjected to Staflo sedimentation or sedimentation-electrophoresis, as desired. The aliquot of the initial sample which had been set aside then was evaluated in the hemocytometer to determine the motility or lack thereof at the temperature of the experiment, and was characterized with regard to the percentage of capped and capless spermatozoa by phase contrast microscopy.

2. Staflo Apparatus

The Staflo apparatus, characteristics of operation and accessory equipment are described in Chapter III. Therefore, this section makes note only of the equipment used in the multi-characterization studies.

a. Flow-Cells. The 3 x 16 flow-cell (No. XI) was used in 1 sedimentation experiment and in all sedimentation-electrophoresis experiments discussed below. The 3 x 12 flow-cell (No. XIII) was used only for 2 sedimentation experiments. The principles of operation of the 2 flow-cells are the same in all respects.

b. <u>Optical Accessories</u>. The gross migration patterns were observed
by eye, using the slit lamp technique[229,263]. Observations of individual
spermatozoa and the microscopic steady-state migration pattern were made
with the microscopic system described in Chapter III.

Using dark field illumination, observation of individual spermatozoa
was possible up to magnifications of 640 X. In addition to observing the
orientation and movement, if any, of spermatozoa, the microscope was used
to measure the vertical migration distances in the centerline of the flow-
chamber.

3. Media and Density Gradient

a. <u>Glycine-Phosphate Buffer (GPB)</u>. This buffer is the primary medium
for the density gradient solutions used in this series of experiments.
The composition and initial evaluation, and additional studies on the
glycine-phosphate buffer are reported in Chapters V and VI, respectively.

b. <u>Sucrose Density Gradient</u>. The density gradient contains sucrose by
weight/volume percent in the flowing layers numbered 1 through 16 from top
to bottom, as follows: Nos. 1-3 contained no sucrose. A higher conduc-
tivity solution with 3 times as much modified Baker's medium was used in
these layers and also in the top electrode wash compartment as a "conduc-
tivity barrier"[229,263]; No. 4, 0.05%; No. 5, 0.20%; No. 6, 0.35%; No. 7,
0.5%; No. 8, 0.65%; No. 9, 1.0%; No. 10, 1.15%; No. 11, 1.3%; No. 12, 1.45%;
No. 13, 1.60%; Nos. 14-16, and the bottom electrode compartment contained
the same high conductivity medium used in the top electrode wash compartment
plus 2.0% sucrose.

Figure 53 shows the following characteristics of the glycine-phosphate
buffer and this density gradient at 3°C: specific gravity, viscosity and
osmolarity.

CHARACTERISTICS OF DENSITY GRADIENT
Multicharacterization studies
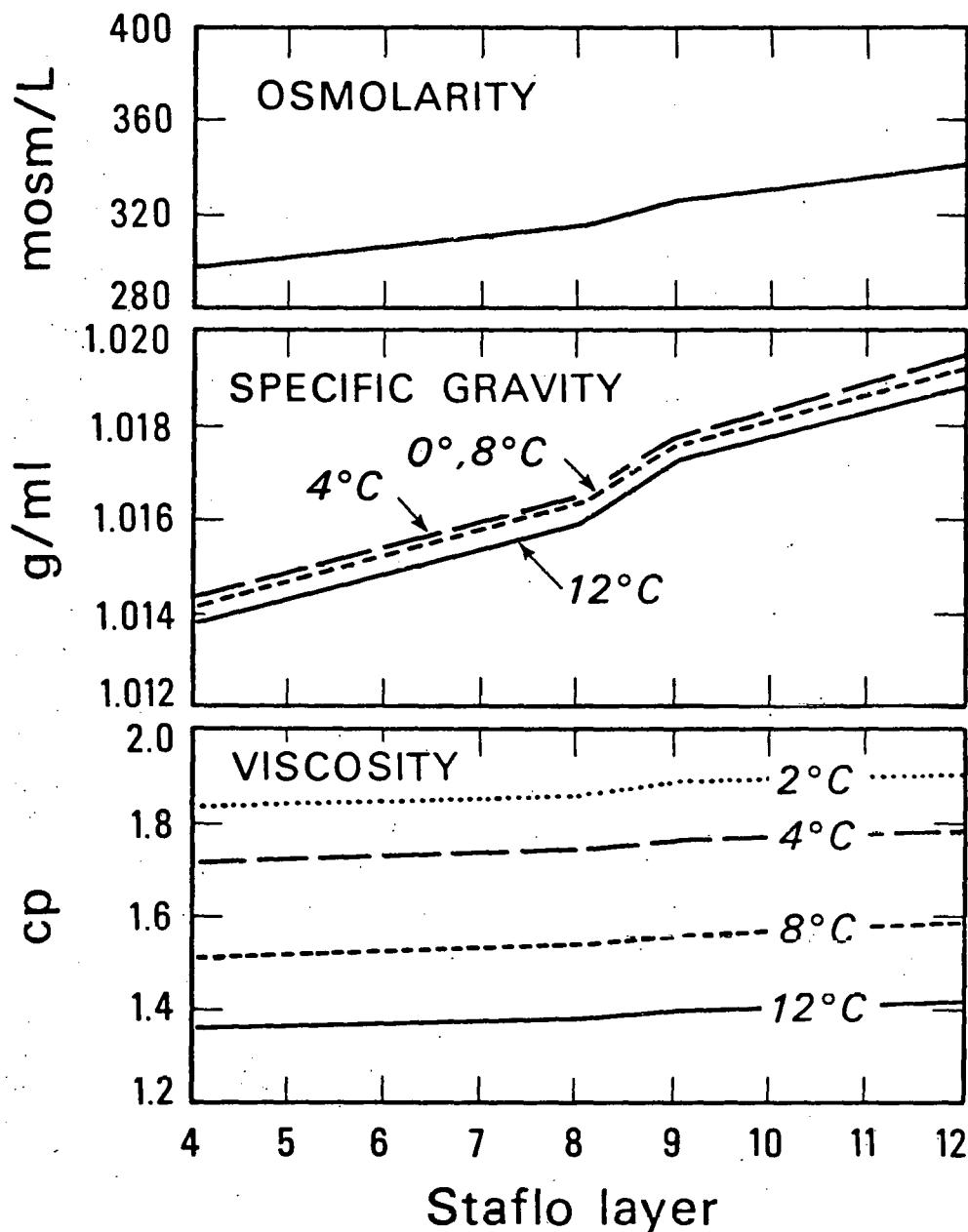
DBL 7011 5992

Fig. 53. Characteristics of the glycine-phosphate buffer and sucrose density gradient for multi-characterization studies of rabbit spermatozoa.

c. <u>Egg Yolk Medium (EYM)</u>. The egg yolk medium used in these experiments was the same as described in Chapter VI. Because of the ability of the egg yolk medium to maintain the viability, motility and fertilizing capacity of rabbit spermatozoa, the spermatozoa in the collection fractions were reconcentrated by centrifugation and resuspended in egg yolk medium. This eliminated the necessity for immediate artificial insemination (AI) and provided more time for evaluation of the spermatozoa prior to AI.

4. <u>Temperature Control</u>.

All experiments were conducted in a refrigerated room which was large enough for the entire Staflo apparatus, accessory equipment and several investigators. In the sedimentation-electrophoresis experiments with high electric field strengths, the average and maximum temperature rises in the flow-chamber were 0.5°C and 1.0°C, respectively. For sedimentation-electrophoresis experiments with low electric field strengths there was essentially no change in temperature. Flow-chamber temperatures reported below are corrected for the estimated temperature rise depending upon the electric field strengths and other operating conditions.

5. <u>Evaluation of Spermatozoa</u>

a. <u>Morphology, Motility and Viability</u>. Immediately after completion of the Staflo fractionation phase of the experiment, the suspensions in the central row of collection containers were evaluated in a hemocytometer for spermatozoa concentration and for percentage of capped and capless spermatozoa by phase contrast microscopy. Each collection fraction, the 1.0 ml initial sample and any remaining stirred suspension (SS) then was concentrated by centrifugation for 5 minutes at 1,400 g in a clinical centrifuge in the refrigerated room. The supernatant was removed and the spermatozoa were re-suspended in egg yolk medium to a concentration of

approximately 20 x 10^6/ml. The re-suspended spermatozoa then were eval-
uated at room temperature for motility, percent capped and capless by
phase contrast microscopy and for vital staining characteristics by a
nigrosin-eosin staining technique. The stained spermatozoa were cate-
gorized as unstained (U), morphologically normal but stained (MNS) and
stained (S). All work with spermatozoa was accomplished using minimal
lighting in order to reduce the deleterious effects of visible light[176].

 b. Fertilizing Capacity. If 10% or more of the spermatozoa in each
collection fraction had a high degree of motility at room temperature,
artificial insemination was attempted. Ovulation was induced by an intra-
venous injection of 0.5 mg/kg of luteinizing hormone (ovine or bovine),
generally less than 1 hour prior to insemination. A 0.5-1.0 ml aliquot
of each suspension, containing 10-20 x 10^6 spermatozoa, was inseminated
in as many rabbits as there was suspension, using a plastic pipette
(Falcon Plastics, Division of B-D Laboratories, Inc.), on which the tip
had been rounded. A control insemination was made with 0.5 ml of the
spermatozoa suspension initially diluted and stored in egg yolk medium.

C. Results

The order of presentation of the results is as follows: (1) sedimen-
tation experiments, (2) sedimentation-upward-electrophoresis (SUE) experi-
ments with high electric field strengths, and (3) sedimentation-upward-
electrophoresis (SUE) and sedimentation-downward-electrophoresis (SDE)
experiments with low electric field strengths.

Because the migration distances were small in all experiments, the
migration pattern shown in Figure 54 (SUE with low electric field strength)
is representative of that encountered in all 3 series of experiments. The
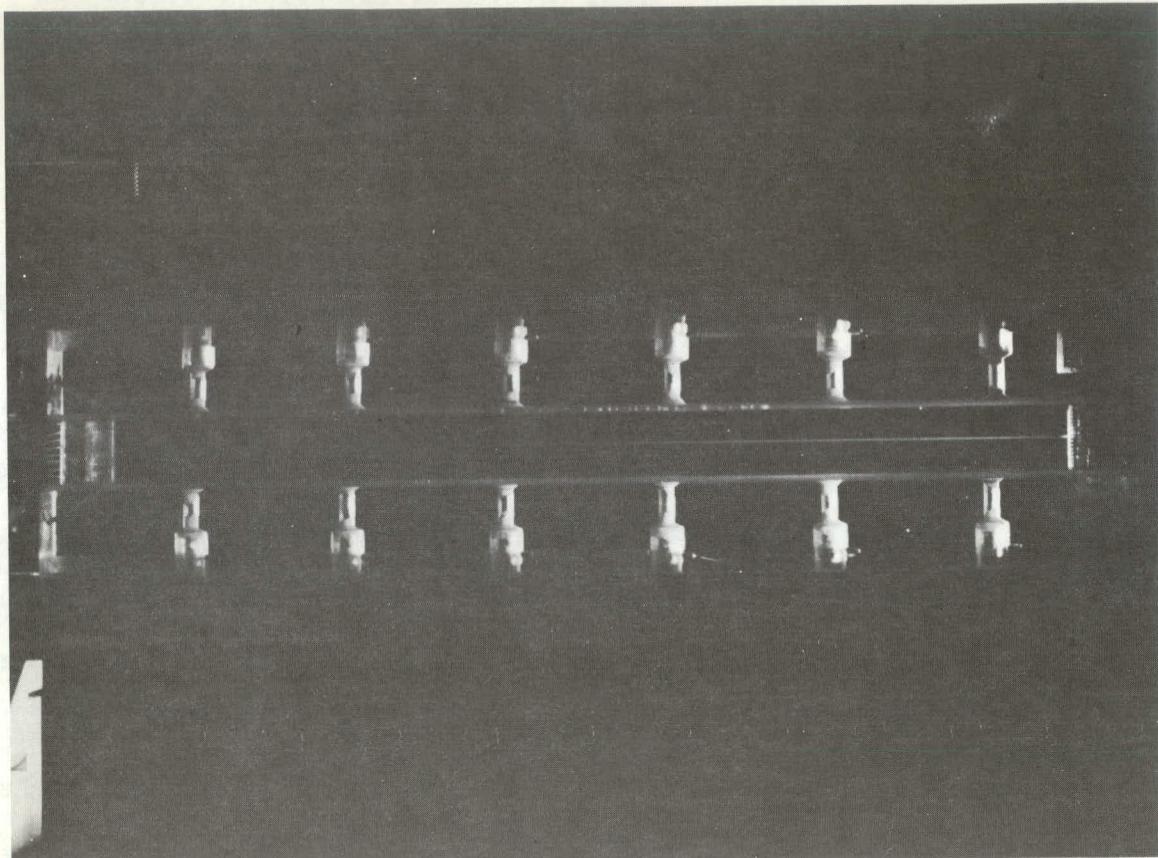
XBB 7011-5007

Fig. 54. Photograph of the migration pattern in a sedimentation-
upward-electrophoresis experiment with rabbit spermatozoa using a
low electric field strength in the 3 x 16 Staflo apparatus.

orientation of the paraboloid migration profile (e.g. Figure 49) de-
pended upon the direction of net migration.

1. Sedimentation

Table XX is a summary of the characteristics of the spermatozoa
sample and Staflo operating parameters for 3 sedimentation experiments.
The quality of the spermatozoa samples was high initially only in Experiment
8-10-67 and that sample deteriorated rapidly during the course of the ex-
periment. Consequently, motility is considered to have contributed little,
if anything, to the migration patterns in this series of experiments.
There was no opportunity for artificial insemination at the conclusion of
any of the 3 experiments because of the poor quality of the spermatozoa.

Experiment 12-26-67 was conducted with the 3 x 16 flow-cell. The
sample stream initially was 0.7 mm high at the bottom of layer 8, which is
the desired location for downward migration, as discussed in Chapter III.
The 3 x 12 flow-cell, in which the inlet solutions are provided by thirty-
six 20 ml syringes, was used for the other two experiments. The sample
was introduced into layer 6 with a single 20 ml syringe, giving a 0.4 mm
high sample stream which was in the middle of layer 6 at steady-state
conditions. Thus, even though technically acceptable migration patterns
were observed in both experiments with the 3 x 12 flow-cell, three-layer
fractionation was not achieved because the spermatozoa had to sediment
through part of layer 6 and all of layer 7 before reaching layer 8. If
the sample migration had started at the bottom of layer 6, a significant
number of spermatozoa would have been recovered in collection fraction 8.

a. Three Individual Experiments.

(1) Experiment 12-26-67. Immediately after collection, the ejaculate
had less than 50% forward motion and tail-wagging combined at room temperature

Table XX. Spermatozoa Sample and Staflo Operating Parameters for
Multi-Characterization Sedimentation Experiments

Characteristics of Spermatozoa Sample							General Staflo Operating Parameters					
Experiment Number	Rabbit	Breed	Medium for Cooling[a]	Quality Initial	Final	Initial Sperm Conc.(units of 10^6/ml)	pH	Temperature Room (°C)	Staflo (°C)	τ_{ave} (min)	τ_{min} (min)	Remarks
12-26-67	Karl	Dutch Belt	GPB	Less than 50% tail-wag-ging and forward motion.	Very Poor	20	7.1	3	3	43	28.7	Sample 0.7 mm high at inlet in bottom of layer 8. (3 x 16 flow-cell)
7-29-67	Otto	Dutch Belt	GPB	Less than 40% tail-wagging and forward motion	Poor	17	7.1	5	5	34	22.7	Sample 0.4 mm high at inlet in middle of layer 6. (3 x 12 flow-cell)
8-10-67	Psi	Dutch Belt	EYM	60% for-ward motion	Poor (ques-tion of toxin in apparat-us).	26	7.1	6	6	34	22.7	Sample 0.4 mm high at inlet in middle of layer 6. (3 x 12 flow-cell)

a. See Glossary for explanation of abbreviations.

in GPB. No motility was observed at 3°C in a hemocytometer. After steady-state conditions had been reached, there was no change in the migration pattern. The migration profile was paraboloid throughout the experiment.

Observations in the flow-chamber with the Staflo microscope showed the following: (1) spermatozoa were at the top of the migration pattern but at a lower concentration than in the middle; (2) there was no rolling, tail-wagging or other indication of intrinsic motility; (3) there were a small number of microscopic streamers, in which spermatozoa were spread well apart, at the bottom of the migration pattern only; (4) spermatozoa were oriented randomly from a horizontal to a vertical position with the head down and the tail up. (Most tails were oriented within 45° of the vertical position and no spermatozoa were seen with the tail down and the head up.) and (5) there was no effect of location within the flow-chamber upon the orientation of the spermatozoa.

Table XXI summarizes the distribution of spermatozoa among the collection fractions and the phase contrast observations and results of nigrosin-eosin staining thereof. Despite the range of concentrations from 0.72-2.91 x 10^6/ml, there were no significant differences among the 3 fractions with regard to the distribution of capped spermatozoa immediately after collection. Deterioration of the spermatozoa sample at a concentration of 20 x 10^6/ml is shown by the change from 79.2% capped in the initial sample (IS) to 52.4% capped in the stirred sample (SS) at the conclusion of the experiment. After reconcentration and suspension in egg yolk medium, no motile spermatozoa were observed at room temperature. There were no significant differences in the nigrosin-eosin staining results.

(2) <u>Experiment 7-29-67</u>. As shown in Table XXI, there was no significant difference between the 2 collection fractions with regard to the per-

Table XXI. Phase Contrast Observations and Nigrosin-Eosin Staining
of Spermatozoa in Sedimentation Experiments.

Experiment Number	Spermatozoa Sample[a]	Sperm Collected Concentration (units of 10^5/ml)	(%)	Temp. (°C)	Phase Contrast Observations[b]				Nigrosin-Eosin Staining[b]		
					C (%)	CL (%)	TW (%)	FM (%)	U (%)	MNS (%)	S (%)
12-26-67	IS	–	–	3	79.2	20.8	0	0	29.4	1.6	68.7
	CF 8	1.09	23.1	3	44.5	55.5	0	0	31	9	60
	CF 9	2.91	61.6	3	47.2	52.8	0	0	26	5	69
	CF 10	0.72	15.3	3	48.7	51.3	0	0	25	1	74
	SS	–	–	3	52.4	47.6	0	0			
7-29-67	IS	–	–	5	85.6	14.4	0	0			
	CF 6	1.90	60.7	5	90	10	0	0	–	–	–
				23	–	–	5.2	2.0	21.5	7	71.5
	CF 7	1.23	39.3	5	92.1	7.9	0	0	–	–	–
				23	–	–	10.5	2.3	29	7	64
	Control			23	–	–	–	–	45	2	53
8-10-67	IS	–	–	6	43.0	14.7	38.9	0	–	–	–
				23	11.5	19.1	48.2	21.2	90	1	9
	CF 6	5.62	88.7	6	73.3	25.2	1.5	0	–	–	–
				23	13.1	29.9	56.5	0.5	93	2	5
	CF 7	0.71	11.3	6	70.0	27.5	2.5	0	–	–	–
				23	11.8	57.6	28.6	2.0	92	3	5

a. CF: Collection Fraction; IS: Initial Sample; SS: Stirred Suspension;

b. See Glossary for explanation of abbreviations.

centages of capped spermatozoa at 5°C or of forward motility at room temperature. There were slightly higher percentages of both tail-wagging and unstained spermatozoa in fraction 7 relative to 6. The large difference between the percentage of capped spermatozoa at 5°C and the percentage of unstained spermatozoa at 23°C is attributed primarily to deterioration during centrifugation. In the control sample, 53% of the spermatozoa stained by the nigrosin-eosin technique, indicating that the initial quality of the sample was poor.

(3) Experiment 8-10-67. Despite the 8-fold difference in spermatozoa concentration, there was no significant difference between the 2 fractions with regard to the percentages of capped spermatozoa at 6°C or of unstained spermatozoa at room temperature. Although the preservation of motility and viability was better in this experiment than in either of the other 2 sedimentation experiments, the motility was almost all tail-wagging. The lower percent tail-wagging in fraction 7 is probably due to the lower concentration in that fraction.

b. Calculation of Sedimentation Velocities. A sample calculation of sedimentation velocities for Experiment 12-26-67 is presented in Appendix F. This experiment was selected as a sample for the calculations because the spermatozoa suspension entered the flow-cell at the bottom of the inlet layer, satisfying the requirements for the formulae discussed in Chapter III. Table XXII presents the minimum, maximum and average sedimentation velocities for the spermatozoa in each collection fraction. The weighted average sedimentation velocity at 3°C was calculated to be 0.53 $\times 10^{-4}$ cm/sec.

Table XXIII presents the minimum, maximum and weighted average sedimentation velocities, normalized to 3°C, for all 3 experiments. The maximum sedimentation velocity ranges from 0.87-1.24 $\times 10^{-4}$ cm/sec and the

Table XXII. Sedimentation Velocities for Spermatozoa in Collection Fractions;
Sedimentation Experiment 12-26-67, 3°C.

Collection Fraction	Percent of Sperm Collected	Sedimentation Velocities and Rates (in units of)					
		Minimum		Maximum		Average	
		Velocity[a]	Rate[b]	Velocity[a]	Rate[b]	Velocity[a]	Rate[b]
8	23.1	0	0	0.465	0.475	0.157	0.161
9	61.6	0	0	0.987	1.01	0.530	0.593
10	15.3	0.725	0.74	0.987	1.01	0.90	0.918

a. in units of 10^{-4} cm/sec; b. in units of 10^6 Svedbergs

Table XXIII. Summary of Calculated Sedimentation Velocities
Normalized to Values Expected at 3°C.

Experiment Number	Temperature	$t$min (minutes)	Sedimentation Velocity (units of 10^{-4} cm/sec)			Average Sedimentation Rate (units of 10^6 Svedbergs)
			Minimum	Maximum	Weighted Ave.	
12-26-67	3.0°C	28.7	0	0.99	0.53	0.54
7-29-67	5.0°C	22.7	0	1.24	0.43	0.44
8-10-67	6.0°C	22.7	0	0.87	0.44	0.45

weighted average sedimentation velocity from $0.43-0.53 \times 10^{-4}$ cm/sec.

2. Sedimentation-Upward-Electrophoresis: High Electric Field Strengths

The characteristics of the spermatozoa sample and Staflo operating parameters for this series of experiments are given in Table XXIV. In all experiments in this series, the spermatozoa sample stream initially was 0.7 mm high and, during steady-state conditions, usually was at the bottom of layer 8 at the inlet. Most experiments were conducted near pH 6.6 or pH 7.1; one was conducted at pH 6.0. Generally, the upward migration pattern spread over 2 layers and exited the flow-chamber in layers 6 and 7.

Unless it was desired to use the intrinsic motility of spermatozoa as a downward migration vector against the upward electrophoretic force, the experiment was conducted as close to 1°C as possible. On many occasions, the lowest temperature maintained by the cold room was from 4-6°C, which was higher than desired. Table XXV summarizes the distribution of spermatozoa among the collection fractions and the phase contrast observations and results of nigrosin-eosin staining thereof, in all experiments of this series. Offspring were obtained after artificial insemination of spermatozoa from the collection fractions in the 4 experiments listed last in Tables XXIV and XXV. Individual experiments, including the results of artificial insemination, will be considered in the order listed in those tables. The calculation of electrophoretic mobilities will be considered separately.

a. Ten Individual Experiments

(1) Experiment 10-2-67. This experiment was conducted at pH 6.0 with a spermatozoa sample having a high initial quality. Observations with the Staflo microscope showed no motility in the flow-chamber and that, after

Table XXIV. Spermatozoa Sample and Staflo Operating Parameters for Sedimentation-Upward-Electrophoresis Experiments Using High Electric Field Strengths.

Experiment Number	Characteristics of Spermatozoa Sample					General Staflo Operating Parameters								
	Rabbit		Cooling Medium[a]	Quality		Initial Sample Conc. (units of 10^6/ml)	pH	Temp. RT (°C)	Staflo (°C)	τave (min)	τmin (min)	τE (min)	L (cm)	$\bar{E}$ (V/cm)
	Name	Breed		Initial	Final									
10-2-67	Psi	Dutch	GPB	Excellent	Poor	24	6.0	2.4	3.3	20	13.3	8.84	20	7.6
8-18-67	Otto	Dutch	EYM	Fair	Poor	20	6.60	3.5	4.1	20.5	13.7	9.10	20	6.2
7-26-67	Otto	Dutch	GPB	Fair	Poor	18.5	7.05	4.5	4.9	21.7	14.5	9.64	20	5.4
7-29-67	Larry	Calif.	GPB	Excellent	Fair	22.8	7.05	5.0	5.4	21.3	14.2	9.48	20	5.4
8-10-67	Psi	Dutch	EYM	Excellent	Poor	26	7.10	6.0	6.4	20.5	13.7	9.10	20	5.3
9-22-67	Sam	NZW	GPB	Fair	Poor	23.0	7.10	7.0	7.2	20	13.3	8.87	20	4.0
8-29-67	Otto	Dutch	GPB	Excellent	Good	22.8	6.63	3.5	4.2	20.7	13.8	9.10	20	6.3
9-13-67S	Snoopy	Calif.	GPB	Superior	Excellent	21.1	6.55	2.1	2.8	20.5	13.7	9.10	20	8.7
9-13-67P	Psi	Dutch	GPB	Excellent	Fair	20.8	6.55	2.7	3.1	20.5	13.7	9.10	20	5.1
9-18-67	Psi	Dutch	GPB	Excellent	Fair	25.6	7.15	2.8	3.2	20.5	13.7	9.07	20	5.5

a. See Glossary for explanation of abbreviations.

Table XXV. Summary of Distribution, Phase Contrast Observations and Nigrosin-Eosin Staining of Spermatozoa in Sedimentation-Upward-Electrophoresis Experiments Using High Electric Field Strengths.[a]

Experiment Number	pH	Spermatozoa Sample	Sperm Collected Conc.[b]	(%)	Temp. (°C)	Phase Contrast Observations				Nigrosin-Eosin Staining		
						C (%)	CL (%)	TW (%)	FM (%)	U (%)	MNS (%)	S (%)
10-2-67	6.0	CF 6	0.5	3.3	3.3	50	50	0	0	–	–	–
					23	13.5	83.0	3.5	0	30.5	0.6	68.9
		CF 7	6.0	91.7	3.3	79.7	20.3	0	0	–	–	–
					23	38.1	40.2	21.7	0	66.5	0	33.5
		IS			3.3	91.5	8.5	0	0	–	–	–
					23	41.1	36.5	22.1	$\ll 1.0$	68	0.5	31.5
8-18-67	6.60	CF 5	1.36	30.5	3.8	67.5	32.5	0	0			
					22			Poor	Poor	29	2	69
		CF 6	3.10	59.5	3.8	62.2	37.8	0	0			
					22			Poor	Poor	45.5	0	54.5
		IS			3.8	67.7	32.3	0	0			
					22			Poor	Poor	44.5	11.5	44.0
7-26-67	7.05	CF 5	0.70	11.6								
		CF 6	4.0	56.2	4.5	86.5	13.5	0	0			
					22	23.9	51.8	10.8	13.5			
		CF 7	1.34	22.2	4.5	84.5	15.5	0	0			
					22	21.0	48.9	11.3	18.8			
		IS			4.5	92.5	7.5	0	0			
7-29-67	7.05	CF 6	5.04	58.6	5.4	65.4	10.7	23.9	0			
					23	26.9	16.1	48.7	8.3	47	3	50
		CF 7	3.53	41.4	5.4	57.0	6.6	36.4	0			
					23	32.2	26.2	24.3	17.3	45	1	54
		IS			5.4	64.8	4.4	30.7	0			
					23					46	6	48

Table XXV. (Continued)

Experiment Number	pH	Spermatozoa Sample	Sperm Collected Conc.[b]	(%)	Temp. (°C)	Phase Contrast Observations				Nigrosin-Eosin Staining		
						C (%)	CL (%)	TW (%)	FM (%)	U (%)	MNS (%)	S (%)
8-10-67	7.10	CF 6	5.0	99	6	71.8	28.2	0	0			
					23	11.7	34.1	50.0	4.2	91	1	8
		IS			6	43.0	14.7	38.9	0			
					23	11.5	19.1	48.2	21.2	90	1	9
9-22-67	7.10	CF 6	3.42	47.9	7	71	29	0	0	–	–	–
					23	32.2	63.5	4.3	0	34	1.5	64.5
		CF 7	3.72	52.1	7	74	26	0	0	–	–	–
					23	34.8	58.7	6.5	0	35	0.5	64.5
		IS			7	68	28.2	3.8	0	–	–	–
					23	25.3	71.5	3.2	0	32.5	4	64.5
8-29-67	6.63	CF 6	1.71	23.4	4.2	96.3	3.7	–	–	–	–	–
					23	–	–	–	40	59	3	38
		CF 7	5.60	76.6	4.2	97.5	2.5	–	–	–	–	–
					23	–	–	–	60	75	3.5	21.5
		IS			4.2	93.8	6.2	–	–	–	–	–
					23	–	–	–	> 50	64	1	35
9-13-67S	6.55	CF 6	1.9	19.2	2.8	92.8	7.2	–	–	–	–	–
					22	–	–	–	>50	87	1.0	12
		CF 7	8.0	80.8	2.8	97.0	3.0	–	–	–	–	–
					22	–	–	–	>50	72.5	6	21.5
		IS			2.8	96.3	3.7	–	–	–	–	–
					22	–	–	–	>50	67	4	29
9-13-67P	6.55	CF 7	4.25	45.0	3.1	88.3	11.7	–	–	–	–	–
					22	–	–	–	~30	52.5	1.5	46
		CF 8	5.20	55.0	3.1	92.5	7.5	–	–	–	–	–
					22	–	–	–	"	59.5	1.5	39
		IS			3.1	–	–	–	–	–	–	–
					22	–	–	–	"	51	0	49
9-18-67	7.15	CF 6	2.5	35.2	3.0	80	20	–	–	–	–	–
					23			–	24	61.5	0	38.5
		CF 7	4.6	64.8	3.0	91.5	8.5	–	–	–	–	–
					23			–	40	75.5	0.5	24
		IS			3.0	87.4	12.6	–	–	–	–	–
					23	–	–	–	50	66.5	2	31.5

a. See Glossary for explanation of abbreviations; b. in units of 10^6 sperm/ml.

passing into the region of the electric field, essentially all spermatozoa were oriented vertically with their tails pointing toward the top (positive) electrode. The migration pattern was difficult to control and only 8% of the spermatozoa (0.5×10^6/ml) exited in layer 6. In Table XXV, it is seen that the percentage of capped spermatozoa at 3.3°C and the percentages of tail-wagging and unstained spermatozoa at 23°C were significantly less in CF 6 than in 7. This is attributed primarily to the low spermatozoa concentration in CF 6. Although the percentage of capped spermatozoa at 3.3°C was slightly less in CF 7 than in the initial sample, the percentages of tail-wagging and unstained spermatozoa after centrifugation were essentially the same in those 2 samples. There were no motile spermatozoa at room temperature in CF 6 or 7 and less than 1% in the initial sample. Because there was little change in the control sample which had been stored in egg yolk medium, the deterioration of the spermatozoa in the Staflo apparatus and in the initial sample is believed to be due to an irreversible loss of motility at pH 6.0.

(2) <u>Experiment 8-18-67</u>. This experiment was conducted with a spermatozoa sample of poor initial quality. The migration pattern was highly acceptable throughout the experiment with 30.5% and 69.5% of the spermatozoa recovered in fractions 5 and 6, respectively. As shown in Table XXV, there were no significant differences between the percentages of capped spermatozoa in fractions 5 and 6 and among those fractions and the initial sample at 3.8°C. After centrifugation, too little supernatant was removed and the addition of the egg yolk medium resulted in a precipitate which hindered the evaluation of the low percentage and poor degree of motility present. CF 6 had a much higher percentage of unstained spermatozoa than CF 5 and was comparable to the initial sample.

(3) <u>Experiment 7-26-67</u>. Because inlet syringe No. 6 was left off inadvertently, the inlet sample stream reached a steady-state position at the boundary between layers 8 and 9. Otherwise, the migration pattern was technically acceptable. No observations were made with the Staflo microscope. The spermatozoa were distributed among fractions 5, 6 and 7, but fraction 5 was not evaluated. There was no significant difference between fractions 6 and 7 with regard to the percentage of capped spermatozoa at 4.5°C or the percentages of capped, tail-wagging and motile spermatozoa at 22°C. The large increases in the percentages of capless spermatozoa between the observations at 4.5°C and 22°C in fractions 6 and 7 are attributed to damage during centrifugation.

(4) <u>Experiment 7-29-67</u>. Immediately after collection of the ejaculate, the spermatozoa swam in very small circles, but eventually straightened out. Because a 3 x 12 sedimentation experiment was being conducted simultaneously, no microscopic observations were made in the flow-chamber. The migration pattern was stable throughout the experiment and the concentrations of spermatozoa recovered in fractions 6 and 7 were 5.04×10^{6}/ml and 3.53×10^{6}/ml, respectively. At 5.4°C, 23.9% and 34.6% of the spermatozoa in fractions 6 and 7, respectively, had tail-wagging. The 5% and 20% increases in capless spermatozoa in fractions 6 and 7, respectively, because of centrifugation were less than usually encountered in this series of experiments. At room temperature, after centrifugation, the spermatozoa in CF 6 had a higher percentage of tail-wagging, but a lower percentage of forward motion than CF 7. With regard to nigrosin-eosin staining results, there was no significant difference between the 2 fractions or between those fractions and the initial sample. However, the percentages of stained spermatozoa were much greater than the percentages of capless spermatozoa for both collection fractions, suggesting deterioration during the staining

procedure.

(5) **Experiment 8-10-67.** Inlet syringe No. 6 was turned off because
of mechanical difficulty and, therefore, the sample stream at the inlet
was high in layer 8. After steady-state conditions had been reached,
the migration pattern remained stable. Essentially 100% of the spermatozoa
were oriented vertically with the tails toward the top (positive) electrode.
A low percentage of the spermatozoa showed slight rolling and tail-wagging
at a temperature of 6.4°C. Approximately 99% of the intact spermatozoa
exited in layer 6 while fractions 5 and 7 contained many tails and heads,
respectively. At 6°C, 38.9% of the spermatozoa in the initial sample
showed tail-wagging but no motility was observed at that temperature in
CF 6. After centrifugation and re-suspension in egg yolk medium, the
initial sample had about the same percentage of tail-wagging as CF 6, but
had a higher percentage of motile spermatozoa. However, the percentages
of unstained spermatozoa were essentially the same in the 2 samples.

(6) **Experiment 9-22-67.** This experiment was conducted at 7.5°C with
the intention of separating the highly motile spermatozoa from the remainder
of the sample by allowing downward swimming of the spermatozoa against the
electric field. The initial percentage and degree of motility of the
spermatozoa was excellent, but the sample deteriorated markedly during
the course of the experiment, both in the GPB and in the egg yolk medium.
No motility was observed in the flow-chamber at 7.4°C and essentially all
spermatozoa were oriented vertically with the tail pointing toward the top
(positive) electrode. Only 3.8% of the spermatozoa in the initial sample
had tail-wagging at 7°C and all samples had less than 7% tail-wagging at
room temperature. After collection, there were no differences among the
samples. The centrifugation resulted in a 33%-43% increase in capless
spermatozoa.

(7) <u>Experiment 8-29-67</u>. Inlet 6 did not flow because of mechanical difficulty. However, after reaching equilibrium conditions, the migration pattern appeared stable and resulted in concentrations of 1.71 x 10^6 and 5.60 x 10^6 spermatozoa/ml in fractions 6 and 7, respectively. No observations were made in the flow-chamber. Evaluation in a hemocytometer at 3.5°C showed no motile spermatozoa in the initial sample. As shown in Table XXV, there were no significant differences among the samples at 4.2°C. After centrifugation and resuspension in egg yolk medium, the spermatozoa in fractions 6 and 7 had 40% and 60% forward motion, respectively, as compared to slightly greater than 50% in the initial sample. Thus, CF 7 was slightly enriched with regard to motile sperm. The percentage of unstained spermatozoa was greater in CF 7 than in either CF 6 or the initial sample, again, suggesting absolute enrichment. The insemination of spermatozoa from CF 6 and the control sample resulted in no offspring. However, as shown in Table XXVI, the insemination of spermatozoa from CF 7 resulted in 2 litters with a total of 5 offspring.

(8) <u>Experiment 9-13-67S</u>. The initial quality of the spermatozoa sample used in this experiment was superior. No observations were made in the flow-chamber with the microscope; however, at 2°C, no motile spermatozoa were observed in a hemocytometer in GPB. Even though the spermatozoa concentrations were 1.9 x 10^6 and 8.0 x 10^6/ml in fractions 6 and 7, respectively, there were no significant differences among the fractions and the initial sample with regard to capped spermatozoa at 2.8°C and motile spermatozoa at room temperature. The percentage of unstained spermatozoa was greater in CF 6 than in CF 7 and both were greater than the initial sample. Having both fractions better than the initial sample suggests an artifact of staining.

Table XXVI. Summary of Artificial Insemination (AI) Results.

Experiment Number	Spermatozoa Sample[a]	Artificial Insemination			Insemination Results				
		No. of Females	Sperm per Female	Comments	Litters (#)	Total	Males	Females	Comments
8-29-67	CF 6	1	8×10^6	Good AI	0	0			One eaten.
	CF 7	2	8×10^6	Good AI	2	5	4	0	
	Control	1	16×10^6	Good AI	0	0			
9-13-67 S	CF 6	1	5×10^6	Urinated during AI	0	0			
	CF 7	3	8×10^6	1 urinated during AI	0	0			
				2 partial loss of inseminate	2	8	4	4	
	Control	1	15×10^6	Good AI	1	1	1	0	
9-13-67 P	CF 7	1	14×10^6	Re-insemination after sample was squirt out	1	3	1	1	One eaten.
	CF 8	1	25×10^6(?)	Entire sample squirt out by female.	0	0			
	Control	1	15×10^6	Good AI	0	0			Pseudopregnant
9-18-67	CF 6	2	7×10^6	Lost 1/2 of 1 inseminate	0	0			
	CF 7	2	13×10^6	Good AI	1	2	1	1	
	Control	1	14×10^6	Good AI	0	0			

a. CF: Collection fraction

Five rabbits were inseminated with spermatozoa of the highest quality seen in any collection fractions in any Staflo experiment. As shown in Table XXVI, the single insemination with spermatozoa from CF 6 gave no offspring. However, two of the three inseminations with spermatozoa from CF 7 gave 2 litters with a total of 8 offspring; 4 males and 4 females. The single control insemination gave 1 male offspring.

(9) **Experiment 9-13-67P.** The initial quality of the spermatozoa sample used in this experiment was excellent, but not quite so good as that used in Experiment 9-13-67S. Because the electric field strength was lower than that used in Experiment 9-13-67S, the spermatozoa were distributed between fractions 7 and 8, in concentrations of 4.25×10^6 and 5.20×10^6 /ml, respectively. No observations were made in the flow-chamber. There were no motile spermatozoa in either of the 2 fractions at 2.6°C at the termination of the experiment. However, it cannot be stated that there was no motility in the flow-chamber where the average temperature was 3.1°C. There was no significant difference between fractions 7 and 8 with regard to capped spermatozoa at 3.1°C, or unstained spermatozoa at room temperature.

Because of slight precipitation of the egg yolk medium in the phosphate buffer, it was difficult to assess the motility of the spermatozoa at room temperature. Because only about 30% of the spermatozoa in each fraction and in the initial sample had an excellent degree of motility, only 1 rabbit was inseminated with spermatozoa from each sample. Despite difficulty in insemination, 1 litter of 3 offspring was obtained from CF 7. No offspring were obtained from the control sample or CF 8; however, both samples were rejected by the females during insemination.

(10) **Experiment 9-18-67.** The spermatozoa sample used in this experiment initially had approximately 60% forward motion at room temperature, but had

no motility at 2.8°C in GPB. No observations were made in the flow-chamber with the microscope. The percentage of capped spermatozoa in CF 7 was slightly higher than in either CF 6 or the initial sample. At room temperature, the spermatozoa in CF 7 had approximately 40% motility compared to 24% in CF 6 and 50% in the initial sample. CF 7 had a higher percentage of unstained spermatozoa than CF 6 or the initial sample. Thus, CF 7 contained higher quality spermatozoa than CF 6 by all criteria, and was almost as good as the initial sample.

Two rabbits were inseminated with spermatozoa from each fraction and one from the control sample. The only litter was obtained from the insemination of spermatozoa from CF 7 and consisted of 2 offspring, 1 male and 1 female.

b. <u>Calculation of Electrophoretic Mobilities</u>. Table XXVII presents the minimum, maximum and average observed and theoretical electrophoretic mobilities for the SUE experiments with high electric field strengths. Appendix G is a sample calculation of the electrophoretic mobilities for Experiment 7-29-67, using the formulae presented in Chapter III. All values in Table XXVII have been normalized to 3°C to facilitate comparison within this series and with other experiments. The observed minimum, maximum and average electrophoretic mobilities are calculated from the minimum and maximum SUE migration distances. Thus, they are not corrected for the contribution of sedimentation to the net migration.

The theoretical electrophoretic mobilities are corrected for the expected sedimentation component to the migration pattern. The corrections were made using the sedimentation velocity range of 0-0.99 x 10^{-4} cm/sec and the weighted average sedimentation velocity of 0.53 x 10^{-4} cm/sec from Experiment 12-26-67. As shown in Figure 19, (Chapter III), the minimum electrophoretic migration distance is equal to the sum of the minimum SUE

Table XXVII. Observed (Uncorrected) and Theoretical Electrophoretic Mobilities of Rabbit Spermatozoa; Sedimentation-Upward-Electrophoresis Experiments Using High Electric Field Strengths.

Experiment Number	pH	Flow-Chamber Temperature (°C)	Electrophoretic Mobility at 3°C [units of 10^{-4}(cm/sec)/(V/cm)]					
			OBSERVED			THEORETICAL		
			Minimum $(\lambda_{SUE_{min}})^a$	Maximum $(\lambda_{SUE_{max}})$	Average $(\lambda_{SUE_{ave}})$	Minimum $(\lambda_{SUE_{min}} + \lambda_{S_{max}})$	Maximum $(\lambda_{SUE_{max}} + \lambda_{S_{max}})$	Average $(\lambda_{SUE_{ave}} + \lambda_{S_{ave}})$
10-2-67	6.0	3.3	0.24	0.56	0.40	0.44	0.76	0.51
8-18-67	6.60	4.1	0.83	1.04	0.93	1.08	1.28	1.06
7-26-67	7.05	4.9	0.32	0.88	0.60	0.60	1.16	0.75
7-29-67	7.05	5.4	0.56	0.90	0.71	0.84	1.18	0.86
8-10-67	7.10	6.4	0.72	0.84	0.78	1.00	1.13	0.94
9-22-67	7.10	7.2	0.87	1.25	0.98	1.20	1.59	1.18
8-29-67	6.63	4.2	0.33	0.63	0.40	0.57	0.87	0.53
9-13-67S	6.55	2 8	0.42	0.54	0.46	0.59	0.71	0.55
9-13-67P	6.55	3 1	0.32	0.53	0.40	0.61	0.82	0.55
9-18-67	7.15	3 0	0.61	0.83	0.69	0.86	1.06	0.89

a. Migration distances used in calculations are shown in parentheses.

and maximum sedimentation migration distances. The maximum theoretical
electrophoretic migration distance is equal to the sum of the maximum SUE
and maximum sedimentation migration distances, assuming independence of
the 2 properties. The weighted average electrophoretic migration dis-
tance is equal to the sum of the weighted average SUE and weighted
average sedimentation distances.

The effect of motility is not considered in the calculations of either
the observed or the theoretical electrophoretic mobility. A comparison of
the electrophoretic mobilities and an interpretation of the results are
presented in the Discussion.

3. <u>Sedimentation-Electrophoresis: Low Electric Field Strengths.</u>

Table XXVIII is a summary of the characteristics of the spermatozoa
sample and the Staflo operating parameters for sedimentation-upward- and
-downward-electrophoresis experiments with low electric field strengths.
Table XXIX summarizes the distribution of spermatozoa among the collection
fractions and the phase contrast observations and results of nigrosin-eosin
staining thereof, in those experiments.

a. Upward Electrophoresis

(1) <u>Experiment 7-9-67</u>. The spermatozoa sample used in this experiment
initially had a fair degree of forward motion at room temperature and a
slight degree of tail-wagging in a hemocytometer at 3.5°C. Observations
with the Staflo microscope showed that after the spermatozoa entered the
electric field, all had a vertical orientation with head down and tail up.
Mild twisting and rolling of spermatozoa was observed in the lower part of
the migration pattern only. The migration pattern at pH 7.1, 3.9°C and an
electric field strength of 1.2 V/cm was stable throughout the experiment
and gave spermatozoa concentrations of 4.32 x 10^6 and 2.65 x 10^6/ml in

Table XXVIII. Spermatozoa Sample and Staflo Operating Parameters for Sedimentation-Electrophoresis Experiments Using Low Electric Field Strengths.[a]

Experiment Number	Characteristics of Spermatozoa Sample					General Staflo Operating Parameters								
	Rabbit		Cooling Medium	Quality		Initial Sample Conc. (units of 10^6/ml)	pH	Temp. RT Staflo (°C) (°C)		τ_{ave} (min)	τ_{min} (min)	τ_E (min)	L (cm)	$\tilde{E}$ (V/cm)
	Name	Breed		Initial	Final									
A. SUE														
7-9-67	Larry	NZW	EYM	Fair	Poor	20.5	7.10	3.8	3.9	30.7	20.5	17.1	25	1.2
10-3-67	Snoopy	Calif.	GPB	Poor	Poor	21.7	6.60	2.2	2.3	37	24.7	20.6	25	1.7
B. SDE														
9-29-67 (2 expts.)	Old Man	NZW	GPB	Good	Fair	19.6	7.05	2.7	2.7	30.0	20.0	16.7	25	0.6

a. See Glossary for explanation of definitions.

Table XXIX. Summary of Distribution, Phase Contrast Observations and Nigrosin-Eosin Staining of Spermatozoa; Sedimentation-Electrophoresis Experiments Using Low Electric Field Strengths.[a]

Experiment Number	pH	Spermatozoa Sample	Sperm Collected (10^6/ml)	(%)	Temp. (°C)	Phase Contrast Observations C (%)	CL (%)	TW (%)	FM (%)	Nigrosin-Eosin Staining U (%)	MNS (%)	S (%)
A. SUE												
7-9-67	7.10	CF 7	4.32	61.8	3.9	86.5	13.5	0	0	–	–	–
					22	–	–	36.4	1.0	74	1.5	24.5
		CF 8	2.65	38.2	3.9	88.7	12.3	0	0	–	–	–
					22	–	–	31.2	1.0	75	7	18
		IS			3.9	86	14	0	0	–	–	–
					22	–	–	41.7	3.0	79.5	2	18.5
10-3-67	6.60	CF 8	5.45	83	2.2	76	24	0	0	–	–	–
					23	32.0	56.4	10.5	1.0	65	4	31
		CF 9	1.05	17	2.2	77.5	22.5	0	0	–	–	–
					23	36.0	47.5	10.2	6.3	68	2.5	29.5
		IS			2.2	83.6	16.4	0	0	–	–	–
					23	39.4	46.8	10.5	3.3	57.0	1.5	41.5
B. SDE												
9-29-67 (Two Parts)	7.05	CF 8-1	3.44	49.2	2.7	56.8	43.2	0	0	–	–	–
					22	–	–	–	~50	63	0.5	36.5
		CF 9-1	3.55	50.8	2.7	58.0	42.0	0	0	–	–	–
					22	–	–	–	~50	66	0.7	32.3
		CF 8-2	1.5	37.5	2.8	47.2	52.8	0	0	–	–	–
					22	–	–	–	~50	64.5	0.5	35
		CF 9-2	2.5	62.5	2.8	48.3	51.7	0	0	–	–	–
					22	–	–	–	~50	72.5	1.0	26.5
		IS			2.7	83.6	12.2	0.2	0	–	–	–
					22	–	–	–	~50	70.5	0	29.5

a. See Glossary for explanation of abbreviations.

fractions 7 and 8, respectively. At room temperature, the spermatozoa in CF 7 had a slightly higher percentage of tail-wagging than CF 8, and a slightly lower percentage than in the initial sample. There were no other differences among the spermatozoa samples at room temperature or at 3.9°C, as shown in Table XXIX A.

(2) Experiment 10-3-67. This experiment was conducted at pH 6.6, 2.3°C and an electric field strength of 1.7 V/cm. Even though the sample stream at the inlet was in the same location as in Experiment 7-9-67, the migration pattern was lower and gave spermatozoa concentrations of 5.45×10^6 and 1.05×10^6/ml in fractions 8 and 9, respectively. In the region of the electric field, spermatozoa were oriented vertically with the tail up and head down. No motility was observed in the flow-chamber or in a hemocytometer at 2.2°C.

There were no significant differences among the collection fractions and the initial sample with regard to the percentages of capped spermatozoa at 2.2°C or capped and tail-wagging spermatozoa at 23°C. Because of the extremely poor motility, the differences among the samples is not believed to be significant. The slightly higher percentages of unstained spermatozoa in the fractions compared to the initial sample are probably due to the staining procedure.

The differences between Experiment 7-29-67 and 10-3-67 are considered in the Discussion.

b. Downward-Electrophoresis. Experiment 9-29-67 consisted of 2 separate Staflo fractionations using the same spermatozoa sample and the same operating conditions. Even though there was no change in the location of the sample stream at the inlet during the second experiment, there is a slight possibility of a change in the migration pattern during that experiment because of the very slow rate at which equilibrium is re-established in

in the flow-cell after emptying the collection containers. This may explain the slight difference in distribution of spermatozoa between the 2 collection fractions in the 2 experiments as shown in Table XXIX B. Approximately 50% of the spermatozoa had a high degree of forward motion at room temperature before the initial cooling, but no motility was observed in a hemocytometer at 2.7°C. Except for a slightly higher percentage of unstained spermatozoa in CF 9-2 compared to CF 8-2, there were no differences between the respective fractions in the 2 parts of the experiment at 2.8°C or at room temperature.

In the region of the electric field, with the bottom electrode positive, there was a slight differential orientation of the spermatozoa in the top and bottom of the migration profile. Near the top, the orientation ranged from nearly horizontal to a 45° angle from the horizontal with the tail angled downward. Near the bottom, the orientation ranged from the last mentioned 45° angle to just beyond the horizontal so that the head was in a slightly downward position. A higher percentage of spermatozoa was obliquely or horizontally oriented than in any other type of Staflo experiment reported in this thesis.

The expected average sedimentation migration distance is greater than the observed average SDE migration distance. This unexpected result and the quantitative aspects of the experiment are considered in the Discussion.

D. Discussion

1. Sedimentation

a. Comparison of Three Experiments. As shown in Table XXI, there were no significant differences among the collection fractions in individual experiments with regard to the distribution of capped spermatozoa at low temperatures or of unstained spermatozoa at room temperature. Insufficient

observations were made to evaluate fractionation with regard to motility. Thus, within the limitations of the two- and three-layer fractionations of spermatozoa samples in this series of experiments, there does not appear to be any influence of morphology or of viability on the sedimentation velocity. Because the quality of the spermatozoa was inadequate for artificial insemination, the relationship between sedimentation velocity and chromosomal content of the spermatozoa could not be evaluated.

In Experiment 12-26-67, the minimum sedimentation velocity was estimated to be 0 cm/sec inasmuch as some spermatozoa were observed in the top part of the migration pattern. The maximum sedimentation velocity for the three experiments ranges from $0.87-1.24 \times 10^{-4}$ cm/sec. This wide range may be a partial reflection of the difficulty in accurately measuring small migration distances in the flow-chamber. The minimum and maximum sedimentation velocities calculated for the $2.5°C$ sucrose series of early sedimentation experiments (Chapter IV), in a similar medium, are 0 and 1.07×10^{-4} cm/sec, respectively, indicating good agreement between the two series of experiments. As expected, the weighted average sedimentation velocity range of $0.43-0.53 \times 10^{-4}$ cm/sec for the three experiments at $3°C$, encompasses the average of 0.49×10^{-4} cm/sec for the $2.5°C$ sucrose experiments (Chapter IV). The agreement between the two sets of sedimentation experiments in the low density-low viscosity media strengthens the impression stated in Chapter IV that the apparent sedimentation velocities in the experiments by Bhattacharya[35] and by others[29,205] are high.

In Experiment 12-26-67, the average sedimentation velocity calculated from the minimum and maximum sedimentation distances is 0.523×10^{-4} cm/sec compared to the weighted average sedimentation velocity of 0.53×10^{-4} cm/sec. The excellent agreement between these 2 methods of calculating the average

sedimentation velocity suggests a uniform distribution in the migration pattern. However, the small migration distance is a limiting factor in this interpretation of the results.

b. **Migration Comparison: Actual vs. Hypothetical Spermatozoa Populations**

Figure 55 is a comparison of the actual distribution of spermatozoa in Experiment 12-26-67 with the expected distribution of hypothetical spermatozoa populations characterized by uniform, bimodal and normal (Gaussian) distributions of sedimentation velocities over the range $0-0.99 \times 10^{-4}$ cm/ sec. As shown in Figure 55, the observed distribution of spermatozoa among collection fractions 8, 9 and 10 was 23.1%, 61.6% and 15.3%, respectively. With a uniform population, one would expect 23.6%, 68.9% and 7.5% of the spermatozoa in fractions 8,9 and 10, respectively, under the same Staflo operating conditions. The 2 distributions compare favorably except for the higher percentage of spermatozoa in fraction 10 in Experiment 12-26-67. This might be due either to the observed mild degree of microscopic streaming, or to a skewed distribution of the actual sedimentation velocities toward the maximum.

Comparison of the actual and the hypothetical bimodal distributions in Figure 55 shows good agreement in fraction 10 but not in fraction 8 where there is a higher percentage of spermatozoa in the bimodal distribution. A difference of the same magnitude, but in the opposite direction, is seen in fraction 9. In Figure 55, spermatozoa are shown distributed evenly throughout layer 9 because a sperm count in that collection fraction would not give information on the differential distribution of spermatozoa in the 0.125 cm high layer represented by that fraction.

As shown in Figure 55, the expected distribution of spermatozoa among the collection fractions in the case of a population with a normal

COMPARISON OF ACTUAL SPERM DISTRIBUTION
AND HYPOTHETICAL POPULATIONS
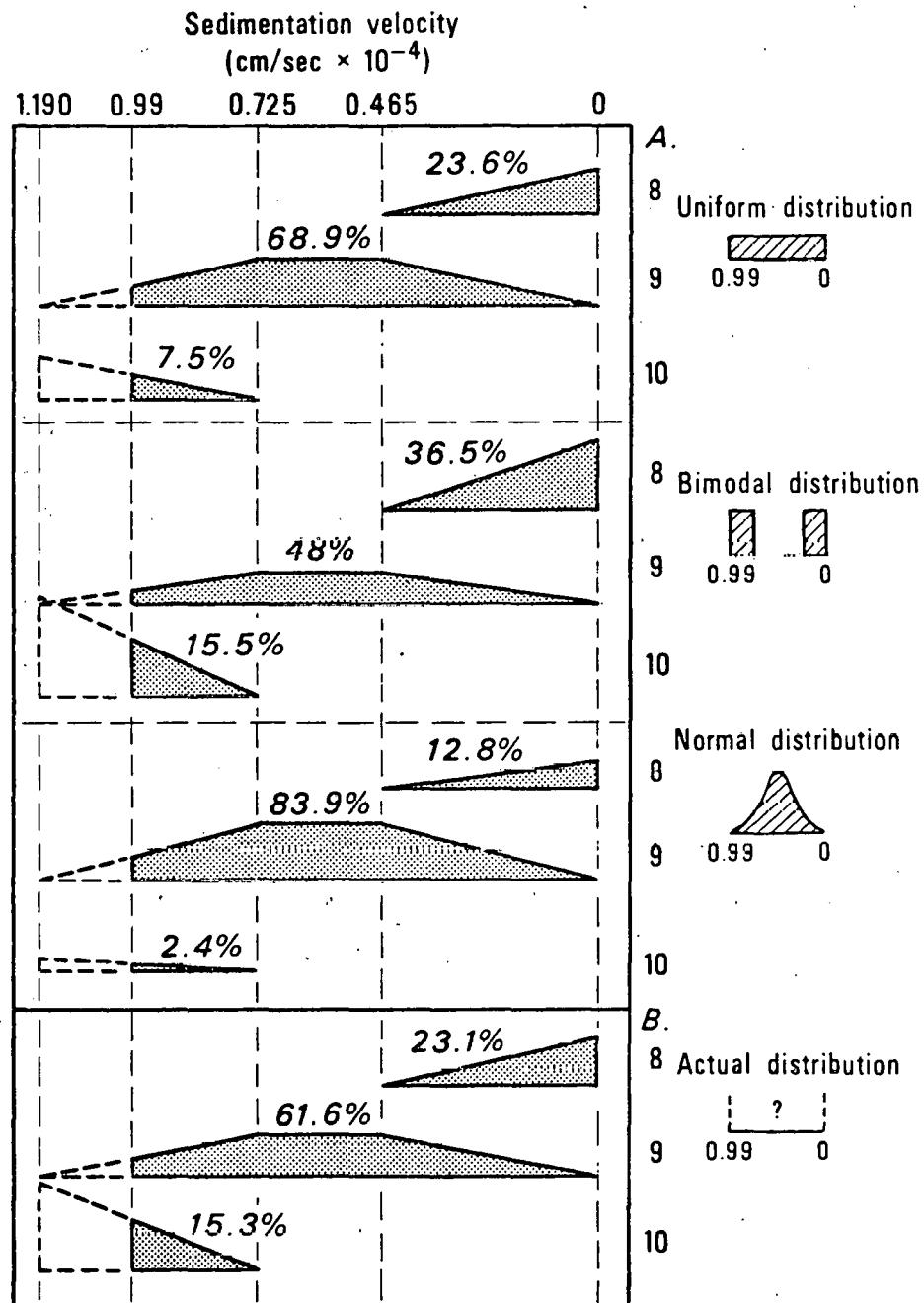
DBL 7011 5993

Fig. 55. Distributions of rabbit spermatozoa for actual, and three
hypothetical distributions of sedimentation velocities.

distribution of sedimentation velocities differs widely from the actual distribution. This is the least likely possibility of the three hypothetical distributions considered. Even recognizing the limitations of a 3-layer fractionation of the spermatozoa population in the actual experiment, the comparison of the normal distribution with the actual distribution is very unfavorable.

Of the three hypothetical distributions in Figure 55, the uniform distribution compares most favorably with the actual experiment. A better evaluation of the type of distribution of sedimentation velocities in a population of spermatozoa would be possible if the sedimentation migration pattern spread over 5 or 6 layers, thereby allowing a more quantitative comparison of actual and hypothetical distributions.

2. Sedimentation-Upward-Electrophoresis (SUE): High Electric Field Strengths.

As shown in Table XXIV, the spermatozoa samples and Staflo operating conditions differed considerably among the experiments in this series. The migration profile generally spread from an inlet sample thickness of 0.7 mm to a final height of 2.0 mm or less in the centerline of the flow-chamber. Except for one experiment, in which essentially all of the spermatozoa exited the flow-chamber in one layer, the fractionation was between two layers. The ten experiments will be evaluated in terms of (1) the distribution of categories of spermatozoa between the collection fractions; (2) the effects of pH, egg yolk medium, breed of rabbit and temperature on the electrophoretic mobilities; and (3) the relationships between the concentration of spermatozoa and biological results.

a. Comparison of Experiments. The distribution of capless, tail-wagging, highly motile and viable (unstained) spermatozoa in 9 experiments was

evaluated using a difference of 10% between the 2 collection fractions as an arbitrary level of significance. In 7 of the 9 experiments, there was no difference with regard to the distribution of capless spermatozoa immediately after collection (at the low temperature). In the 2 other experiments, there was a lower percentage of capless spermatozoa in the lower collection fraction. Thus, the presence of the acrosomal cap appears to have little influence on the distribution of spermatozoa between the 2 collection fractions.

In 8 of the 10 experiments, there are nigrosin-eosin staining results on 2 fractions. In 4 experiments, there was a higher percentage of un-stained spermatozoa in the lower of the 2 collection fractions. In 3 ex-periments, there was less than a 10% difference between the 2 fractions, and, in 1 experiment, the bottom fraction contained a lower percentage of unstained spermatozoa. Although not conclusive, these results suggest that the lower of the 2 collection fractions contained spermatozoa of slightly better quality.

In 8 of the 10 experiments, there was sufficient tail-wagging and/or forward motion to assess the relative distribution of these properties. In Experiment 10-2-67, one of 2 experiments in which there was no forward motion, the percentage of tail-wagging spermatozoa at 23°C was 18.2% higher in the lower collection fraction. In Experiment 9-22-67, the per-cent tail-wagging was less than 7% at room temperature, and there was no significant difference between fractions 6 and 7. Both tail-wagging and forward motion were evaluated in Experiments 7-26-67 and 7-29-67. As shown in Table XXV, there was a slightly higher percentage of spermatozoa with forward motion in the lower fraction (CF 7) in both experiments. In contrast, CF 6 in Experiment 7-29-67 had 24% more tail-wagging spermatozoa than did CF 7. There was no difference in Experiment 7-26-67 in regard to

the distribution of tail-wagging spermatozoa.

In 4 experiments with high quality spermatozoa, only forward motion was evaluated. In Experiments 8-29-67 and 9-18-67, there was a significantly higher percentage of motile spermatozoa in the lower of the 2 collection fractions whereas there was no difference in Experiments 9-13-67S and 9-13-67P. The temperature in the flow-chamber was approximately 4.2°C in Experiment 8-29-67 compared to 2.8 and 3.1°C in Experiments 9-13-67S and 9-13-67P, respectively. This could explain the differences among those experiments; however, there was relative enrichment of the lower fraction in Experiment 9-18-67 even though the flow-chamber temperature was only 3.2°C.

Therefore, the strongest statement that one can make with regard to the distribution of motile spermatozoa is that there is a tendency for the lower of the 2 collection fractions to have a higher percentage of spermatozoa with forward motion. This is consistent with the idea that in these experiments the intrinsic motility vector is directed downward against the upward force of the electric field. With spermatozoa samples of the same initial quality, the important variable then becomes the flow-chamber temperature which determines the magnitude of the intrinsic motility vector.

b. _Interpretation of Electrophoretic Mobilities_. It is seen in Table XXVII that the theoretical average electrophoretic mobilities (Column 9) are significantly greater than the uncorrected (observed) average SUE electrophoretic mobilities in column (6). As shown in Appendix G, the correction for sedimentation in the calculation of the weighted average electrophoretic migration distance in Experiment 7-29-67 is 20.8% of the observed average migration distance. In other experiments, this correction ranges from 12-28%. As expected, the average, uncorrected electrophoretic

mobility (6) calculated from the observed migration distance lies be-
tween the minimum (4) and maximum (5) observed electrophoretic mobilities.

With regard to the theoretical electrophoretic mobility calculations,
the minimum (7) is greater than the average (9) in 7 of the 10 experiments.
The theoretical minimum electrophoretic migration distance was assumed to
be equal to the sum of the minimum observed SUE migration distance and
the expected maximum sedimentation distance. The discrepancy between
columns 7 and 9 suggests that the maximum sedimentation velocity from
Experiment 12-26-67, which was used in the calculations, is too great.
The small degree of microscopic streaming observed at the bottom of the
sedimentation migration pattern in that experiment may have given a false
maximum sedimentation velocity. This supports the suggestion that the
difference between the actual and uniform distributions of spermatozoa in
Figure 55 is due to a small degree of microscopic streaming.

The theoretical electrophoretic mobility ranges from 0.51×10^{-4} (cm/
sec)/(V/cm) at pH 6 to 1.18×10^{-4} (cm/sec)/(V/cm) at pH 7.10. Three of
4 electrophoretic mobilities determined at pH 6.6 range from $0.53-0.55 \times$
10^{-4} (cm/sec)/(V/cm), while one measurement at that pH gave a value of
1.06×10^{-4} (cm/sec)/(V/cm). Similarly, 4 of the 5 values of electrophore-
tic mobility determined at pH 7.05-7.15 range from $0.75-0.94 \times 10^{4}$ (cm/sec)/
(V/cm), while the fifth value is 1.18×10^{-4} (cm/sec)/(V/cm). Inaccuracy
in measuring the migration distance may explain some, but not all, of the
discrepancy in the 2 values of electrophoretic mobility which appear to
be somewhat higher than other values in the series of measurements. Other
variables to consider are pH, the effect of egg yolk medium, the breed
of the rabbit and temperature.

(1) <u>Effect of pH</u>. The two apparently high values of electrophoretic

mobility in Experiments 8-18-67 and 9-22-67 were obtained at pH 6.6 and pH 7.1, respectively. The agreement among the other experiments at each of those pH's was quite good. There is no question about the pH of the medium in those 2 experiments.

Experiments 10-2-67, 9-13-67P and 9-18-67 were conducted with spermatozoa from rabbit Psi at pH 6, pH 6.55 and pH 7.15, respectively. The calculated theoretical average electrophoretic mobilities for these 3 experiments are 0.51, 0.55 and 0.89 x 10^{-4}(cm/sec)/(V/cm), respectively. The 3 experiments were conducted at temperatures ranging from 3.1°C-3.3°C, and, therefore, the normalization to 3°C was a minor correction. A similar increase in the electrophoretic mobility with increase in pH was observed in the other experiments in this series. Because only one experiment was conducted at pH 6, the small increment in electrophoretic mobility from pH 6 to pH 6.55 compared to the increment between pH 6.55 and pH 7.15 is of questionable significance.

(2) <u>Effect of Egg Yolk Medium.</u> Spermatozoa from rabbit O were used in Experiments 8-18-67, in which egg yolk medium was used for the initial dilution and cooling, and in 8-29-67, in which the initial dilution and cooling was with the glycine-phosphate buffer. Both experiments were conducted at approximately pH 6.6 and gave electrophoretic mobilities of 1.06 and 0.53 x 10^{-4} (cm/sec)/(V/cm), respectively. This suggests an increased electrophoretic mobility for spermatozoa cooled and diluted in egg yolk medium. However, comparison of the electrophoretic mobility of the spermatozoa from rabbit Psi in Experiment 8-10-67, using egg yolk medium, and in Experiment 9-18-67, using glycine-phosphate buffer for cooling shows only a difference between 0.94 and 0.89 x 10^{-4} (cm/sec)/(V/cm), respectively. In Experiments 7-26-67 and 9-22-67, the spermatozoa were cooled in glycine-phosphate buffer and electrophoretic mobilities of 0.75 and 1.18 x 10^{-4}

(cm/sec)/(V/cm), respectively, were obtained at pH 7.10. Therefore, factors other than egg yolk medium are responsible for the two high values of electrophoretic mobility observed in this series of experiments. As noted in Chapter II, Nevo did not observe any influence of egg yolk medium on the electrophoretic mobilities of rabbit and bull spermatozoa[174].

(3) <u>Effect of Breed of Rabbit</u>. In Experiments 9-13-67S and 9-13-67P, identical Staflo operating conditions except for the strength of the electric field were used with spermatozoa from S, a California rabbit, and Psi, a Dutch Belt rabbit. The calculated average theoretical electrophoretic mobilities were 0.55×10^{-4}(cm/sec)/(V/cm) at pH 6.55 for the spermatozoa from both rabbits. Experiment 7-29-67, at pH 7.05 with spermatozoa from L, a California rabbit, and Experiment 9-18-67, at pH 7.15, with spermatozoa from Psi gave electrophoretic mobilities of 0.86 and 0.89 $\times 10^{-4}$ (cm/sec)/(V/cm), respectively. The agreement between these two sets of experiments at different pH's with spermatozoa from California and Dutch Belt rabbits suggests that there is no differential electrophoretic mobility because of breed, at least within the range of accuracy of these measurements.

(4) <u>Effect of Temperature</u>. In approximately one-half of these experiments, there was a higher percentage of motile and/or tail-wagging spermatozoa and of unstained spermatozoa in the lower of the two collection fractions. Experiment 9-22-67 was conducted at 7°C in a deliberate attempt to enrich the lower collection fraction with regard to motile spermatozoa. However, because the spermatozoa sample as a whole lost motility rapidly during the experiment, no enrichment was obtained. Inadvertent enrichment with regard to both forward motion and unstained spermatozoa was obtained in Experiment 8-29-67. At 4.2°C, 60% of the spermatozoa in CF 7 had for-

ward motion compared to only 40% in CF 6. A similar differential distribution of motile spermatozoa was observed in Experiment 9-18-67 at 3.2°C. The role of intrinsic motility in the latter experiment is questionable because observations in a hemocytometer initially at 2.8°C and later at 3°C showed no motility. Also, there was no enrichment of the lower collection fraction in Experiments 9-13-67S and 9-13-67P, at 2.8°C and 3.1°C, respectively, in which the spermatozoa were of extremely high initial and final quality. These observations suggest that with electric field strengths of about 6 V/cm, the intrinsic motility of spermatozoa becomes a significant factor at temperatures of approximately 4°C and above. In addition to temperature control, it is necessary to have high quality spermatozoa in order to achieve enrichment of collection fractions.

c. <u>Concentration of Spermatozoa: Effect on Artificial Insemination</u>. The higher quality of spermatozoa in the lower of the two collection fractions is also demonstrated by the relatively greater number of successful inseminations with spermatozoa from that fraction. As shown in Table XXX, 4 of 5 inseminations with spermatozoa from the upper collection fraction were adequate technically, but only 1 resulted in a litter (3 offspring). Six of 8 inseminations with spermatozoa from the lower fraction were adequate and 5 resulted in litters with a total of 15 offspring. All 4 control inseminations were good but only 1 resulted in a litter.

On further analysis, all of the inseminations with "Staflo spermatozoa" which resulted in offspring were from collection fractions in which the spermatozoa concentration was always greater than 4×10^6/ml, even in the Staflo apparatus. In Experiment 9-13-67, in which the only litter resulted from the insemination of spermatozoa from the upper collection fraction, the concentration in the collection container was 4.25×10^6/ml

Table XXX. Analysis of Artificial Insemination Results
by Spermatozoa Sample Source.

Sperm Sample	Number of Inseminations		Litters (No.)	Offspring (No.)			
	Total	Good		♂	♀	Unk	Total
Upper Fraction	5	4	1	1	1	1	3
Lower Fraction	8	6	5	9	5	1	15
Control	4	4	1	1	0	0	1
Totals	17	14	7	11	6	2	19

before centrifugation. In the other 3 experiments, no offspring were obtained from the insemination of spermatozoa from the upper collection fractions which had pre-centrifugation concentrations ranging from 1.71-2.5 x 10^6/ml. In the 3 experiments in which insemination of spermatozoa from the lower collection fraction resulted in offspring, the concentrations in the fractions prior to centrifugation were 4.6, 5.6 and 8.0 x 10^6/ml. In the fourth experiment, the spermatozoa concentration in the lower fraction was 5.2 x 10^6/ml. Unfortunately, the inseminate in the latter experiment was rejected by the female and no litter was obtained. In summary, 6 of the 7 inseminations with spermatozoa from collection fractions having initial concentrations of greater than 4 x 10^6/ml gave litters and the one insemination that did not was rejected by the female rabbit. Inasmuch as only 1 of 4 control inseminations, in which the spermatozoa had been stored in egg yolk medium, gave litters, the success rate of inseminations with the "Staflo spermatozoa" is considered excellent. Thus, it appears that the minimum concentration of spermatozoa in a Staflo collection fraction at which fertilizing capacity is preserved is approximately 4 x 10^6/ml.

The loss of fertilizing capacity at concentrations of less than 4 x 10^6 spermatozoa/ml in the collection fractions is in marked contrast to the preservation of motility at concentrations as low as 1.71 x 10^6/ml (Experiment 8-29-67). In addition, in Experiment 9-13-67S, a superior degree of motility was preserved in fraction 6 at a spermatozoa concentration of 1.9 x 10^6/ml for over 2 hours.

As noted in Table XXX, 14 of the 17 inseminations in the 4 experiments with high quality spermatozoa were considered to be technically adequate. Litters were obtained in 7 of the 14 good inseminations (50%) but the number of offspring was disappointingly low. This is believed to be a

reflection of the anestrous condition of many female rabbits which had
not littered in the preceding year.

3. Sedimentation-Electrophoresis: Low Electric Field Strengths

a. Upward Electrophoresis (SUE). The operating parameters and distribu-
tion of spermatozoa among the collection fractions in Experiments 7-9-67
and 10-3-67 are presented in Table XXXI. Despite the higher electric field
strength and longer electrophoresis time in Experiment 10-3-67, the mi-
gration pattern was significantly lower in the flow-chamber than in
Experiment 7-9-67. The difference is not completely explained by the lower
electrophoretic mobility of spermatozoa at pH 6.6. Even though no abnor-
mality in the migration pattern was observed, it is probable that a slight
shift in the pattern accounts for the discrepancy in these results. Thus,
these experiments illustrate the necessity for unequivocal steady-state
conditions in the Staflo apparatus, particularly when the migration dis-
tances are very small. In addition, in experiments such as these, it would
be desirable to fractionate the samples with greater resolution than the
1.25 mm layer height in current flow-cell models.

In Experiment 7-9-67, the estimated average sedimentation distance was
approximately 42% of the total estimated electrophoretic migration distance.
In Experiment 10-3-67, the estimated average sedimentation distance was
significantly greater than the weighted average SUE migration distance.
This confirms the impression that steady-state conditions did not prevail
in the flow-chamber during the experiment.

The strength of the electric field required to balance the sedimenta-
tion of spermatozoa in the Staflo apparatus has been calculated under con-
ditions of a temperature of 3°C using an average transit time of 28 minutes,
an electrode length of 25 cm, and a weighted average sedimentation velocity

Table XXXI. Comparison of Staflo Operating Parameters and
Results of Sedimentation-Upward-Electrophoresis Experiments
Using Low Electric Field Strengths.

Parameter	Experiment Number	
	7-9-67	10-3-67
$\tilde{E}$ (V/cm)	1.2	1.7
pH	7.1	6.6
Temperature (°C)	3.9	2.3
Electrophoresis Time (min)	17.1	20.6
Motility in Staflo	Slight	None
Distribution		
Collection fraction 7	61.8%	2%
Collection fraction 8	38.2%	82%
Collection fraction 9	0	15.8%
Calculated Electrophoretic Mobility (cm/sec/V/cm)	1.23×10^{-4}	0.49×10^{-4}

of 0.53×10^{-4} cm/sec. The weighted average electrophoretic mobilities used in the calculations are 0.55 and 0.90×10^{-4} (cm/sec)/(V/cm), at pH 6.6 and pH 7.10, respectively. The electric field strengths required under these conditions are 0.96 and 0.59 V/cm at pH 6.6 and 7.10, respectively.

b. <u>Downward Electrophoresis (SDE)</u>. The differences in the migration pattern and in the distribution of the various categories of spermatozoa among the respective collection fractions in the 2 parts of Experiment 9-29-67 are not remarkable. They may be due to a delayed return to steady-state conditions in the second part of the experiments after emptying the collection containers between runs, as mentioned earlier.

The expected average sedimentation distance of 0.064 cm is greater than the observed average SDE migration distance of 0.046 cm in part 1 of Experiment 9-29-67. Inasmuch as the electrophoretic migration was in the same direction as sedimentation, one would have expected a greater combined (SDE) migration distance than with either the sedimentation or electrophoretic component alone. It is believed that the change from the primarily vertical orientation seen in the sedimentation experiments to the more horizontal orientation observed in this experiment significantly changed the hydrodynamic properties of the migrating spermatozoa. Thus, the increased frictional force due to the "broadside" orientation of the spermatozoa to the direction of migration may have reduced the net migration rate to less than that expected with either sedimentation or electrophoresis in a more vertical orientation.

4. <u>Orientation of Spermatozoa</u>

The differential orientation of spermatozoa in the flow-chamber under conditions of different electric field strength and direction of migration is shown in Figure 56. In SUE experiments with both high and

ORIENTATION OF SPERMATOZOA IN STAFLO EXPERIMENTS
(3-5°C)

Experiment	Direction of electric field and migration		$\tilde{E}$ (v/cm)	pH	Orientation of spermatozoa Predominant	Range
7-26-67	+ −	↑	5.4	7.05		
7-9-67	+ −	↑	1.2	7.10		
12-26-67	none	↓	0	7.10		
9-29-67	− +	↓	0.6	7.05	Top Bottom	Rare
7-14-66	− +	↓	6.3	7.10		

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Fig. 56. Orientation of rabbit spermatozoa in Staflo sedimentation and sedimentation-electrophoresis experiments at 3-5°C.

low electric field strength, essentially all of the spermatozoa were oriented in a vertical direction with the tail pointing toward the top (positive) electrode. An orientation different from this was extremely rare at low temperatures, even when there was a slight degree of twisting or other expression of intrinsic motility of spermatozoa in the flow-chamber. No difference in orientation was observed at different locations within the migration pattern in the region of the electric field.

In sedimentation experiments, the spermatozoa were oriented with the heads down and tails upward, ranging up to 45° on either side of the vertical position. Usually the head pointed in the direction of the flow with the tail trailing slightly.

In SDE experiments with high electric field strengths, the spermatozoa were oriented vertically with the tail pointing toward the bottom (positive) electrode. In SDE experiments with low electric field strengths, however, the orientation of the spermatozoa differed slightly at the top and bottom of the migration profile. At the top, it ranged from a horizontal position to primarily a 45° angle with the head up and the tail pointing obliquely toward the bottom (positive) electrode. Very few spermatozoa were observed in positions outside of this range. At the bottom of the profile, some spermatozoa heads were pointing slightly downward as shown in Figure 56. Because SDE experiments were not conducted with electric field strengths between 0.6-6.0 V/cm, the minimum electric field strength required to give a vertical orientation with the tail pointing downward toward the bottom (positive) electrode was not determined.

The partially horizontal position assumed by spermatozoa in the SDE experiments with low electric field strengths, shown in Figure 56, is believed to result from the competition between the force of gravity acting

on the very dense head and the electrophoretic force on the tail which has a relatively greater negative charge than the head at pH 7. This orientation is believed to explain the discrepancy between the expected SDE migration distance and the observed migration distance in that series of experiments. Thus, it appears that in order to test the hypothesis presented in Chapter III with regard to the separation of spermatozoa characterized by the fastest sedimenting-fastest electrophoresing and alternate combinations of these properties, it will be necessary to determine the minimum electric field strengths at which the spermatozoa assume a vertical orientation in SDE experiments.

5. Quality of Spermatozoa

On many occasions, spermatozoa initially had a high percentage and degree of forward motion which was lost shortly after starting the experiment. The effect of the environment and other factors on the initial quality of spermatozoa and factors influencing the preservation of viability, motility and fertilizing capacity are considered in Chapter IX.

The effects of spermatozoa concentration on the preservation of viability, motility and fertilizing capacity were discussed in 2,c above. In experiments of this series, with concentrations as different as 1.05 x 10^6 and 5.45 x 10^6 spermatozoa/ml, there was no difference between the 2 collection fractions with regard to the percentage of capless and unstained spermatozoa. In Experiment 9-13-67S, spermatozoa in CF 6 with a concentration of 1.9 x 10^6/ml had essentially the same percentage and degree of motility as the spermatozoa in CF 7 with a concentration of 8 x 10^6/ml. In Experiment 8-29-67, CF 6 with a concentration of 1.71 x 10^6/ml had the same percentage of capped spermatozoa as CF 7 with 5.60 x 10^6/ml. However, after reconcentration and suspension in egg yolk medium,

CF 6 had a lower percentage of motile and unstained spermatozoa, suggesting that the stress of centrifugation was more detrimental to the spermatozoa which had been exposed to the lower concentration. Thus, morphology, viability and motility appear to be preserved in the Staflo apparatus at spermatozoa concentrations above 1×10^6/ml. This is considerably lower than the concentration of 4×10^6/ml apparently required for the preservation of fertilizing capacity.

In several SUE experiments with short average transit times, the percentage and degree of motility of the spermatozoa in the collection fractions was essentially the same as in the initial sample which had been stored at a concentration of approximately 20×10^6/ml. The longest time during which spermatozoa were exposed to low concentrations in the Staflo apparatus and maintained their motility was approximately 6 hours. This was observed in Experiment 9-13-67S in which the spermatozoa quality was superior. In 4 experiments with high quality spermatozoa, artificial insemination of "Staflospermatozoa" gave litters with viable offspring. Thus, this series of experiments shows that glycine-phosphate buffer preserves the motility and fertilizing capacity of rabbit spermatozoa for several hours during Staflo sedimentation-electrophoresis experiments if the initial quality of the spermatozoa is excellent.

Reconcentration of the spermatozoa in the collection fractions by centrifugation prior to insemination resulted in 5-40% increases in the percentages of capless spermatozoa with the greatest increases occurring in the samples of poorest quality. This confirms the general observation that a spermatozoa sample of high initial quality remains good throughout the procedure involved in Staflo experiments, whereas spermatozoa of poor initial quality deteriorate rapidly. This observation is discussed

in more detail in Chapter IX.

Clumping rarely was a problem in sedimentation or sedimentation-
electrophoresis experiments. No clumping was observed in the glycine-
phosphate buffer at any temperature, or in the egg yolk medium at cold
room temperatures. In Experiment 9-29-67, after resuspension in egg yolk
medium, the spermatozoa were not clumped in the cold room but clumped
immediately upon warming to room temperature. The spermatozoa used in
that experiment were from an old New Zealand White rabbit whose sample
happened to be the highest quality on that particular day. Such clumping
was not observed in any other experiment.

E. Summary and Conclusions

The glycine-phosphate buffer evaluated in Chapters V and VI was used
in all of these experiments. In a 3-layer sedimentation-fractionation of
rabbit spermatozoa at 3°C with concentrations in the collection containers
ranging from 0.72-2.91 x 10^6/ml, there were no differences among the
fractions with regard to morphology (evaluated by phase contrast micro-
scopy) or viability (judged by nigrosin-eosin staining). The distribution
of the spermatozoa among the collection fractions compared favorably to
that expected with a hypothetical population of cells having a uniform
distribution of sedimentation velocities. The slight difference between
the actual and hypothetical distributions may be due to a small degree of
microscopic "cluster sedimentation" observed in the flow-chamber. Compari-
son of the actual with hypothetical bimodally or normally distributed pop-
ulations showed poor agreement.

For 3 experiments, the minimum sedimentation velocity was 0 cm/sec,
the average ranged from 0.43-0.53 x 10^{-4} cm/sec, and the maximum from
0.87-1.24 x 10^{-4} cm/sec at 3°C. Average values for all 3 experiments were

0, 0.48 x 10^{-4} and 1.06 x 10^{-4} cm/sec, respectively. Within the limits

of measurement in the Staflo apparatus, these averages are the same as

those obtained in the 2.5°C sucrose experiments in a similar low density-

low viscosity medium (Chapter IV): 0, 0.49 x 10^{-4} and 1.07 x 10^{-4} cm/sec,

respectively.

In these particular experiments, the calculation of electrophoretic

mobilities included a correction for sedimentation which was estimated to

be 12-28% of the average "true" electrophoretic migration distance. After

making these corrections, the average electrophoretic mobilities of rabbit

spermatozoa at pH 6.0, 6.6 and 7.10 are 0.51, 0.54 and 0.86 x 10^{-4}(cm/sec)/

(V/cm), respectively, corrected to 3°C. The latter value is essentially

the same as that obtained in Chapter V for both upward- and downward-

electrophoresis. The numerical values of electrophoretic mobility were

not influenced by the breed of the rabbit or by prior exposure of the

spermatozoa to egg yolk medium.

In 7 of 9 sedimentation-upward-electrophoresis (SUE) experiments

with high electric field strengths, no differences were found in percentages

of capped spermatozoa between the different fractions from a given experi-

ment, evaluated immediately after collection. The evaluation at room tem-

perature suggested that the lower collection fraction was better with

respect to morphology, viability, motility and fertilizing capacity. As

was previously suggested in Chapter VI, this may be a reflection of motility

in the flow-chamber and/or generally higher spermatozoa concentrations

therein. No differential distribution of motile spermatozoa between

fractions was found where the average flow-chamber temperature was 2.8°C,

even with superior quality spermatozoa. However, at an average temperature

of 4.2°C, absolute enrichment of the lower fraction was achieved. Thus,

in the glycine-phosphate buffer with electric field strengths of about 6 V/cm,

intrinsic motility appears to become a significant factor at about 4°C
and becomes increasingly important at higher temperatures.

Although motility was well preserved in this glycine-phosphate buffer
at concentrations of 1 x 10^6 spermatozoa/ml for 2-3 hours during Staflo
runs, fertilizing capacity was lost at concentrations of less than 4 x 10^6/
ml. Because of this loss, the number of offspring was too small to attach
any significance to the slightly greater number of males resulting from
the insemination of lower-fraction spermatozoa.

In the low electric field strength sedimentation-upward-electrophoresis
(SUE) and sedimentation-downward-electrophoresis (SDE) experiments, no
differences were found between the respective collection fractions with re-
gard to capped, viable, tail-wagging or highly motile spermatozoa. In the
SUE experiments, all spermatozoa were oriented vertically. The calculated
electric field strengths needed to exactly balance the average contribution
of sedimentation to the observed migration at 3°C were 0.96 and 0.59 V/cm
at pH 6.6 and 7.1, respectively.

In the low electric field strength SDE experiments, the observed
average migration distances were less than either of the expected average
sedimentation or electrophoretic migration distances. This discrepancy is
attributed to the more horizontal orientation of the spermatozoa in these
experiments (Figure 56) as compared to the orientation observed in sedimen-
tation experiments or SDE experiments with high electric field strengths.

Because of the small spread of the migration pattern in both the SUE
and SDE experiments, it was not possible to obtain sub-populations having
the combinations of sedimentation and electrophoretic properties (E·S, E·s,
e·S and e·s) discussed in Chapter III. Furthermore, the apparent influence
of spermatozoa orientation on SDE experiments indicates that in such experi-
ments, a sufficiently high electric field must be used to orient all sperm-
atozoa "tail down" to eliminate the differential migration effect of
orientation.

IX. THE QUALITY OF RABBIT SPERMATOZOA POPULATIONS:
A CRITICAL DISCUSSION

Contents

A. Introduction

One of the most perplexing and frustrating problems encountered

in this study of spermatozoa has been the unpredictability of the quality

of ejaculates from different rabbits on the same day, of ejaculates from

the same rabbit on different days and of consecutive ejaculates from the

same rabbit on the same day. Observations over a year suggested that the

quality of ejaculates was consistently good during the winter and early

spring, and was extremely variable in the summer and autumn months. In

addition, spermatozoa samples which initially appeared to be of high quality

with regard to percentage and degree of motility, demonstrated variable
resistance to the stresses encountered in Staflo experiments. This
variable durability of spermatozoa samples contributed to many unhappy
days because spermatozoa which initially appeared to be adequate for an
experiment lost their motility before the completion of the experiment.
Thus, they were inadequate for artificial insemination and the biological
assay system of most interest was lost. The purpose of this section is
to review the many factors which influence the quality of spermatozoa,
and, insofar as possible, to draw conclusions from the many observations
of spermatozoa quality made in this study, most of which were opportunis-
tic in conjunction with Staflo experiments. In the review of the biolog-
ical properties of spermatozoa presented in Chapter II, much information
pertinent to the subject of this chapter was presented. It is appropriate
at this time to refer to or present additional information from those
studies.

Doggett found a cyclic variation of and correlation between the ejacu-
late volume and the concentration and motility of spermatozoa therein[68].
The total sperm count varied approximately ± 50% from the mean with an
apparent correlation of good and poor motility with high and low sperm
counts, respectively. With maturation of the rabbit, the periods generally
shortened and showed a predominance of 3 and 4-day intervals. The timing
of the peaks appeared to be an inherent characteristic of the rabbit and
not related to the environment [68].

Degerman and Kihlström found cyclic variations in the body temperatures
of male rabbits. The cycles varied in length from 3-9 days with a mean of
7 days, and were correlated with variations in the daily semen volume[63].
The motility of the spermatozoa was not reported.

Asdell and Salisbury studied the duration of spermatogenesis in the rabbit by observing the regeneration of the spermatogenetic process in testes which had been damaged by exposure to the relatively higher temperature of the abdominal cavity, by anchoring testes therein, for 1-7 days[12]. Although their technique was not so precise as current methods using tritiated thymidine, their studies are enlightening. The effect of exposure of testes to the intra-abdominal temperature for periods less than 1 day was not reported. After exposure for 1 day to the elevated intra-abdominal temperature, testes showed clumping and detachment of spermatids, some derangement in lacunae formation in spermatocytes and cessation of spermatogenesis even in the spermatogonial layer. In testes exposed to the elevated temperature for 7 days, there were no spermatozoa or spermatids, spermatocytes were clumped and the layers of spermatogonia showed some signs of damage. The rate of recovery of the spermatogenic process after return of the testes to the scrotum was roughly proportional to the length of stay in the abdominal cavity. They estimated that 35 days were necessary to observe new spermatozoa in the testes[12]. Remembering that the male rabbit can, and often does, pull his testes into his abdomen when handled or frightened, the significance of exposure to intra-abdominal temperatures as noted above is of great concern in regard to animal care.

The influence of the environment on the reproductive performance of rabbits has been reported by Sittman and co-workers[216] and is discussed in Chapter II. It is interesting to note that Ruffer has observed a marked decrease in the percentage of litters obtained from rabbits mated from early morning to late afternoon on very hot days; however, he experienced fair success with matings in the evenings of those same days[267]. A major section which follows presents observations during this study on the effects

of the environment on sperm quality. However, the first section is concerned with differences among several methods of evaluating the quality of spermatozoa.

B. Comparison of Optical-Cytological Methods Used to Evaluate Spermatozoa

In the experiments reported in Chapter VIII, spermatozoa in the collection fractions and the initial sample were evaluated in the cold room immediately after collection, and again at room temperature after re-concentration by centrifugation. Evaluation in the cold room and at room temperature, by phase contrast microscopy, determined the percentages of capped (C), capless (CL), tail-wagging (TW) and highly motile (FM) spermatozoa. At room temperature, the reconcentrated spermatozoa were also evaluated by the nigrosin-eosin vital staining technique. By light microscopy, stained spermatozoa preparations were categorized as unstained (U), morphologically normal but stained (MNS), and stained (S). It was assumed that capless spermatozoa would take up the vital stain, and, therefore, that the percentage of capless (CL) spermatozoa by phase contrast microscopy would be approximately equal to the percentage of stained (S) spermatozoa (e.g., CL = S). However, differences ranged from S being 33.9% greater than CL (CF 6, Experiment 7-29-67, Table XXV) to S 52.6% less than CL (CF 7, Experiment 8-10-67, Table XXI). In the former, the spermatozoa concentration was 5.04 x 10^6/ml and in the latter, 0.71 x 10^6/ml, suggesting a differential effect of concentration. However, in another experiment (CF 6, Experiment 8-10-67, Table XXV), S was 22.1% less than CL even though the spermatozoa concentration was 5 x 10^6/ml. Thus, there was a marked lack of correlation between the 2 methods of evaluating spermatozoa and it was not explained, solely at least, by differences in cell concentration. Further evaluation of these discrepancies includes(1) a comparison of scoring nigrosin-eosin stained preparations by both ordinary

light and phase contrast microscopy, and (2) a comparison of phase contrast observations of unstained spermatozoa and nigrosin-eosin stained preparations.

1. **Comparison of Scoring Nigrosin-Eosin Preparations with Ordinary Light and Phase Contrast Microscopy.**

As noted above, scoring of nigrosin-eosin stained preparations by light microscopy led to 3 categories of spermatozoa (U, MNS and S), whereas scoring by phase contrast microscopy gave 4 categories of spermatozoa as follows: (1) unstained and capped (UC), (2) unstained and capless (UCL), (3) morphologically normal but stained (MNS) and (4) stained and capless (SCL). One would expect agreement between the unstained categories on slides assayed by both techniques, (e.g., U = UC + UCL). This hypothesis was tested by comparing light microscope and phase contrast scoring of 23 pairs of slides with 100 spermatozoa being scored on each slide by each method, or 200 spermatozoa per sample. The results are shown in Table XXXII. Columns 10 and 11 compare the percentages of unstained spermatozoa representing the "U" category (3) from light microscope scoring and the "UC + UCL" categories (6+8) from the phase contrast scoring, respectively. Using a one-tail test[90], the differences were significant at the $P \leq 0.01$ level in only 3 (13.6%) of the 22 samples, whereas thero was no difference ($P > 0.10$) in 16 (72.7%) of the samples. In the 3 other samples, the differences were significant at the $P = 0.03$-0.05 level.

Generally, there was a higher percentage of MNS spermatozoa by phase contrast scoring. This is consistent with the better observation of the acrosomal cap region with this method. Despite these differences, there is fair agreement between the 2 methods of scoring in regard to determining the percentage of unstained spermatozoa in the sample.

2. **Comparison of Phase Contrast Observations of Unstained and Nigrosin-Eosin Stained Spermatozoa.**

Table XXXII. Comparison Light and Phase Contrast Microscopy Scoring of Nigrosin-Eosin Stained Rabbit Spermatozoa.[a]

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Experiment Number	Sample	Ordinary Light			Phase Contrast				Unstained	
		U (%)	MNS (%)	S (%)	UC (%)	MNS (%)	UCL (%)	SCL (%)	(3) (%)	(6+8) (%)
9-13-67S	CF 6	87	1.0	12	24.3	2.3	63.4	10	87	87.7
	CF 7	72.5	6	21.5	21	3	44	32	72.5	65
	IS	67	4	29	30.5	4	30	35.5	67	60.5
9-13-67P	CF 7	52.5	1.5	46	18	11	30	41	52.5	48
	CF 8	59.5	1.5	39	31.5	3	33	32.5	59.5	64.5
	IS	51	0	49	28	9	21	42	51	49
9-18-67	CF 6	61.5	0	38.5	38	15.5	23	23.5	61.5	61
	CF 7	75.5	0.5	24	63	12	12.5	12.5	75.5	75.5
	IS	66.5	2	31.5	67	3	16	14	66.5	83
9-22-67	CF 6	34	1.5	64.5	8.5	10.5	22	59	34	30.5
	CF 7	35	0.5	64.5	14.5	9	26.5	50	35	41
	IS	32.5	4	64.5	17.5	2.5	25	55	32.5	42.5
9-29-67	CF8-1	63	0.5	36.5	24.5	6.5	44.8	24.2	63	69.3
	CF8-2	66	0.7	32.3	32.6	4	36.4	27	66	69.0
	CF9-1	64.5	0.5	35	25.4	8.2	40.7	25.7	64.5	66.1
	CF9-2	72.5	1.0	26.5	35	7.7	37.4	20	72.5	72.4
	IS	70.5	0	29.5	34.5	11	30.8	23.7	70.5	65.3
10-2-67	CF 6	30.5	0.6	68.9	7.3	22.6	37	33.1	30.5	44.3
	CF 7	66.5	0	33.5	44.5	16	24.5	15	66.5	69
	IS	68	0.5	31.5	39	8	30	23	68	69
10-3-67	CF 8	65	4	31	13.5	13.5	43.5	29.5	65	57
	CF 9	68	2.5	29.5	15	10.5	53	21	68	68
	IS	57.5	1.5	42	14	9.5	42	34.5	57.5	56

a. See Glossary for explanation of abbreviations.

Twelve ejaculates were diluted 1:2 with modified Baker's medium. In less than 1 hour, both unstained and stained preparations were evaluated by phase contrast microscopy. As noted above, with phase contrast scoring one gets 4 categories of spermatozoa in fresh specimens (C,CL,TW and FM) and 4 categories in nigrosin-eosin preparations (UC, UCL, MNS and SCL). The results of scoring the 12 specimens are given in Table XXXIII. In this table, capped spermatozoa are represented by columns 3, 5 and 6 in phase contrast observations and columns 7 and 8 in the nigrosin-eosin stained preparations. Capless spermatozoa by phase contrast are listed in column 4 and by the nigrosin-eosin staining technique in columns 9 and 10. For convenience, a comparison of the percentages of capless spermatozoa is given in columns 11 and 12. The same difference would be observed between the total percentages of capped spermatozoa.

The percentages of capless spermatozoa determined by the 2 methods agree within 4% in 9 of the first 10 ejaculates listed in Table XXXIII. In the first 10 ejaculates, the percentages of tail-wagging and forward motion were very low. In the last 2 ejaculates listed in Table XXXIII, the percentages of capless spermatozoa by phase contrast microscopy (11) are much less than those of the capless spermatozoa by nigrosin-eosin staining (12). The percentage and degree of motility in the last 2 ejaculates was considerably better than all but 1 of the 10 previous ejaculates. Thus, agreement between the 2 methods of evaluation appears to be good only for poor spermatozoa samples.

The question of correlation between the quality of spermatozoa samples, judged by motility alone, and the discrepancy between the percentages of capless spermatozoa determined by the 2 methods of evaluation was explored by analyzing 61 ejaculates. A difference of ± 5% between the percentages of capless spermatozoa determined by the 2 techniques

Table XXXIII. Comparison of Phase Contrast Observations of Unstained Spermatozoa and Nigrosin-Eosin Stained Spermatozoa.[a]

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
Date	Rabbit	Phase Contrast Observations				Nigrosin-Eosin Staining				Capless	
		C (%)	CL (%)	TW (%)	FM (%)	UC (%)	MNS (%)	UCL (%)	SCL (%)	(4) (%)	(9+10) (%)
9-22-67	Chi	10	90	0	0	3.5	15.5	43.5	37.5	90	81
9-22-67	Larry	5.0	92.8	2.2	0.5	4.5	4	0.5	90.5	92.8	91
9-22-67	Old Man	11.5	84.5	1	3	2.5	10.5	0.5	86.5	84.5	87
9-22-67	Otto	11.9	83	1.7	3.4	9.5	6.5	32.5	51.5	83	84
9-22-67	Snoopy	3	96	1	0	5.5	1.5	0	93	96	93
9-27-67	Chi	20.7	78.8	0.5	0	19	6	8.5	66.5	78.8	75.0
9-27-67	Otto	26.4	67.3	5.3	1.0	18.5	14	28	39.5	67.3	67.5
9-27-67	Psi	16.3	80.8	2.2	0.7	8	8.5	2	81.5	80.8	83.5
10-2-67	Otto	16.7	71.0	2.4	10.3	22.6	5.3	48.6	23.3	71.0	71.9
10-6-67	Otto	17.2	53.1	8.1	21.6	36	9	40	15	53.1	55
9-22-67	Sam	17.5	47.8	4.7	30	26.5	6.5	35	32	47.8	67
10-2-67	Psi	9.9	29.6	5.0	55.5	43	7.5	30.5	19	29.6	49.5

a. See Glossary for explanation of abbreviations.

was considered to be within the accuracy of counting. Three relationships
between the 2 evaluation techniques are presented in Table XXXIV. In the
first, the percentage of capless spermatozoa by phase contrast observation
is less than the percentage of capless spermatozoa by the nigrosin-eosin
staining technique. Twelve of 61 ejaculates or 19.7% fell within this
category, 8 (66.7%) of which had 20% or more motile spermatozoa. Twenty-
two of the 61 ejaculates fell into the classification of approximately
equal percentages of capless spermatozoa by both methods. Of the 22, 6
(27.3%) had 20% or more motile spermatozoa. Of the 27 ejaculates falling
into the third category (CL > UCL + SCL), only 1 (3.7%) had more than 20%
motile spermatozoa.

The conclusion from this comparison is that, if the spermatozoa
sample is of good quality judged by forward motion, the percentage of
capless spermatozoa by phase contrast observation of unstained spermatozoa
is less than by phase contrast scoring of nigrosin-eosin stained spermatozoa.
This is illustrated by the last 2 ejaculates listed in Table XXXIII, both
of which had a fair percentage of spermatozoa with forward motion. This
suggests that staining has a detrimental effect, possibly mechanical,
which causes some unstained spermatozoa to lose their acrosomal caps,
thereby increasing the UCL category. When the spermatozoa are of very poor
quality, a higher percentage appears to be capless by phase contrast obser-
vation of unstained spermatozoa.

C. Environmental and Procedural Factors Affecting Spermatozoa Quality

1. Miscellaneous Factors

a. Animal Care. The environment, illness, feeding and type of pellet,
unnecessary moving while cleaning and possibly other peculiarities of animal
care can influence the initial quality of ejaculates. Even when all of these

Table XXXIV. Correlation of Scoring of Caplessness by Phase Contrast Observations
of Unstained (CL) and Nigrosin-Eosin Stained (UCL+SCL) Spermatozoa.[a]

Relationship of Categories of Spermatozoa	Samples (No.)	(%)	Samples with 20% or More Motile Sperm (No.)	(%)
CL $<$ UCL + SCL	12	19.7	8	67
CL $\approx$ UCL + SCL	22	36	6	27.3
CL $>$ UCL + SCL	27	44.3	1	3.7
Totals	61	100	15	100

a. See Glossary for explanation of abbreviations.

items were under close supervision, variations in the quality of sperm-atozoa in ejaculates were observed.

b. Sperm Collection. Polge reported that rabbit spermatozoa will not regain motility if more than approximately 2.5% glycerol is introduced rapidly into the semen[188]. Inasmuch as I used glycerol as a lubricant in the artificial vagina in most experiments, it is conceivable that excessive lubrication contributed to the deterioration of spermatozoa samples early in the study. However, later on, a standard number of drops of glycerol was used to lubricate each lumen, thereby eliminating the possibility of excess glycerol.

A constant danger in the collection of spermatozoa is too high a temperature in the artificial vagina. Brackett, Weinman and Williams observed a decrease in motility after exposure of spermatozoa to 48°C temperature for only 40 seconds[265]. The artificial vagina shown in Appendix C was generally used with a temperature of 40-42°C. Temperatures below 38°C discouraged the bucks, but did not appear to affect the sperm-atozoa. Occasionally, temperatures as high as 45°C were used, but, unfortunately, no correlation of that temperature and the quality of the sample was made. However, Walton recommended using a temperature of 45°C[239].

Because of the well known phenomenon of "cold shock", which occurs with rapid cooling of some mammalian spermatozoa, great care was taken to avoid this problem. Since the same procedure was used for all samples (see Appendix B), the great variability in sample quality noted above does not appear to be attributable to the cooling procedure.

c. Staflo Handling Procedures. An attempt was made to avoid low sperm-atozoa concentrations, because of the deleterious effects thereof on moti-lity, viability and fertilizing capacity. Dilution was accomplished slowly

and in several stages. The medium generally was prepared by the same individual and, whenever there was a question of contamination of glassware, it was washed by the author.

In numerous Staflo experiments, spermatozoa which initially had a high degree of motility lost essentially all motility before the end of the experiment. At the conclusion of other Staflo experiments, both fowl and rabbit spermatozoa appeared to have motility suggestive of good fertilizing capacity but insemination produced no offspring. During the period in which these experiments were conducted, other Staflo experiments in this laboratory, using bone marrow cells, showed a marked loss of colony formation using procedures which previously had led to a high percentage of colony formation. Studies conducted with the bone marrow cells showed that exposure to a very short segment of new polyethylene tubing resulted in a marked decrease in colony formation. Other observations by me showed that spermatozoa suspended in egg yolk medium, when stored for several hours in new polyethylene syringes, lost essentially all motility. In contrast, spermatozoa from the same ejaculate stored in the same medium in a clean glass vial maintained a high degree of motility for over 48 hours. These problems with polyethylene tubing and syringes were avoided by using old tubing and old syringes which apparently had lost the toxic substance.

Another toxin, Roccal (benzalkonium chloride) could have caused deterioration of spermatozoa samples on occasion. It was used to prevent bacterial growth in the Staflo apparatus between experiments. Evaluation of its effects in saline solutions showed that a 1:1,000 concentration of Roccal caused immobilization of all spermatozoa within 45 seconds. In a 1:2,500 concentration, after 10 minutes, spermatozoa showed reduced degree of motility. After 80 minutes, a high percentage of spermatozoa

had only a poor degree of tail-wagging. After 90 minutes, only 27% of the spermatozoa were unstained (nigrosin-eosin stain) compared to 64% of the control sperm. At a Roccal concentration of 1:5,000, after 80 minutes, the percentage and degree of motility was less than in the saline control suspension. After 90 minutes, 50% of the spermatozoa were unstained (viable) compared to 64% of the control sperm. At concentrations of 1:10,000 and less, there appeared to be no effect of Roccal within 90 minutes. The fertilizing capacity of spermatozoa exposed to Roccal was not studied.

2. Frequency of Collection.

A desire to have the best possible spermatozoa for an experiment led to the collection of a second ejaculate within an hour, on some occasions, or the next morning from rabbits which had given the best spermatozoa sample on a particular day. Hopes of obtaining an abundance of excellent spermatozoa for experiments were shattered by the results shown in Table XXXV. It is seen that there were marked variations in the quality of consecutive ejaculates from the same rabbit collected at intervals of 24 hours or of 15-60 minutes during the summer and early autumn.

Table XXXV A shows a striking difference in the quality of consecutive ejaculates with an interval of 15-60 minutes between collections. All rabbits were housed in outdoor hutches with a sprinkler system for limited temperature control. Even though the percentage of motile spermatozoa in the first ejaculate ranged from 13.6%-85%, there were no motile spermatozoa in any of the second ejaculates. The absolute increases in the percentages of capless spermatozoa in 4 of the pairs of ejaculates ranged from 26.4%-82.5%.

Table XXXV. Variations in the Quality of Consecutive
Ejaculates from the Same Rabbit.

Interval Between Collections and Date	Rabbit	Percent Forward Motion			Percent Capless		
		Ejaculate 1st	2nd	Difference (2-1)	Ejaculate 1st	2nd	Difference (2-1)
A. 15-60 Min.							
6-24-67	Whitey	65	0	-65			
7-7-67	Bill	41	0	-41			
7-7-67	Otto	13.6	0	-13.6	24.4	50.8	+26.4
7-8-67	Larry	85	0	-85	7.5	90	+82.5
7-8-67	Robert	73.4	0	-73.4	15.7	91.5	+75.8
7-15-67	Red	24(TW)[a]	0	-24(TW)	48.2	86	+37.8
B. 15-60 Min.							
10-13-67	Otto	50	50	0	25	22.5	-2.5
10-26-67	Psi	85	17.5	-67.5	10.5	37.5	+27
C. 24 Hours							
8-30-67	Otto	60.0	16.0	-44.0	18.4	45.0	+26.6
9-18-67	Larry	16.7	1.0	-15.7	50.0	77.0	+27.0
9-18-67	Otto	67.2	16.5	-50.7	11.6	39.1	+27.5
9-18-67	Psi	59.4	18.8	-40.6	27.1	47.1	+20.0

a. TW: Tail wagging.

The pairs of ejaculates listed in Table XXXV B were collected approximately 2 months after the rabbits had been placed in an air-conditioned room. The second ejaculate collected from rabbit Otto less than 1 hour after the first was essentially unchanged, whereas the second ejaculate collected from Psi showed a 67% decrease in the percentage of motile spermatozoa and a 27% increase in the percentage of capless spermatozoa. In the 2-week interval between the collection of the ejaculates from Otto and Psi, there was a transient temperature rise of about 13°F in the air-conditioned room when the air-conditioner was inadvertently turned off. The spermatozoa samples from other rabbits in the air-conditioned room on 10-26-67 showed a decrease in quality which suggests that relative, as well as absolute, temperature changes are important. Thus, it is believed that the relative temperature rise on 10-17-67 was the primary cause of the difference in the quality of the 2 ejaculates from Psi on 10-26-67.

Table XXXV C shows that the absolute loss of forward motion ranged from 15.7%-50.7% and the relative loss from 68.4%-94% in 4 pairs of ejaculates collected from rabbits on consecutive days. The absolute increase in the percentage of capless spermatozoa ranged from 20-27.5%. At the time of collection, rabbits Otto and Psi had been in an air-conditioned room for about 5 weeks, whereas Larry was in an outdoor hutch cooled by a sprinkler system. Thus, although being in the cool room did appear to improve the quality of the first ejaculates from Otto and Psi, even 5 weeks in a controlled environment did not prevent a significant decrease in the quality of the second ejaculates.

These observations showed that in hot weather there was a marked decrease in the quality of the second ejaculate collected from a rabbit within

one hour after the first, and that there was a significant decrease in
the quality of ejaculates collected approximately 24 hours later. This
suggests that there is a selective process within the genital duct system
of the male rabbit enhancing the migration of the highest quality sperm-
atozoa toward the distal portion of the duct. This may be related to
the observation by Orgebin-Crist that, during epididymal passage, young
and old sperm cell populations are mixed[181]. Consequently, the first
ejaculate is of the best possible quality. In view of the frequent dev-
elopment of pseudo-pregnancy in female rabbits following a sterile mating,
one might speculate that "getting off a good first shot" so to speak,
is very important for the survival of the species, especially in hot
weather. However, one could offer a counter-argument to the effect that
such a phenomenon is designed to minimize the reproductive rate in the
rabbit population, at least in the summer. Under these circumstances, an
overly energetic young buck, despite his ambitions, could fertilize only
1 female a day, if that often.

3. Centrifugation.

As noted in Chapter VIII, the percentages of capped, capless and motile
spermatozoa in each collection fraction were determined by phase contrast
microscopy, immediately after Staflo operation ceased. In order to have
a sample with a high concentration of spermatozoa for artificial insemina-
tion, each collection fraction was centrifuged for 5 minutes at 1,400 g
in a clinical centrifuge in the refrigerated room. After the supernatant
was removed and the spermatozoa resuspended in egg yolk medium, they were
warmed to room temperature for evaluation which included a second deter-
mination of the percentages of capped, capless and motile spermatozoa,
and nigrosin-eosin vital staining.

In most experiments, it was observed that the percentage of capless

spermatozoa increased significantly between the evaluations prior to and after centrifugation, a period of approximately 30 minutes. Because the spermatozoa were in egg yolk medium for about half of that interval, the deterioration is attributed solely to centrifugation. Even though the centrifugation time and speed were the same in all experiments, the changes in the percentages of capless spermatozoa were not constant from sample to sample. As shown in Table XXXVI, the increases in the percentages of capless spermatozoa in selected experiments ranged from 4.4%-43.3%. In assessing these changes in the initial samples (IS), it should be noted that the first determination of the percentages of capless spermatozoa was made shortly after the initial dilution which was generally 2-3 hours prior to centrifugation. Therefore, some deterioration could have occurred in the initial sample prior to centrifugation, whereas the collection fractions were assayed immediately prior to and after centrifugation.

With regard to Table XXXVI, the following observations on individual experiments are pertinent:

a. Experiment 7-26-67. Despite the much lower spermatozoa concentration, CF 7 had a slightly smaller increase in capless spermatozoa. This suggests that with a spermatozoa sample that undergoes significant deterioration during centrifugation, there is no differential effect of concentrations as low as 1.34×10^6/ml. Even though the cold room temperature was 4.5°C, no motile spermatozoa were seen in the collection fractions. Thus, it is unlikely that CF 7 was enriched by motile spermatozoa during electrophoresis.

b. Experiment 7-29-67. In this experiment, both collection fractions had relatively high spermatozoa concentrations. Thus, the greater increase in capless spermatozoa in CF 7 is not believed to be related to the slightly lower sperm concentration in that fraction.

c. Experiment 8-10-67. The increase in capless spermatozoa was less

Table XXXVI. Effect of Low Temperature Centrifugation for 5 Minutes
at 1400 g on the Morphology of Rabbit Spermatozoa.[a]

Experiment Number	Flow-Chamber Temp.[b] (°C)	Sample	Initial Concentration (units of 10^6/ml)	Capless Spermatozoa		
				After (%)	Before (%)	Increase (%)
7-26-67	4.9	CF 6	4.0	51.8	13.5	38.3
		CF 7	1.34	48.9	15.5	33.4
7-29-67	5.4	CF 6	5.04	16.1	10.7	5.4
		CF 7	3.53	26.2	6.6	19.6
8-10-67	6.4	CF 6S	5.62	29.9	25.2	4.7
		CF 7S	0.71	57.6	27.5	30.1
		IS	20	19.1	14.7	4.4
		CF 6EP	5.0	34.1	28.2	5.9
9-22-67	7.2	CF 6	3.42	63.5	29	34.5
		CF 7	3.72	58.7	26	32.7
		IS	20	71.5	28.2	43.3
10-2-67	3.3	CF 6	0.5	83	50	33
		CF 7	6.0	40.2	20.3	19.9
		IS	20	36.5	8.5	28.0
10-3-67	2.3	CF 8	5.45	56.4	24	32.4
		CF 9	1.05	47.5	22.5	25.0
		IS	20	46.8	16.4	30.4

a. See Glossary for explanation of abbreviations.
b. Room temperature was 0.5°C lower.

than 5.9% for 3 samples with spermatozoa concentrations equal to or greater than 5×10^6/ml prior to centrifugation. There was a 30.1% increase in CF 7S which had an initial concentration of only 0.71×10^6/ml, suggesting that, in this experiment with relatively high quality spermatozoa, the low concentration was detrimental.

d. <u>Experiment 9-22-67</u>. Even though the concentrations of spermatozoa in the collection fractions prior to centrifugation were 3.42 and 3.72 $\times 10^6$/ml, the absolute increases in the percentage of capless spermatozoa were very large, 34.5% and 32.7%, respectively. A slightly greater change was observed in the initial fraction, part of which may be explained by the time difference between evaluations.

e. <u>Experiment 10-2-67</u>. In addition to a greater increase during centrifugation, CF 6 also had a higher pre-centrifugation percentage of capless spermatozoa than did CF 7. This suggests a detrimental effect of a concentration of 0.5×10^6/ml even with the highest quality spermatozoa of any experiment listed in Table XXXVI.

f. <u>Experiment 10-3-67</u>. As observed in Experiment 7-26-67, the lower collection fraction had a smaller increase in capless spermatozoa, despite a lower concentration. The sample as a whole experienced a significant increase in capless spermatozoa, suggesting poor spermatozoa quality. Thus, with poor quality sperm, a concentration as low as 1.05×10^6/ml is not detrimental. The flow-chamber temperature of 2.3°C was too low for the spermatozoa to have had any intrinsic motility, thereby ruling out enrichment in CF 9 with regard to motility.

Conclusions from these results, pertaining to the centrifugation of spermatozoa under the conditions specified above, are as follows: (1) With spermatozoa concentrations of less than 1×10^6/ml, there is a dispropor-

tionate increase in the percentage of capless spermatozoa compared to samples of higher concentrations if the initial quality of the sample as a whole is good. (2) With spermatozoa concentrations of 1.05×10^6 and 1.34×10^6/ml, there is no differential increase in capless spermatozoa as compared to higher concentrations if the quality of the sample as a whole is poor. This observation appears unrelated to possible effects of intrinsic motility on spermatozoa distribution. (3) With spermatozoa concentrations ranging from $3-6 \times 10^6$/ml, the increases in the percentage of capless spermatozoa were generally comparable in the 2 collection fractions and ranged from 4.4%-38.3%. The magnitude of the increase is believed to reflect the quality of the spermatozoa. (4) The changes in the percentages of capless spermatozoa do not appear to be related to the initial percentages thereof or to the temperature over the range 2.3°C-7.2°C.

4. <u>Environmental Temperature</u>

Observations over several summers showed a marked variation of ejaculate quality from extremely good to very poor in an irregular manner until well after cool weather had returned. This variation in quality persisted even when a roof sprinkler was used to cool the rabbit hutches. In an attempt to improve the quality of spermatozoa available for Staflo experiments and to gain information concerning the effect of environmental temperature on sperm quality, a study was designed with 6 rabbits divided between 2 subgroups matching breed and history. Three rabbits remained out-of-doors in the hutches cooled by the sprinkler, and hereafter are referred to as the "outside" group. The other 3 rabbits, the "inside" group, were moved into an air-conditioned room with controlled artificial lighting and 1 small window without direct sunlight. The temperature was recorded continuously in both locations. Insofar as

possible, ejaculates were collected from matched pairs on the same day, usually within minutes of each other.

Prior to August 15, all 6 rabbits were housed out-of-doors and experienced the same temperatures to that date. From the period July 1-August 15, significantly elevated temperatures were as follows: 87°F on July 3; 91°F on July 11; 83°F on July 20, 26 and 28, and 90°F on August 14, the day before the "inside" group was moved into the air-conditioned room. Ejaculates were collected from each of the rabbits on August 15 prior to moving the "indoor" group into the air-conditioned room. For administrative reasons, the "outdoor" group was moved into the air-conditioned room on October 14.

All ejaculates were evaluated by phase contrast microscopy for percentages of capped, capless, tail-wagging and highly motile spermatozoa and by the nigrosin-eosin vital staining technique described in Appendix B. Figures 57 and 58 show the percentages of motile and capless spermatozoa, respectively, for individual rabbits of the "indoor" and "outdoor" groups. The curves for Otto in Figures 57 and 58 will be discussed in detail and then generalizations will be made with reference to the information on the other rabbits in both groups.

Before discussing the figures, it is necessary to know the relative lifespan of each of the stages of spermatogenesis. Swierstra and Foote arbitrarily divided the cycle of seminiferous epithelium of the rabbit into 8 stages and determined the relative duration of each stage[222]. They observed that the Type A spermatogonia divided to produce 2 Type A spermatogonia, 1 of which remained the starting cell for the next generation, while the other underwent 4 spermatogonial divisions to give (theoretically) 16 primary spermatocytes and, in turn, 64 spermatids. However, they estimated that, because of degenerative processes, only 15.6 primary spermatocytes
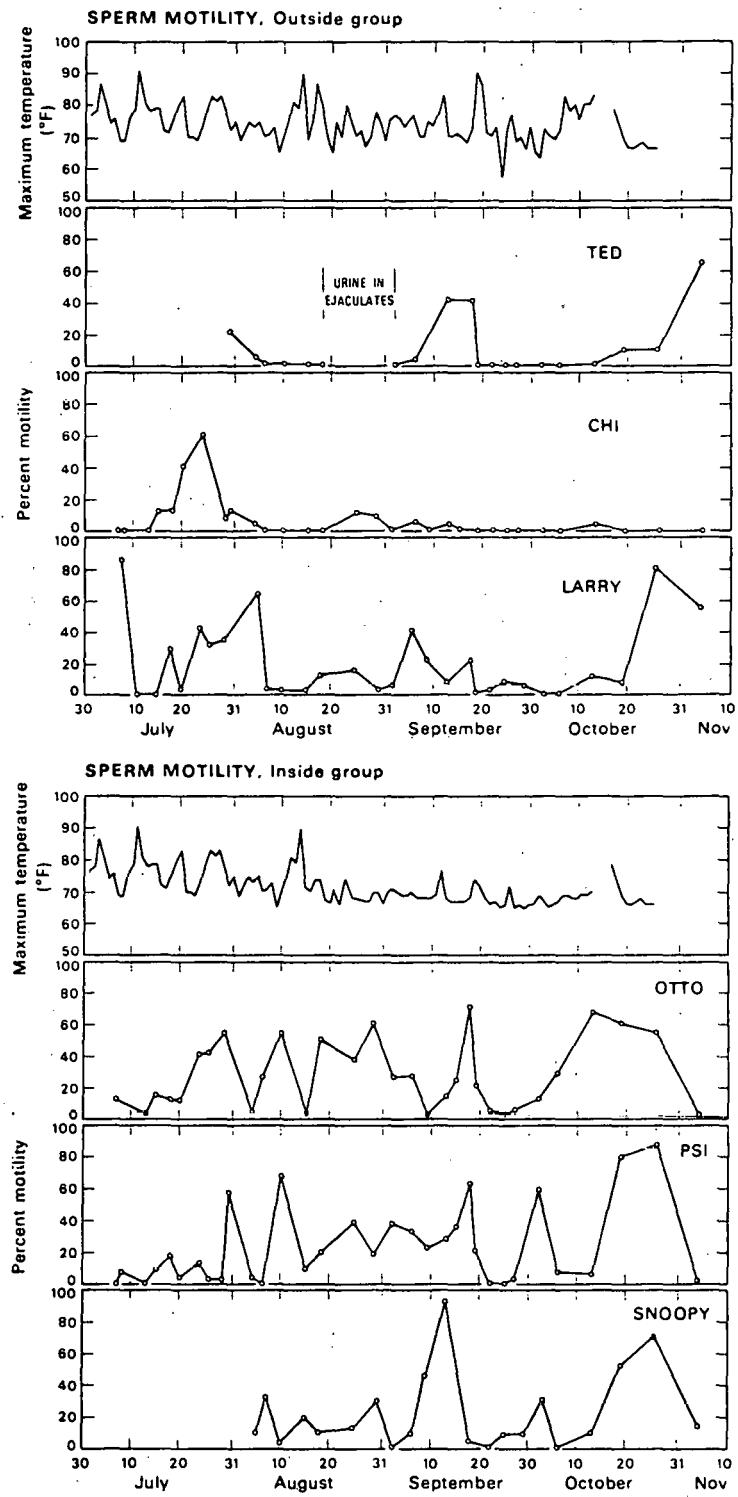
Fig. 57. Effects of environmental temperature on the motility of rabbit spermatozoa. A. "Outside" group. B. "Inside" group. Lifespan scale (Figure 59 reduced) in rear-cover envelope may be used with this figure.
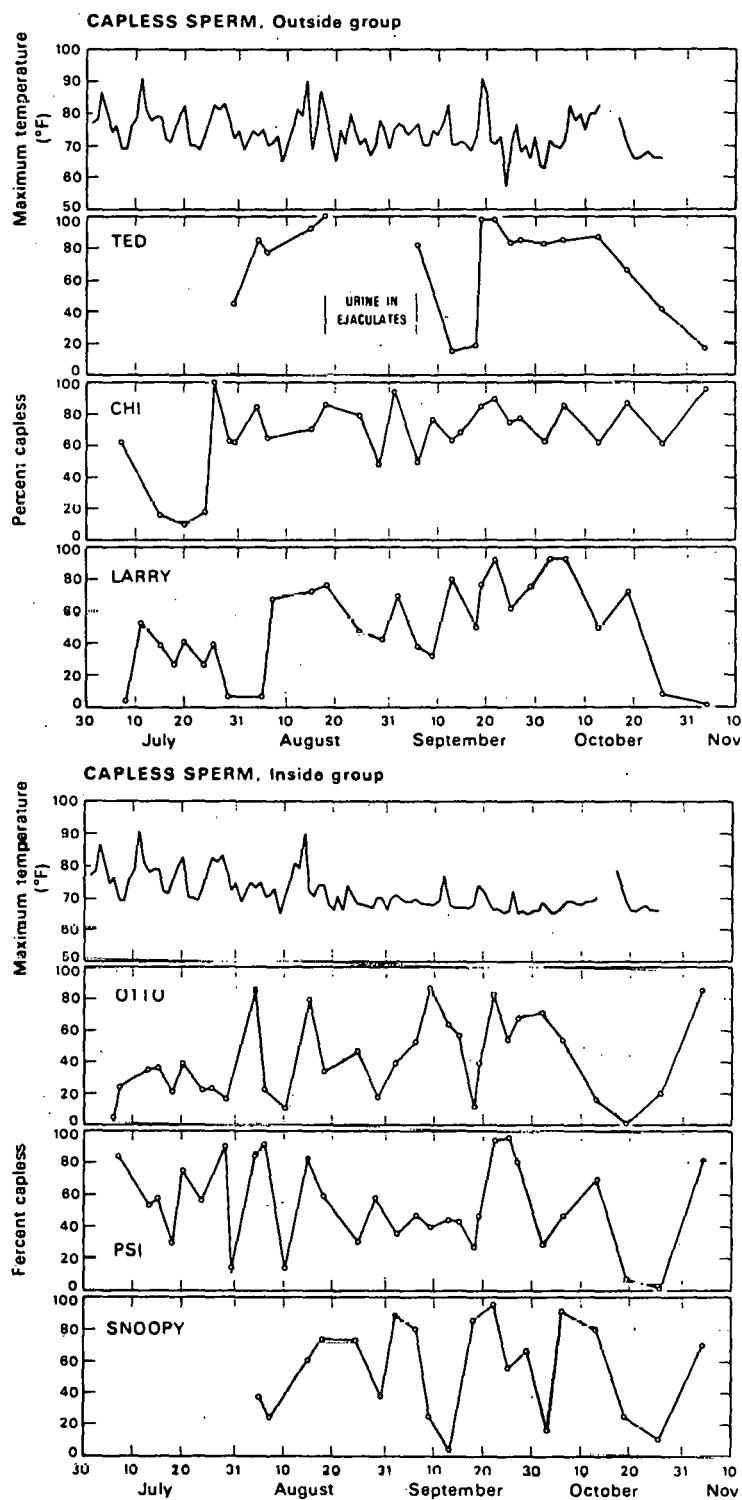
Fig. 58. Effects of environmental temperature on the occurrence of capless spermatozoa in rabbit ejaculates. A. "Outside" group. B. "Inside" group. Lifespan scale (Figure 59 reduced) in rear-cover envelope may be used with this figure.

and 47.5 spermatids were produced[222]. Subsequently, they studied the duration of spermatogenesis and of spermatozoa transport in the male rabbit using tritiated thymidine[223]. The estimated life span of the different cells in the spermatogenetic process are given in Table XXXVII. After formation from spermatids, spermatozoa spend approximately 5.6 days in the lumen of the seminiferous tubule and then 8-10 days in transport through the ducts. As noted by the authors, the estimate of the total duration of spermatogenesis depends upon the point chosen as the onset of the process. If it is considered to begin with the first of the series of spermatogonial divisions leading to the production of primary spermatocytes, the duration is approximately 43.6 days. If it is assumed that spermatogenesis starts with the formation of spermatogonial stem cells, and that the life span of these cells is 1 cycle of the seminiferous epithelium, then spermatogenesis would extend over approximately 51.8 days. Thus, adding 10 days for transport in the duct system would make the total time from the onset of spermatogenesis to the appearance of spermatozoa in the ejaculate either 54 or 62 days. Figure 59 R is a linear representation of the time scale for spermatogenesis and sperm transport on the same scale as Figures 57 and 58. When the right border of the time scale is placed at the date on which an ejaculate was collected, one can visualize the relationship between individual environmental temperature peaks and the corresponding stage of development of the spermatozoa in that particular ejaculate.

Swierstra and Foote found that the first labeled spermatozoa appeared in the ejaculate from 39-42 days after injection of tritiated thymidine[223]. The earliest observation of labeled spermatozoa in the testis by Orgebin-Crist was 30.6 days after thymidine injection[181]. Adding time for spermatozoa release and transport in the duct system to the 30.6 days would

Table XXXVII. Estimated Lifespan of Rabbit Spermatogenetic Cells[223]

Type	Estimated Lifespan (Days)
Spermatogonia	
A	3.4
Intermediate-1	0.6
Intermediate-2	2.8
B	1.1
Primary Spermatocytes	16.5
Secondary Spermatocytes	0.8
Spermatids	10.0
Spermatozoa	
In Testes	5.6
In Transport	10.0
	50.8

LIFESPAN OF SPERMATOGENETIC CELLS
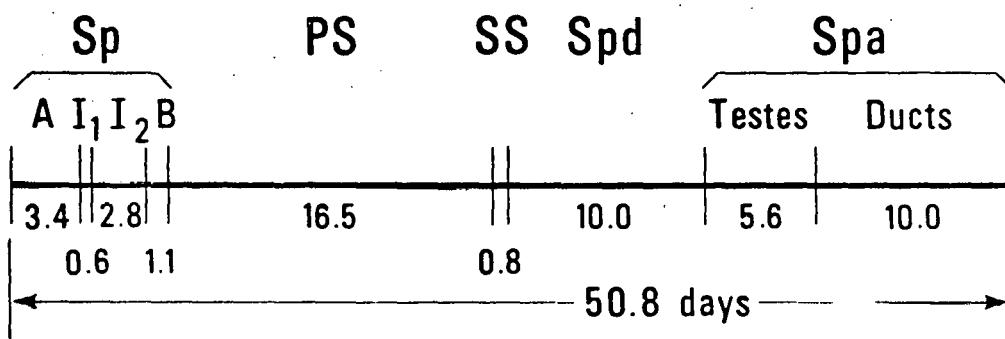
DBL 7011 5995

Fig. 59. Lifespan of rabbit spermatogenetic cells. See also Figure 59 (reduced) in rear-cover envelope, for use with Figures 57 and 58.

give a time of appearance in the ejaculate similar to that observed by

Swierstra and Foote. Takeda, Lutwak-Mann and Mann labelled mammalian sperm-

atozoa with 32 p in order to study the so-called leakage of intracellular sperm

compounds which is known to accompany sperm aging or senescence[225]. The

level of radioactivity in rabbit spermatozoa separated from seminal

plasma by centrifugation and washed 3 times with Ringer's solution re-

mained low until 40-42 days after the injection of P^{32}, at which time the

radioactivity rose steeply[225]. This time span corresponds favorably to the

results reported by other investigators.

Referring to Figures 57 and 58, the ejaculate from Otto on August

15, the day after the temperature was 90°F, contained less than 4% motile

spermatozoa and 80% capless spermatozoa. Ejaculates collected from then

until September 6 contained from 25-60% motile spermatozoa and from 18-52%

capless spermatozoa. Spermatozoa appearing in the ejaculates from August

15-29 should have been in the spermatogonial stage at the time of the

91°F temperature on July 11 and in varying primary spermatocyte stages

during the 83°F days on July 20, 26 and 28. The spermatozoa appearing in

ejaculates from August 29-September 6 would have been in spermatogonial

stages during the 83°F days in the latter part of July; therefore, the

91°F temperature on July 11 would have had no effect.

On September 9, the quality of Otto's ejaculate was very poor, with

less than 4% motile spermatozoa and 87% capless spermatozoa. Most of the

spermatozoa in that ejaculate would have been undergoing the second

maturational (meiotic) division on August 14 when it was 90°F and in the

spermatogonial division stages on July 26 and 28 when it was 83°F. The

effects of increased environmental temperature on either of these stages

could have contributed to the decreased motility and high percentage of

capless spermatozoa in that ejaculate. This suggests that spermatozoa

and spermatids are relatively resistant to increased environmental temperatures. It appears that spermatozoa in the intra-abdominal part of the duct system are damaged, thereby demonstrating the importance of the lower scrotal temperatures in sperm survival.

After slight improvement in the next 2 ejaculates, the ejaculate on September 18 contained 72% motile spermatozoa and only 10% capless spermatozoa. The decrease in quality on the following day to only 22% motile and 40% capless spermatozoa is probably an example of the second ejaculate phenomenon discussed in subsection 2, above. The spermatozoa in this transient peak of high motility would have been in the middle stages of primary spermatocyte development on August 14 (90°F) and may well have started their development after the elevated temperatures on July 26 and 28. This suggests that elevated environmental temperatures during the intermediate stages of primary spermatocyte development have little effect on the subsequent motility of the spermatozoa.

The downward trend observed on September 19 continued such that 3 ejaculates between September 22-27 had less than 6% motile spermatozoa and from 55-84% capless spermatozoa. Spermatozoa appearing in those ejaculates would have been in the early spermatocyte and late spermatogonial division stages of development on August 14 when it was 90°F. This suggests that the spermatogonial and early spermatocyte stages of development are very sensitive to increased environmental temperature.

The improvement in the quality of the ejaculates after October 5 reflects the appearance of spermatozoa which started their spermatogonial development after August 14. One can speculate that the failure of the quality of the spermatozoa to reach a superior level, e.g., approaching 90% motile, reflects a prolonged influence of that particular high environmental temperature on the stem cell layer.

Although the curves for Psi and Snoopy in Figures 57 and 58 are not identical to those for Otto, the similarity is evident and analyses of those curves lead to similar conclusions.

Spermatozoa samples collected from the "indoor" group on November 4 showed a significant decrease in quality with an average of 8% motile spermatozoa and an increase in percentage of capless spermatozoa to 80%. This is attributed to a transient relative temperature rise to 79°F in the air-conditioned room, which was generally kept at 66°F, due to an error on the part of the caretaker. On the 79°F day (October 17), the spermatozoa would have been in the final stages of morphogenesis from spermatids.

In general, the quality of the ejaculates from the rabbits in the "outdoor" group was extremely poor until after they had been moved into the air-conditioned room on October 14. Shortly thereafter, there was a marked increase in the quality of the ejaculates from Ted and Larry, whereas those from Chi remained poor. However, the average quality of the ejaculates increased considerably and did not show a drop on November 4, as did the ejaculates from the "indoor" group. It is probable that the relative temperature rise of 13°F did not seem significant to the rabbits in the "outdoor" group which had been exposed to much higher temperature ranges prior to coming into the air-conditioned room 3 days previously. This introduces the concept of relative, as compared to absolute, temperature changes. Thus, for rabbits acclimatized to consistently low temperatures, a relative temperature rise can be significant. In contrast, rabbits accustomed to wide daily temperature variations are not affected by moderate elevations of temperature.

In retrospect, this ancillary study on the influence of the environmental temperature on the quality of spermatozoa is one of the most interest-

ing and most important parts of this thesis. Putting rabbits in an air-conditioned room greatly improved the quality of spermatozoa available for Staflo experiments. In addition, the observations strongly suggest that a very high environmental temperature for part of one day can cause marked variations in the quality of spermatozoa of rabbits, kept thereafter in a controlled environment, for 7-8 weeks and probably to some degree for several weeks longer. There were no changes in animal care or sperm collection to explain the variations in quality. The probable differential effects of the environmental temperature on the stages of spermatogenesis explain the random variation in spermatozoa quality previosly observed in warm weather.

Within the obvious limitations of this study, it appears that several stages of spermatogenesis are sensitive to increased testicular and abdominal temperatures secondary to elevated environmental temperatures. It is probable that the spermatogonial divisions are critical periods considering the improvement in sperm quality of the "indoor" group after October 5. However, it is difficult to separate that phase of development from the early primary spermatocyte stage in this study because of the several hot days to consider. The maturational division stages and the very early (and possibly very late) phase of spermatid development appear to be sensitive to elevated temperatures. Whereas late spermatids and immature spermatozoa appear to be protected to some degree in the scrotum, mature spermatozoa in the intra-abdominal duct system are very sensitive to temperature changes. For instance, nearly complete disintegration of spermatozoa was observed in many ejaculates collected the morning after a day with a 90°F or higher temperature.

The difficulty in dissecting the effects of various temperature peaks during the period shortly after the rabbits were moved into the air-conditioned

room emphasizes the need for a controlled study. In general, it should include the following: (1) establishing a steady-state baseline with regard to sperm quality and collection frequency in a controlled low temperature (60-65°F) environment, (2) exposure of the experimental group to a temperature of 85-90°F for 6-10 hours on one day only, and (3) daily collection of ejaculates from both control and experimental groups until full reproductive performance returns or at least for 60 days. Daily spermatozoa collection is necessary to avoid the second ejaculate phenomenon which would mask less dramatic actual changes in sperm quality. Such a study would give a comparison of the differential sensitivity of the stages of spermatogenesis to temperature, determined in this manner, and also to external radiation of the testes.

D. Summary and Conclusions

Even after eliminating obvious possible adverse factors in animal care and sperm handling, the quality of ejaculates showed marked and unpredictable variability in warm weather. In addition to differences in the quality of ejaculates from different rabbits, a dramatic decrease in the quality of consecutive ejaculates from the same rabbit was observed if the interval between collections was 60 minutes or less. Only partial recovery was observed after an interval of 24 hours, suggesting a selective process in the genital duct system favoring ejaculation of the highest quality spermatozoa with the first emission.

Substantial differences in measured sperm quality were found, according to the methods used for evaluation. For example, there was a lack of correlation between the percentages of capless (CL) spermatozoa determined by phase contrast microscopy and of stained (S) spermatozoa from light microscopy scoring of nigrosin-eosin stained preparations. Capless

percentages ranged from 33.9% less than to 22.1% more than "stained"
percentages, with the differences apparently unrelated to spermatozoa
concentration.

Subsequent studies showed fair agreement between phase contrast and
light microscope scoring of nigrosin-eosin stained spermatozoa. Comparison
of phase contrast scoring of unstained spermatozoa with nigrosin-eosin
stained preparations of the same sample showed differences related to
spermatozoa quality. With poor quality ejaculates, the unstained samples
showed a higher percentage of capless spermatozoa than did the nigrosin-
eosin preparations (e.g., CL $>$ UCL + SCL in Table XXXIV). The reverse
was true for high quality samples. The deterioration of spermatozoa
samples during centrifugation is a function of the inherent quality of
the sample even at concentrations above 4 x 10^6/ml. It does not appear
to be related to the initial percent of capless sperm in the sample or
to temperature, over the small range of 2.3-7.2°C.

It was shown in this chapter that elevated environmental temperatures
caused marked variations in sperm quality for 7-8 weeks (and possibly
longer) after exposure, even if the rabbits were placed in an air-condi-
tioned room at 66°F right after the high temperature exposure. Therefore,
the relatively sensitive stages of spermatogenesis would appear to be the
spermatogonial divisions, the maturational divisions and the early
spermatid stage (Figure 57 and 59). The late spermatid and spermatozoa
stages appear relatively resistent to elevated body temperatures except
for the mature spermatozoa in the intra-abdominal part of the genital
duct system. The latter spermatozoa undergo marked deterioration as
evidenced by loss of motility and increased percentages of caplessness
in the ejaculate. The delayed and intermittent expression of the effects
of environmental temperature on spermatozoa quality explains, at least

partially, the observations mentioned in previous chapters and in the first paragraph of this section.

X. SUMMARY

Since a number of detailed conclusions and recommendations were presented in the individual chapters, only the most important are recapitulated here, along with some summary findings.

The motility and fertilizing capacity of rabbit spermatozoa were preserved for several hours during separation experiments at $3^{\circ}C$ at concentrations of approximately 1×10^6 and 4×10^6/ml, respectively. These experiments were carried out in low ionic strength glycine-phosphate buffer. Fowl spermatozoa motility was preserved at concentrations as low as 8×10^6/ml for 6 hours at $3^{\circ}C$ in 310 mosm solutions of both low ionic strength glycine-citrate and glycine-phosphate buffers. Under the same media and temperature conditions, fertilizing capacity was preserved for about 2 hours at concentrations down to 90×10^6 sperm/ml. The establishment of these conditions rendered it possible to conduct analytical biophysical experiments on low concentration preparations of rabbit and fowl spermatozoa, and to exploit the results of such studies at higher concentrations in preparative Staflo experiments with maintenance of vital cellular functions, especially the fertilizing capacity.

In Staflo experiments at $3^{\circ}C$ or lower, migration was not significantly influenced by intrinsic motility and there was no differential distribution of live and dead rabbit spermatozoa among collection fractions in three-layer sedimentation and two-layer electrophoresis migration experiments. Under these temperature conditions a most important variable in experiments using low density-low viscosity media is the cell concentration. For instance, concentrations above 25×10^6 rabbit spermatozoa/ml may result in "cluster sedimentation",

i.e., cooperative rather than individual cell sedimentation.

At 4°C and higher, the intrinsic motility vector may have a significant contribution to the migration pattern. Sperm orientation was influenced by gravity in all Staflo experiments and by the strength and direction of the electric field in electrophoresis experiments. By proper selection of the medium, pH, density gradient, temperature and electric field strength (if used), the intrinsic motility vector of both rabbit and fowl spermatozoa was controlled so as to give enrichment of certain collection fractions with regard to motility, viability and fertilizing capacity.

The best values obtained for selected physical properties of rabbit and fowl spermatozoa are given in Table XXXVIII. The sedimentation and electrophoretic values were determined in Staflo experiments, the specific gravities in sodium iothalamate gradient. Within the single (specific gravity) band of fowl sperm, a higher percentage and degree of motility were found in the top of the band. Among the different bands of rabbit spermatozoa, there was a marked differential viability distribution with the low density band containing nearly all of the viable sperm. Thus centrifugation in a sodium iothalamate density gradient provides a ready means for separating live and dead rabbit spermatozoa. Although there was no evident bimodal distribution of viable sperm in the low density band, (as suggested by others) it is possible that, with an expanded density gradient of physiological osmolarity, different groups of spermatozoa might be found in this band. The high density band, containing essentially only non-viable sperm, appeared to migrate farther with increasing centrifugation time suggesting a non-constant effective density.

Table XXXVIII. Best Values of Physical Properties of Rabbit and Fowl Spermatozoa[a].

Sperm	Property	Medium	pH	Avg. Value
	Sed. Rate	GPB[b]	7.0	0.53×10^{-4} cm/sec/cm/sec^2
	Elect. Mobil. ($\uparrow$)[c]	GPB	6.6	0.54×10^{-4} cm/sec/V/cm
Rabbit	Elect. Mobil. ($\uparrow$)	GPB	7.1	0.85×10^{-4} cm/sec/V/cm
	Elect. Mobil. ($\downarrow$)	GPB	7.1	0.81×10^{-4} cm/sec/V/cm
	Specific Gravity	SI	---	1.19-1.21 (upper band: viable sperm)
				1.26-1.29 (middle band: mixed quality)
				1.33-1.37 (lower band: poor quality sperm)
	Elect. Mobil. ($\uparrow$)	GCB	7.0	1.65×10^{-4} cm/sec/V/cm
Fowl	Elect. Mobil.($\downarrow$)	GCB	7.0	1.68×10^{-4} cm/sec/V/cm
	Specific Gravity	SI	---	1.19-1.21 (single band: all sperm)

a) normalized to conditions of 3°C and 1% sucrose.

b) GPB= glycine-phosphate buffer; GCB= glycine-citrate buffer; both of resistivity 960 ohm cm.

   SI = sodium iothalamate density gradient.

c) $\uparrow$ = upward migration. $\downarrow$= downward migration.

The number of offspring obtained from the artificial insemination of separated rabbit spermatozoa was insufficient for the correlation of physical properties with the X- and Y-chromosome content of the spermatozoa. Observations in these experiments strongly suggest that the partial separation of the two types of spermatozoa reported by other investigators is not a simple function either of differential sedimentation rates or of differing electrophoretic mobilities. Thus the partial separation of X- and Y-chromosome bearing spermatozoa in their sedimentation experiments must have been influenced by "cluster sedimentation" or some process other than individual cell sedimentation, such as an aqueous polymer two phase separation as described by Albertson[270]. My observation of a significant effect of motility on electrophoretic migration, at temperatures as low as 4°C depending upon the medium, suggests that the X-Y separations reported by Schröder[206] and by Gordon[91] using temperatures generally 10-15°C, were most likely a consequence of a differential swimming rate of, rather than an opposite charge on, the two types of spermatozoa.

A frequent obstacle to obtaining offspring from separated spermatozoa was the marked variability and unpredictability in the quality of rabbit ejaculates, especially during warm weather. The effects of elevated environmental temperatures appeared to be intermittent over a 7-8 week period. Relative as well as absolute elevations of temperature may be important in this regard. The most sensitive phases of spermatogenesis appeared to be the spermatogonial and maturational divisions, and the early spermatid stage. In addition, mature spermatozoa in the intra-abdominal part of the genital duct system appeared to undergo marked deterioration because of elevated

environmental temperatures, as evidenced by the immediate loss of motility and increased caplessness of ejaculate sperm.

Several theoretical analyses applicable to free solution zonal migration and fractionation processes were made. Range and distribution diagrams for particle migration distances were developed which make possible visualization of the requirements for optimum fractionation, and subsequent quantitation thereof. The diagrams also provide a means for analyzing the distribution of migration properties among particle populations. They emphasize the fact that migration patterns spreading over only two adjacent layers can be meaningful but that a three-layer pattern is the minimum that will give distinct sub-populations in the extreme fractions.

As in all investigative fields, each project generates innumerable ideas for additional work. Many suggestions for expanding the different methods of studying spermatozoa used in this thesis are made in individual chapters. It is hoped that some of the suggestions will be implemented in the near future.

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Because I lived and studied at Stanford throughout most of the time during which this work was done, my dependence upon and appreciation of the assistance provided by the persons in Berkeley, mentioned below, is more than ordinary.

I wish to express my deepest appreciation to my advisor, Dr. Howard C. Mel, for his continued encouragement, guidance and assistance, and for making available the invaluable and frequent help from his research assistants, Mrs. Gretchen Hylton, Mrs. Anne Poley and Mr. Ralph Reynolds.

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GLOSSARY

Symbol or Term	Meaning	Units	Where First Used
AI	Artificial insemination	-	p 234
C	Capped (spermatozoa)	-	Table XVIII, p 218
CF	Collection fraction. The cells or particles in an individual collection container	-	p 65
CL	Capless (spermatozoa)	-	Table XVIII, p 218
Collection container	Receptacle in collection system for outflow from one outlet channel	-	p 65
cp	Centipoise (viscosity)	$(\frac{g}{cm \cdot sec}) \times 10^{-2}$	p 21
CPD	Cytoplasmic droplet (sperm)	-	p 161
$\frac{dx}{dt}$	Sedimentation velocity	cm/sec	Eq (4), p 84
E	Electric field strength	V/cm	Fig 16, p 87
e·s	Slowest electrophoresing-slowest sedimenting (particle)	-	p 86
e·S	Slowest electrophoresing-fastest sedimenting (particle)	-	p 86
E·s	Fastest electrophoresing-slowest sedimenting (particle)	-	p 86
E·S	Fastest electrophoresing-fastest sedimenting (particle)	-	p 86
EWFR	Electrode wash solution flow rate	ml/min	p 67
EYM	Egg yolk medium	-	p 234
Flow-cell	Consists of flow-chamber and upper and lower electrode chambers	-	p 55
Flow-chamber	Chamber in which electrophoretic and/or sedimentation migration occurs	-	p 55

Symbol or Term	Meaning	Units	Where First Used
FM	Forward motion (spermatozoa motility)	-	Table XVIII, p 218
Fraction	Short for "collection fraction"	-	p 66
g	Acceleration of gravity	980 cm/sec^2	App F, p 381
GCB	Glycine-citrate buffer	-	App D, p 358
GPB	Glycine-phosphate buffer	-	p 232
h	Height of sample stream at inlet	cm	p 75
IS	Initial sample. Sample suspension that is introduced into Staflo inlet layer	-	p 231
L	Length of flow-chamber	cm	Table V, p 148
Layer	Horizontally flowing fluid layer in Staflo flow-chamber	-	p 58
MBM	Modified Baker's Medium	-	App D, p 358
Migration pattern	The overall side view appearance of a continuously flowing sample migrating in the flow-chamber	-	p 58
Migration profile	The appearance or shape of the cross section of a migrating sample	-	p 67
MNS	Morphologically normal but stained (spermatozoa)	-	p 112
NH-1,2	Norman-Johnson Solutions 1 or 2	-	p 37
NZW	New Zealand White (rabbit)	-	Table XXIV, p 245
s	Slowest sedimenting (particle)	-	Fig 16, p 87
S	Fastest sedimenting (particle)	-	Fig 16, p 87

Symbol or Term	Meaning	Units	Where First Used
S	Sedimentation coefficient	Svedbergs ($=10^{-13}$sec)	Table III, p 117
S	Stained (spermatozoa)	-	p 112
SCL	Stained, capless (spermatozoa)	-	p 286
SDE	Sedimentation-downward-electrophoresis	-	p 85
SI	Sodium iothalamate (medium)	-	Table XXXVIII, p 317
SS	Stirred sample. Suspension that remains in Staflo sample (feed) syringe at conclusion of experiment (usually stirred magnetically throughout experiment)	-	p 234
Staflo	Short for "Stable-flow free-boundary"	-	p 2
SUE	Sedimentation-upward electrophoresis	-	p 85
TW	Tail-waggling (spermatozoa motility)	-	Table XVIII, p 218
U	Unstained (spermatozoa)	-	p 112
UC	Unstained, capped (spermatozoa)	-	p 286
UCL	Unstained, capless (spermatozoa)	-	p 286
v	Particle migration rate	cm/sec	Eq (13), p 97
V	Volts	Volts	Eq (13), p 97
η	Absolute viscosity	poise $(\frac{g}{cm \cdot sec})$	Eq (3), p 84
λ	Migration distance	cm	p 77

Symbol or Term	Meaning	Units	Where First Used
λ_E, λ_S, etc.	Migration distances: E (electrophoresis), S (sedimentation), SDE (sedimentation-downward-electrophoresis), SUE (sedimentation-upward-electrophoresis)	—	Eq (2), p 83
$\lambda_{9SUE\text{-}min}$	Minimum λ for spermatozoa in collection fraction 9 in sedimentation-upward-electrophoresis	cm	p 86
μ	Electrophoretic mobility	$\dfrac{cm^2}{sec \cdot volt}$	p 91
μ	Micron	10^{-4} cm	p 42
τ_{min}	Minimum residence time; the time any given volume element resides in the flow-chamber	sec	Eq (4), p 84
τ_{av}	Average residence time; time required to pump a volume of fluid equal to the volume of the flow-chamber	sec	Table V, p 148
$\tau_{E_{min}}$	Minimum residence time for electrophoresis	sec	Eq (13), p 97

APPENDIX A

Considerations in Selecting the Rabbit
as a Sperm Donor

1. Rabbit spermatozoa have been studied extensively with regard to both physiology and attempted separation of X- and Y-chromosome bearing sperm.

2. The rabbit is large enough to work with conveniently. Sperm can be collected with an artificial vagina, allowing reuse of the animal. Rats and mice generally are sacrificed to get sperm.

3. Rabbit semen does not coagulate[92] as does that of the rat[168]. Guinea pig spermatozoa agglutinate more rapidly than sperm from other species in all media, including saline[52].

4. Rat spermatozoa survive only 2-3 hours in saline, whereas spermatozoa from other species generally survive for longer time periods[52].

5. The rabbit is usually in estrus if there is sufficient light. Ovulation can be induced in the rabbit, while the mouse and hamster ovulate spontaneously.

6. Normal ovulation in the rabbit yields an average of 10.2 ova. More can be obtained with superovulation.

7. Fertilized rabbit ova spend about 3 days in the oviduct and implant in the uterus in the morula (3 day) stage or blastula (4 day) stage. This is also true for rat and mouse ova.

8. The gestation period of the rabbit is 30-32 days; slightly greater than those of the rat, mouse and hamster which are 22, 19 and 16-19 days, respectively.

9. The litter size of the rabbit averages from 4.0-8.1 offspring, compared to 6.1-11.1 for the rat and 4.5-7.4 for the mouse.

APPENDIX B

Supplemental Information on Work with Germ Cells

Contents

1. <u>Spermatozoa</u>

   a. <u>Collection</u>. Designs of artificial vaginas which have proved success-

ful are discussed by Gordon[92]; Walton[239]; Bredderman, et al[46]; and Pis-

tenma (Appendix C).

Foote used glycerol as a lubricant in an artificial vagina, and

found a large percentage of bull spermatozoa adherent to the rubber sur-

face[81]. Using silicon as a lubricant did not decrease the tendency for

adhesion of spermatozoa to the lumen[81]. Polge observed that rabbit sperm-

atozoa did not regain motility if more than approximately 2.5% glycerol

is rapidly introduced into the semen[188]. Thus, it is conceivable that

liberal lubrication of the lumen of the artificial vagina with glycerol

could be detrimental to the sperm. KY jelly and similar sterile lubri-

cants have been used by me with no observable effect on the spermatozoa.

Fox collected rabbit semen between 5:30 and 7:30 A.M. when distracting

noises and influences were minimal[85]. He evaluated the semen for motility,

concentration (rejected if less than 100×10^6/ml) and live sperm, using

a differential (live-dead) fast-green FCF and Eosin B stain and rejected

the sample if less than 50% were viable.[85]

Williams and Hamner used a blackened Warburg flask for collection,

centrifugation and washing spermatozoa[243]. They observed a transient in-

crease in respiration of rabbit spermatozoa with a 5-30 second exposure

to white light and observed almost a 4-fold increase in respiratory rate

after a 3 minute exposure to white light (100 watt bulb at 20 cm)[243].

Norman and Goldberg showed that a wave length of 4400 A° produced the greatest effect which is believed to be a photosensitized oxidation[176]. Impairment of motility is exponential with exposure as if the light initiated an autocatalytic reaction. Brief exposure to light initiates a reaction which continues at a slower rate in the dark. The red end of the spectrum had little effect on the spermatozoa[176].

Brackett and Williams observed that the temperature of the artificial vagina used in semen collection changed the effect of light on respiration. Exposure to 48°C for 40 seconds reduced motility. Heat stimulated respiration in the absence of light and depressed respiration upon exposure to light later[265]. Walton[239] suggested a temperature of 45°C for the artificial vagina. In view of Brackett and Williams' observation, I would recommend staying well below 45°C in order to avoid the possibility of heat damage to spermatozoa. Male rabbits are not very happy if the temperature of the artificial vagina is below 38°C, so a temperature of 40-42°C appears to be a good compromise.

b. Cooling. The loss of viability and motility of spermatozoa after sudden cooling has been described for many mammalian species, including the rabbit[92] and the bull[101,109].

Tanaka and Okamoto have shown that rapid cooling of fowl spermatozoa from 25°C to 5°C has no effect on fertility of undiluted and diluted spermatozoa[269]. Rapid cooling of spermatozoa to 0 and -2°C resulted in lowered fertility with undiluted semen, but no change in motility with 4-fold dilution of the semen with Wilcox's buffer. Cooling to -2°C at a rate of 0.5°C/minute resulted in no significant decrease in fertility of diluted or undiluted semen[269].

Cooling curves determined for 2 ml of buffer in a 15 ml centrifuge tube in the center of 1 and 2 pound coffee cans filled with sawdust and immersed in ice water have shown maximum cooling rates of less than 0.4°C/min. Cooling from room temperature (23°C) to 3°C takes approximately 90 and 110 minutes in the 1 and 2 pound coffee cans, respectively. This is a simple and convenient way to cool spermatozoa gradually.

c. Counting. Rothschild[197] and Maude[150] observed nonrandom distributions of spermatozoa in a Hawksley hemocytometer. Rothschild observed that gravity did not affect the distribution of the spermatozoa and that bull spermatozoa appeared to be attracted to the large glass surfaces of the cover slip and microscope slide[197]. Freund and Carol reported that in a study of the variance in counts by different technicians using a hemocytometer, pipetting accounted for 22% of the variance, and handling of the chamber 26%[87]. 57.4% of the variance in hemocytometer technique was due to variance among technicians, and 42.6% was due to variance among duplicate hemocytometer determinations by the same technician[86]. The 95% confidence interval for hemocytometer counts among technicians on a single specimen was ± 52%[86,87].

Segal and Laurence analyzed human spermatozoa using a Coulter Counter[209]. The addition of 1 ml of 1% saponin/100 ml of diluted semen to prevent clumping caused immediate loss of motility of the spermatozoa without an observable change in morphology. Estimates of the spermatozoa concentration, manually, with a hemocytometer, and by counting in the Coulter Counter gave agreement within 25% in approximately 1/2 of the samples counted. The variance in counts ranged from 25-50% in approximately 28% of the samples and was greater than 50% in approximately 22% of the samples[209].

I have found a 1% solution of formalin in saline to be excellent

for counting spermatozoa. The solution stops all motility immediately and does not change the morphology of the spermatozoa, so that differential counts of capped and capless spermatozoa can be made conveniently.

d. Evaluation.

(1) Motility. The motility of spermatozoa can be evaluated quantitatively in low concentrations or qualitatively in high concentrations. The quantitative evaluation can be done with either a light microscope or phase contrast microscope. The degrees of motility of individual spermatozoa are categorized as follows: a) immotile, b) feeble tail-wagging, c) vigorous tail-wagging, d) slowly progressive forward motion, and e) vigorous forward motion.

It is customary to evaluate fowl spermatozoa in high concentrations on a scale of I to VI corresponding to essentially no motility (Grade I) and a vigorous swarming motion (Grade VI), respectively, in the specimen under low or intermediate power in the microscope.

(2) Phase Contrast Observations. Using phase contrast microscopy, individual spermatozoa in a sample can be categorized as follows: a) immotile spermatozoa: capped (C) and capless (CL); b) tail-wagging (TW); and, c) forward motility (FM). The tail-wagging and forward motion are assessed degrees as noted in sub-paragraph (1) above. All spermatozoa with tail-wagging or forward motility observed by the author were capped. However, the possibility of motile capless spermatozoa is not doubted for motile tails with midpieces but no heads have been observed.

(3) Nigrosin-Eosin Staining.

a) Procedure. I used the stain recommended by Hancock which is prepared by dissolving 5 grams of water soluble Eosin Y (Gurr) in 300 ml of 10% aqueous Nigrosin (Gurr)[109]. One drop of spermatozoa suspension is placed in 8 drops of the stain for 5 minutes. It is then smeared on

a glass slide and allowed to dry in air. Other methods of differential staining of spermatozoa are presented in references 21, 58,131 and 152.

b) Scoring Stained Spermatozoa. Stained slides can be scored by either light microscopy or phase contrast microscopy, but there is a difference in the 2 methods with nigrosin-eosin stained samples. The correlation of the scoring by the 2 methods is discussed in Chapter IX.

With the light microscope, 3 categories of stained spermatozoa are observed as follows: unstained (U), morphologically normal but stained (MNS) and "stained" (S) spermatozoa. These are illustrated in Figure 60 which shows the acrosomal cap present on the unstained and on the MNS spermatozoa. The MNS spermatozoa are stained only in the post-nuclear cap region, whereas the "stained" spermatozoa have no acrosomal cap and the entire head is stained homogeneously.

Four categories of spermatozoa are observed with phase contrast microscopy as follows: unstained and capped (UC), unstained and capless (UCL), morphologically normal but stained (MNS) and stained and capless (SCL). This categorization differs from the light microscope scoring of the nigrosin-eosin stained spermatozoa by the division of the unstained spermatozoa into capped and capless groups.

(4) Signs of Deterioration or Damage of Spermatozoa. Signs of deterioration of mammalian spermatozoa in order of increasing severity are loss of motility; loss of the acrosomal cap; bending of the tail, usually at the neck; and complete severance of the tail from the head. The heads of mammalian spermatozoa remained intact despite considerable stress.

The deterioration of fowl spermatozoa is somewhat similar to that of mammalian spermatozoa. However, in addition to the loss of motility and bending of the tail (resembling a hairpin in many instances), the heads

SPERM STAINING WITH NIGROSIN-EOSIN

a.   b.   c.
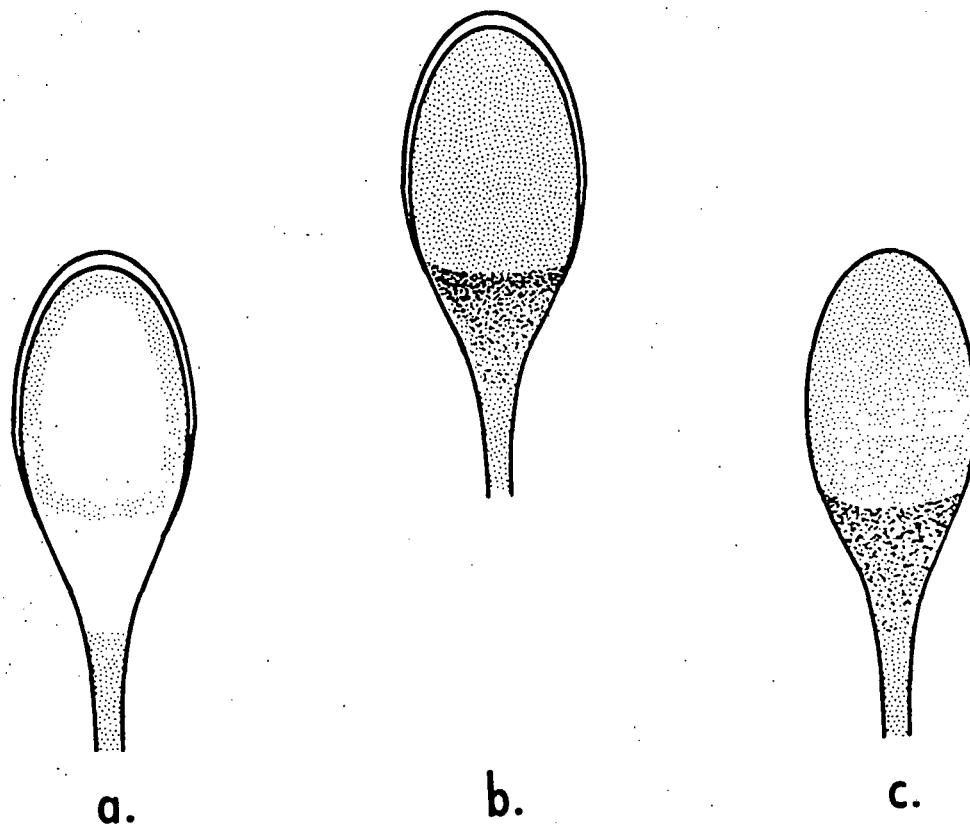
DBL 7011 5996

Fig. 60. Schematic diagram of categories of rabbit spermatozoa

observed with light microscopy scoring of nigrosin-eosin prepara-

tions: unstained (U), morphologically normal but stained (MNS)

and stained (S).

of fowl spermatozoa undergo complete disintegration. This change in morphology is characterized initially by marked swelling of the head to 2-3 times the normal diameter. This is followed by a beady appearance and finally complete disintegration of the head with small beads of chromatin-appearing material scattered in the medium.

(5) Fertilization Capacity. The ultimate test of the spermatozoa of any species is their ability to fertilize ova. This is discussed in other sections below.

2. Ova

a. Normal Fertility of the Female Rabbit. Rabbits are practically 100% fertile when mated 1-4 days after parturition if the offspring have been removed from the nest. They remain in heat up to 36 days after parturition but the fertility is less than days 1-4 thereafter[105]. If the young are to be removed from the nest, they should be removed immediately after birth. The slightest delay may allow letting down of milk and lead to caked breasts in the mother.

Rabbits mated at the end of pseudopregnancy (approximately day 20) have a very low percentage of infertile matings, whereas one gets an average of 31% infertile matings in rabbits which have not been pregnant recently[105]. Foote and coworkers observed that a high proportion of does, even though not pseudopregnant, may refuse to copulate[82].

The age of the female is important for the following reasons: (1) Females less than 100 days give few offspring[170]. (2) The age of the female at the first fertile coitus varies according to season of birth:

Month of Birth	Age at First Coitus
March-April	8.5 months
Oct.-Dec.	5.2 months

Natural copulation is more likely from April through July, whereas anoestrum

normally prevails in late fall and winter[105]. (3) Young does give

an average of 55% normal offspring, whereas 19 month old does give only

36% normal offspring[105].

Staples and Holtkamp found a positive correlation between the number

of Dutch Belt does with corpora lutea and increasing weight of the fe-

males[218]. It is interesting that 1 doe weighing only 1.05 kg maintained

2 fetuses. They suggested that if the age of Dutch Belt does is unknown,

it is important to use does that weigh 1.75 kg or more for reproduction

studies[218].

There is a positive correlation between the number of eggs released

from the left and the right ovaries[170]. The fertilizability of the ovum

is greatest up to 4 hours after ovulation and after approximately 8 hours

is not fertilizable[4]. This may be related in part to the formation of a

mucin coat around the ovum from 5-14 hours after ovulation if the rabbit

is mated normally. Formation of this mucin coat is delayed until about

the 17th hour if ovulation is artificially induced and no mating occurs[15].

Fertilization of aged ova gives a higher mortality rate of the embryos[4].

Shaver and Carr found a marked decrease in the number of blastocysts

recovered when mating of the rabbits took place more than 10 hours after

injection of gonadotropin [212]. They found 7 blastocysts with chromosome

abnormalities from mating 6 or more hours after gonadotropin injection

and 6 blastocysts with chromosome abnormalities with mating 2 hours or

less after injection. A total of 135 blastocysts were examined[212]. Al-

though not specified, it is believed that the 2 groups had approximately

the same number of blastocysts.

Chang and Bedford showed that the cumulus oophorus does not play a

part in fertilization but the corona radiata is important in protecting

the fertilizability of ova[55]. Dickmann discusses the morphology of the

zona pellucida of the rabbit ovum[66]. Szollosi and Ris discuss the disappearance of most of the cortical granules found within the plasma membrane after sperm penetration[224]. Austin presents a thorough review of all aspects of the mammalian egg[15].

Thirty hours after insemination of the doe, eggs should be approaching the 4-cell stage[170]. Additional information on cleavage rates may be found in reference 15. The rate of development of fertilized ova is related to the number of eggs fertilized, the number shed and to the age and weight of the female, in that order of importance[170].

Hafez and Rajakoski have studied the effects of many maternal factors on the growth and survival of blastocysts in the domestic rabbit[103]. The ovulation rate tended to increase with increase in parity. The number of degenerating blastocysts per doe increased in all superovulated groups and the percentage of viable blastocysts among the total number recovered did not vary in normally ovulated groups and in one superovulated group[103]. Hafez showed that salpingectomy, unilateral ovariectomy, subtotal ovariectomy or administration of progesterone in intact rabbits had no significant effect on the viability and size of blastocysts or on the diameter of the embryonic disk[100].

Maurer, Hunt, Van Vleck and Foote studied the developmental potential of superovulated rabbit ova after transfer to recipient females. They found that fertilized ova from the donors exhibited a normal appearance at the time of transfer, revealed no subsequent defects or deficiences and were as capable of developing into viable young as ova obtained by routine ovulation[264].

McGaughey and Daniel have shown that a concentration of 25 μg/ml of estradiol inhibits cleavage of single-cell rabbit eggs IN VITRO while most

eggs are apparently unaffected by an estradiol concentration of 5 μg/ml[153].
Equimolar concentrations of progesterone appear to be antagonistic to the
effects of estradiol[153].

Dukelow showed that the lambing percentages of mated ewes increased
by 12.9% and the number of ewes lambing by 7.6% after oral administration
of extracted wheatgerm oil over 2 breeding seasons[69]. He observed no effect
in Guernsey cows given the wheatgerm oil[69]. He did not evaluate its effect
on rabbits; however, this is an interesting possibility to improve results
of reproductive studies.

b. Inducing Ovulation. Two apparently satisfactory means of inducing
ovulation are the use of a vasectomized buck and the use of hormones.

1) Using a Vasectomized Buck. The advantages of using a vasectomized
buck are that it simulates natural mating and no injection is needed. In
addition, the doe can be mated immediately after parturition without con-
cern about the development of immunity to induction of ovulation by hor-
mones, which is noted below.

The disadvantages of using a vasectomized buck are: (a) The timing
and control of the experiment are subject to an expression of love by the
rabbits and sometimes as many as 50% of the does refuse to mate. (b) One
must check the vasectomized buck often in order to insure that there are
no sperm in the ejaculate. Gordon recommends careful checking for the
first 3 weeks and periodic checks thereafter (Telecon with Mel, March 11,
1964).

2) Use of Hormones. Because a high percentage of does may refuse to
copulate with a vasectomized buck, Foote recommends the use of luteinizing
hormone as a means for carrying out an experiment with sound statistical
design[82]. However, the use of luteinizing hormone to induce ovulation is

reliable only if the female rabbits are maintained in estrus by natural or artificial mating when not being used in experiments. If there is a question as to whether or not a doe is in estrus, Foote has found that a doe that accepts a vasectomized buck will ovulate. Most does that refuse to mate with the vasectomized buck will not ovulate[2]. The use of LH avoids the possibility of inadvertent fertilization by an incompletely vasectomized buck.

Foote, et al., used supplemental lighting to insure 12 hours of simulated daylight to minimize seasonal effects of endogenous hormone production[82]. 0.5 mg/kg of pituitary luteinizing hormone (PLH), (Armour), in 0.9% NaCl, was injected in a marginal ear vein. This was followed immediately by artificial insemination of greater than 3×10^6 sperm in a 0.1-0.2 ml vol. Normal ovulation rates and numbers of offspring were obtained. With injection of a total of 2.5 mg PLH, 91% of 57 does kindled[82]. Following a second injection, 16 does snowed normal fertility and gave a normal litter size. Up to 4 injections over a 2-year period did not affect fertility[82].

Even though Foote observed no decrease in fertility with successive injections of luteinizing hormone, there is evidence that repeated injections of LH leads to immunity in the rabbit. Adams observed a failure of does to produce litters with continued use of LH, as shown below:

Failure of Does to Produce Litters
With Continued Use of LH[1]

Attempted number:	1st	2nd	3rd	4th	5th	6th
Days between injections:	0	87	91	40	37	102
Females failing to produce litters (%)	8	0	20.8	43.5	87.0	100

Some does were mated naturally at the time of the 5th injection of luteinizing hormone and all conceived despite immunity to the hormone[1].

Adams and Chang injected 25 iu of luteinizing hormone (LH)(Lutormone or Follutein), mated does naturally from 5 hours before to 2 hours after ovulation and evaluated results by removing Fallopian tubes and examining ova[4]. They observed from 7 to 15 ovulations (mean of 10.3) per doe and estimated that ovulation occurred 9.5 to 13 hours after LH injection. With mating at 8 hours after LH injection, 85% of the does conceived and 74% of all eggs (including the eggs in does having no fertilized eggs) were fertilized[4].

Adams injected 20 iu of Pregnyl (Organon Laboratories) intravenously and inseminated 20×10^6 fresh spermatozoa in 0.5 ml from 3 hours before to 6 hours after injection of the hormone[1]. 83.6% of the does kindled with an average litter size of 7.3, which is normal[1].

In order to induce superovulation, Foote, et al, injected 0.2 mg FSH (Armour) subcutaneously twice daily for 5 days prior to injecting PLH the morning of the 6th day[82]. Napier gave 2 mg horse anterior pituitary extract for 5 days subcutaneously and, on the 6th morning, he inseminated spermatozoa and injected 25 iu of chorionic gonadotropin[170]. The results at 30 hours showed the mean number of eggs shed and fertilized to be 32.6 and 21.8 per doe, respectively. 15 of 72 does gave no fertilized eggs. He obtained 75% and 88% conception rates with homospermic and heterospermic inseminations, respectively[170].

Mauer, Hunt and Foote studied the development of refractoriness to induction of ovulation by exogenous hormones in Dutch-Belted rabbits using brief administration of FSH-LH or PMSG-HCG at three 16-week intervals followed by one 8-week interval[151]. LH injections were used as a control. The number of ovulation points per doe decreased with both regimens but

the refractoriness was most pronounced in the PMSG-HCG group[151].

c. _Obtaining Ova_. Most workers usually remove the oviducts and uteri, and flush with Krebs-Ringer bicarbonate solution 2-3 hours after ovulation[15]. Chang has a good discussion of this procedure[53]. Moricard discusses removal of ova but does not say that the animal is alive[163]. Avis and Sawin used a surgical technique in order to use the does over again. 81% of ova they transplanted gave normal offspring[16]. This is a feasible technique for getting blastocysts.

In larger animals, such as the cow, a multiple lumina tube can be used to flush the eggs from the uterus.

d. _Culturing Ova IN VITRO_. Gwatkin and Biggers used mouse Fallopian tubes to culture mouse eggs through the morula stage (3rd day) and blastocyst stage (4th day)[97]. Biggers, Gwatkin and Brinster showed that development of blastocysts IN VITRO occurs in the normal time span.[39]

Chang used diluted blood serum to study the maturation of rabbit ova IN VITRO[53]. Biggers, Gwatkin and Brinster found that mouse embryos developed for 4 days IN VITRO in a chemically defined medium containing Fallopian tubes, but did not develop in the chemically defined medium alone[39]. The presence of the ovary had no influence on the development of the embryos[39]. Daniel and Olson found that the first cleavage of the fertilized rabbit ovum will occur in an amino acid-free medium, but that the second and subsequent cleavages require the addition of certain amino acids[59]. Ahlgren transferred fertilized tubal rabbit eggs to millipore filter chambers and compared the effect of emplacement for 48 hours in the abdominal cavity or in the uterus[5]. Development was significantly retarded in the abdominal cavity. He suggests that a tubal factor is necessary for the eggs to develop[5].

e. Storage of Ova. Several different media are discussed by Hafez in conjunction with work primarily related to storage of 2-16 blastomere stage ova at 10°C[99,101,102]. Some observations were that: (1) Serum was indispensible for survival of ova and whether the serum was autologous or heterologous did not change the implantation or survival rates[101,102].

(2) CO_2 did not improve the survival rate of ova[102]. (3) The survival of ova was not affected when stored in media having antibiotics in the following concentrations or lower: streptomycin, 7.5 mg/ml; chloromycetin, 4 mg/ml; paramomycin, 6.5 mg/ml or penicillin G, 23.9 mg/ml. However, at higher concentrations, the survival was partially or completely inhibited[99].

With a 1:1 serum-saline basic solution, Hafez found that 1% and 7% gelatine were better than 5% gelatine[102] and that 2% glycerol proved more helpful than 7% glycerol[101]. The gelatine medium was better for storage than plain liquid and he recommends addition of 1% gelatine even though embryo survival is about the same for gelled or liquid media[102]. The implantation rate is higher for gelled than for liquid media[102].

The importance of temperature control in the survival of ova is illustrated by the following: (1) Unfertilized ova are more resistant to freezing and thawing than are fertilized ova[53]. (2) Slow cooling of ova in a yolk-buffered medium is the best method of preventing temperature shock[101]. (3) Slow warming of stored ova is harmful as shown by an 8% implantation rate for ova warmed slowly after storage for 144 hours at 10°C. In contrast, a rate of 31% was achieved with fast warming (insertion directly into recipient female)[102].

Hafez discusses changes observed in fertilized ova during storage in references 101 and 102. Some of the observed abnormalities are believed

secondary to faulty fertilization or aging of gametes[102]. The mucin coat on fertilized rabbit eggs may enhance their survival IN VITRO. Most other mammalian ova of interest do not have this coat[102]. The implantation rate of abnormal eggs determined at 8 days is similar to that of normal ova; however, the survival rate at 15 days is lower[101]. Cleavage is arrested in rabbit ova stored at 25°C but at 37-39°C one sees 2-16 cell ova develop to the blastocyst stage[101]. Other observations on abnormal development of ova are found in reference 99.

It appears that the ability of stored ova to implant IN VIVO cannot be measured accurately by their ability to cleave IN VITRO. This may be due to the requirement for correlation between endometrial growth and ovum development at the time of transplantation[102].

f. Transplanting Ova. Chang showed that there is little or no chance for a transplanted ovum or blastocyst to develop into young when transferred to the genital tract of the recipient female 1 or 2 days before or after the corresponding corpus luteal stage[50]. Staples has shown that the viability of 5-day rabbit blastocysts was maintained during culture IN VITRO for at least 16 hours, as judged by development of viable fetuses after subsequent transfer to recipient females[218]. There was no significant difference in the percentage of viable fetuses developing from 5-day blastocysts cultured for at least 8 hours at 37°C compared to the development of noncultured blastocysts[218].

Pavlok has reported some improvements in the fertilization IN VITRO, cultivation and transplantation of mouse ova[184]. In some cases, normal embryos were obtained after transplantation[184]. Hunter, Polge and Rowson achieved moderate success in transplanting 7 and 8-day-old pig blastocysts to unmated recipients[118].

3. Fertilization

a. **Fertilization IN VIVO.** In natural mating, ovulation is induced in the rabbit by the act of copulation and occurs from 9.5 to 13 hours thereafter. The time of ovulation after the injection of luteinizing hormone is approximately the same. Fertile mating requires coordination of the capacitation of the spermatozoa, the maintaining of fertilizability of the ovum and spermatozoa, and the transport of an adequate number of spermatozoa to the site of fertilization[15]. Spermatozoa capacitation, transport of spermatozoa in the female rabbit and penetration of ova by spermatozoa are discussed below. Reference 15 contains an excellent discussion of the mammalian egg and the details of oogenesis.

Roche, Dziuk and Lodge evaluated the effectiveness of fertilization of rabbit eggs by fresh and aged spermatozoa[195]. Spermatozoa aged by storage for 24 hours at 5°C in a diluent composed of 0.5% fructose and 2.9% sodium citrate in distilled water fertilized a significantly smaller proportion of eggs than fresh spermatozoa. However, the decreased effectiveness of aged spermatozoa, in competition with fresh spermatozoa, does not appear to be due to a lack of capacitation, transport or fertilizing capacity, or to embryonic losses[195].

Dziuk, by double mating does to 2 different bucks which were distinguishable from each other, showed that when the interval between serving the does to the bucks was 2 hours, the first buck sired 86% of the offspring[72]. When the interval between males was shortened, the first male sired the majority of the offspring, but the proportion was less than after a 2-hour interval. Thus, it appears that spermatozoa which have resided in the genital tract of the doe for up to 10 hours have a distinct competitive advantage over newly inseminated spermatozoa[72].

1) Capacitation of Spermatozoa. Spermatozoa must be in the genital tract of the female rabbit for approximately 6 hours before they are capable of fertilizing eggs[4]. Estimates range from 3-6 hours for the time it takes rabbit sperm to reach the site of fertilization[4,247]. This slowness of transport compared to other species may be related to capacitation[4].

Capacitation in the uterus is more efficient than in the Fallopian tube. This was shown by a higher fertilization rate from deposition of semen into the uterus 10 hours before ovulation than from deposition in the Fallopian tube at the same time[3]. Noyes, Walton and Adams have shown that spermatozoa can be capacitated in the spayed female, in the intact male and in organs unrelated to the genital system[177]. A 41% fertilization rate was achieved with spermatozoa capacitated in an excised rabbit uterus maintained in a saline medium. Small numbers of sperm (2,000-10,000) were inseminated surgically and the evaluation of capacitation was judged by fertilization of ova IN VIVO[177].

Chang showed that seminal plasma is beneficial to both motility and fertility as a medium for artificial insemination[49]. However, after capacitation of spermatozoa in the female genital tract, seminal plasma is detrimental both to motility and fertilizing capacity[49]. Dukelow, Chernoff and Williams showed that seminal plasma from the bull, boar, stallion, rabbit and monkey all contained a decapacitation factor, but human, rooster and dog seminal plasma did not contain this factor[70]. The authors suggested that the presence, or absence, of decapacitation factor in the seminal plasma is indicative of the need for or lack of capacitation in that species. They also discussed the properties of the decapacitation factor[70].

Hamner and Williams showed that rabbit spermatozoa respire at a greater rate in oviduct fluid than in calcium-free Krebs-Ringer phosphate solution[108].

They suggested that the stimulating factor was bicarbonate and that it stimulated maximally at a concentration of 2.36 x 10^{-3} M. It appears to stimulate respiration by increasing the concentration of TCA cycle intermediates[108]. O_2 uptake by sperm increased 4 times after incubation in a rabbit uterus and gave a fertilization rate of 18% compared to 0% for freshly ejaculated sperm[107]. Murdoch and White showed that the metabolism of labelled glucose by rabbit spermatozoa after incubation in the uterus was significantly higher than the rate of metabolism by fresh spermatozoa[167]. The time required for the development of those metabolic changes was similar to that required for the capacitation of rabbit spermatozoa[167].

Howarth, Alliston and Ulberg found no significant difference in fertilization capacity of spermatozoa recovered from females maintained at 21° and 32°C during 6-8 hour capacitation period in the uterus[116]. There was a significant reduction, however, in pre-implantation embryo survival as a result of uterine environment on sperm[116].

Dukelow and Williams showed that spermatozoa incubated for 12 hours in the uterus and inseminated into the rabbit oviduct 4 hours before ovulation gave a fertilization rate of 4.5% compared to a rate of 77% when the spermatozoa had been incubated in the uterus for 16 hours and inseminated at the time of ovulation[71]. This observation and others, using different time factors, suggest that the oviduct has a detrimental effect upon capacitated spermatozoa.

Bedford and Shalkovsky evaluated the species-specificity of rabbit sperm capacitation using the cat, ferret, guinea pig and rat[31]. Spermatozoa did not survive for more than 12 hours in the cat, ferret and guinea pig, and spermatozoa removed from these species prior to that time did not fertilize any rabbit eggs when deposited in the oviducts soon after ovulation. Rabbit spermatozoa survived up to 48 hours in the uterus of estrous

rats, and spermatozoa which had been in the uterus of the rat for more than 14 hours fertilized a significant proportion of tubal rabbit ova. Although some phases of capacitation appear to occur in the uterus of the rat, it appears that functional capacitation involves another phase which is species specific and possibly may be restricted to the female tract of the rabbit[31].

It appears that capacitation has a two-fold effect: (1) alteration of the acrosome, releasing hyaluronidase and thus enabling the spermatozoon to pass through the cumulus and (2) the removal of the acrosome, thus exposing the perforatorium, which is apparently the organelle directly concerned in sperm penetration of the zona pellucida[14,15]. The rate of capacitation may vary under different environmental conditions, indicating the probability of a chemical or physicochemical process which need not be acrosome removal[3,107]. The correlation between the acrosome removal and capacitation is questioned because the decapacitation by seminal plasma does not replace the acrosome[3].

2) <u>Spermatozoa Transport in the Female</u>. Spermatozoa seldom reach the oviduct of the rabbit in less than 1 hour after mating and 5-6 hours may be required before sufficient spermatozoa enter the oviducts of all rabbits to fertilize all of the eggs shed[247]. The mechanism of transport of spermatozoa in the female is attributed to muscular contractions of the vagina and uterus and/or to the intrinsic motility of the spermatozoa.

Krehbiel and Carstens reported the transfer of methylene blue, janus green B and iodochlorol through the reproductive systems of estrous rabbits after artificial stimulation of the vulva to simulate the copulatory reflex (erection of vulvar tissue)[128]. The dyes reached the tubal end of the uterine horns 2-5 minutes after the initiation of stimulation of the vulva which resulted in combined muscular contractions of the vagina and uterus.

It was necessary to place fluids beyond the vaginal sphincter. In the absence of a response to vulvar stimulation, no oil passed into the uterine horns[128].

Noyes, Adams and Walton placed radiopaque oil in the vaginas of rabbits and, even though they observed vaginal contraction upon vulvar stimulation, they did not observe filling of the uterine horns with oil at any time[178]. They even experienced difficulty filling the uterine horns by means of a catheter placed within the uterine lumina. After vaginal insemination of a mixture of 90% dead and 10% motile spermatozoa, an increasing number of spermatozoa in the uterine horns over a period of 4 hours was noted when insemination preceded mating with a vasectomized buck. The percentage of motile spermatozoa decreased from 90-100% in the first hour to 75-85% at the end of 4 hours. When mating preceded artificial insemination, the percentage of motile spermatozoa in the uterine horns ranged from 67-91%. They concluded that living spermatozoa passed through the cervical barrier as a result of intrinsic motility and that the point at which the rate of progress is first influenced by muscular contractions of the female genital tract remains to be identified[178].

Moghissi and coworkers studied the relationship between the motility of spermatozoa and migration through the mucus of the human cervix[161]. In summary, their observations were as follows: (1) Spermatozoa penetrate clear, clean, watery, mid-cycle mucus readily and rapidly. (2) With increasing viscosity of the cervical mucus, the degree of sperm penetration was found to decrease. (3) Acid mucus immobilizes spermatozoa and alkaline mucus enhances migration. (4) Incompatible proteins in mucus may cause death or agglutination of sperm. (5) Proteolysis of cervical mucus by seminal plasma enzymes may play a role in sperm migration. They suggest that an IN VITRO test of the compatibility of mucus with sperm and seminal

plasma may provide clues in infertility problems[161].

3) <u>Penetration of Ova by Spermatozoa</u>. Observations on sperm penetration
are provided by Szollosi and Ris for the rat[224], by Yanagimachi for the
hamster[245], and by Hunter and Dzuik for the pig[119]. Sperm penetration,
the immediate reactions of the mammalian egg upon penetration and the
cytology of fertilization are dicussed in reference 14.

Austin reports that the corona radiata cells need not be off to get
sperm penetration[14]. Rabbit eggs IN VITRO generally cannot be denuded by
treatment with sperm suspension or hyaluronidase solution alone, suggest-
ing that a uterine secretion complements the hyaluronidase released by
the sperm[14]. The importance of the corona radiata in egg fertilizability
is shown by the fertilization of about 85% of ova with an intact corona
radiata but only about 50% without the corona radiata from 2-4 hours after
ovulation[55]. At 6 hours after ovulation, the percentages were 60% and
12%, respectively[55].

Dickmann describes 2 distinct concentric layers in the zona pellucida
of the rabbit ovum[66]. The "outer zona" appears granular and the "inner
zona" appears fairly homogeneous. The width of the "outer zona" varies
relative to the thickness of the "inner zona". Sometimes one can distin-
guish 2 or more concentric layers in the "inner zona". Radial striations
are also seen in this layer on occasion[66]. It has been postulated that
the zona pellucida of the mammalian egg secretes a substance ("fertilizin")
which has the primary purpose of enhancing attachment of the spermatozoon
to the zona pellucida, thereby aiding penetration[14]. This "fertilizin"
also causes agglutination of sperm IN VITRO but not IN VIVO where it may
be neutralized by a secretion of the female genital tract ("antagglutin")[14].
For IN VITRO fertilization, it is necessary to wash freshly ovulated eggs

before introducing the sperm[14]. Soupart has shown that the acquisition

of the ability to penetrate the zona pellucida is associated with a

shortening of the life span and fertilizing ability of rabbit spermatozoa[217].

It is estimated that the fertilizing spermatozoon takes 1/2 hour to

cross the peri-vitelline space[14]. With fertilization IN VIVO, the rabbit

egg usually shows several spermatozoa in the peri-vitelline space[14]. How-

ever, Adams placed as many as 17.6 x 10^6 sperm at the site of fertilization

and got normal fertilization, thereby leading to the belief that a normal

egg can prevent polyspermy[3]. The percentage of eggs fertilized is not

proportional to the sperm concentration[14].

4) <u>Effects of Female Hormones</u>. McGaughey and Daniel have observed that

estrogens cause tubal locking IN VIVO and also cause fragmentation of

fertilized ova IN VITRO[153]. One-stage cells are most susceptible to

fragmentation by this treatment. Subsequent stages of development are

less susceptible as the number of cells increases[153]. Psychoyos has shown

that estrogen is necessary for the dissolution of the zona pellucida in the

rat[190]. His results suggest also that delayed implantation is not related

to the persistence of the zona pellucida[190].

b. <u>Fertilization IN VITRO</u>. The main difficulty in effecting fertilization

IN VITRO is the initiation (penetration) which appears to involve the capaci-

tation of the spermatozoa and the removal or neutralization of egg fertili-

zin[14]. Difficulties in confirming fertilization IN VITRO arise from tech-

nical errors, misinterpretation of artifacts and accidental induction of

parthenogenesis.

1) <u>Parthenogenesis</u>. Reportedly, during culture IN VITRO, rabbit eggs

can be activated by heat shock and by treatment with hypo- and hypertonic

solutions and solutions of butyric acid[14]. Upon sperm penetration, one sees

the contraction of the vitellus, the expulsion of the second polar

body, the formation of 2 pronuclei and the initiation of cleavage. With

a few exceptions, the expulsion of the second polar body occurs only with

activation by sperm entry. When activation occurs by other means, the

second polar body is not expelled and the cells resulting from division

are diploid[14]. Incubation of ova in the presence of spermatozoa increases

the chance of activation without sperm penetration, whereas incubation of

unfertilized ova with Fallopian tube mucosa reduces the incidence of

parthenogenesis[4]. Austin suggests that incomplete vasectomy may account

for some reports of parthenogenesis[4].

2) <u>Example of Procedure used for IN VITRO Fertilization of Rabbit Ova</u>.

A procedure used with some success is as follows:[31]

(a) Eggs were obtained from the Fallopian tube and placed in a

capacitated sperm suspension in a flask and incubated for 3-4

hours at 38°C on a rocking device.

(b) Eggs were removed after 3-4 hours and transferred to an 8 ml

Carrel flask containing fresh homologous serum which earlier

had been heated to 55°C for 20 minutes.

(c) Following an 18-hour incubation period at 38°C in the Carrel

flasks, the eggs were removed and examined.

(d) Of 266 eggs recovered, 55 appeared to have cleaved to the 4-cell

stage. 36 of the 55 fertilized eggs were transferred to 6 females

immediately after examination. Four females gave 15 living young;

2 females did not become pregnant.

(e) Capacitation of sperm was the most important factor; O_2 partial

pressure, the suspending medium and the redox potential appeared

to be less important[31].

In the last few years, investigators have been capacitating spermatozoa

in the female rabbit for fertilization IN VITRO[30,221]. One method for
recovering spermatozoa is to flush the uterine horns with Krebs-Ringer
bicarbonate solution giving a recovery rate of about 10%[15]. Using sperm-
atozoa capacitated in this manner, Bedford and Chang achieved an IN VITRO
fertilization rate of 54% compared to a normal rate of 94%[30].

4. Artificial Insemination

The technique of artificial insemination in the rabbit is discussed
by Adams[1,2]. The advantages and disadvantages of using a vasectomized
buck and hormones to induce ovulation were discussed in Section 2.b. above.

a. Time of Insemination. Ovulation occurs 9.5-13 hours after LH injection
and 10-12 hours after mating with a vasectomized buck. The fertilizability
of the ovum is greatest the first 4 hours after ovulation. It takes 6
hours in the female genital tract for the sperm to be capacitated and sperm
penetration stops about 6-9 hours after ovulation when the ovum becomes
coated with a mucin layer[65]. Fox inseminated 15-30 minutes after using a
vasectomized buck to stimulate ovulation[84]. Using LH and natural mating
as a function of time after injection, Adams observed that 90-100% of the
does yielded fertilized eggs when mated 8-12 hours after LH injection and
that no fertilization of eggs occurred when mating took place 14 hours after
LH injection[4]. Thus, in the rabbit, successful inseminations can be made
from several hours before inducing ovulation to 8-12 hours thereafter.

b. Place and Number of Spermatozoa. Most workers deposit spermatozoa
at the anterior end of the vagina. Because the rabbit has a primitive
reproductive tract (a separate cervix to each horn of the uterus) and it
is easy to inject sperm into the abdominal cavity rather than the uterine
horn when trying to inseminate directly into the latter, it appears best
to use the conventional procedure[105]. With regard to sperm concentrations

required in artificial insemination, Foote, et al, got good results by depositing 3×10^6 motile sperm (in 0.1 or 0.2 ml, either undiluted or diluted with a few drops of 0.9% saline) in the anterior part of the vagina[82]. Napier inseminated 10×10^6 sperm in 0.5 ml of 0.85% saline solution[170]. Adams obtained good results with 0.5 ml inseminate containing 20×10^6 sperm[1]. Both Napier and Adams placed the sperm in the anterior part of the vagina.

Adams and Chang compared the effects of depositing spermatozoa either in the uterus or in the Fallopian tube[3]. They assessed fertilization by examining ova recovered from excised Fallopian tubes. They inseminated at 3 different times after inducing ovulation with 30 mg of LH (Follutein, Squib). The results were as follows:

Effect of Site of Deposition of Spermatozoa[3]

Time after LH Injection (hours)	Sperm Inseminated x 10^6	Ova Fertilized Uterine Insemination (%)	Fallopian Tube Insemination (%)
3/4	0.12-.8	88.5	27.3
6	0.8	100	16.7
8	0.33-0.65	71	10.0

The same low percentage fertilization with insemination into the Fallopian tube was observed with higher sperm concentrations, thereby demonstrating that capacitation takes longer in the tubes[3].

5. Sexing Offspring

Methods of determining the sex of offspring in mammals include examination of the external genitalia, gonads, chromosomes or sex chromatin at appropriate stages of development. Bhattacharya describes a method of

determining the sex of rabbits from the external genitalia at birth without sacrificing them[35]. In order to get feedback on the success of experiments with rabbits, it is desirable to determine the sex of offspring prior to the end of the 31-day gestation period. Thus, determining the sex by studying either the sex chromatin or chromosomes as soon as possible after conception with accuracy and without loss of ova or offspring is a method of reducing this waiting period.

a. <u>Gonads.</u> Bhattacharya found the gonads of fetal rabbits after 18 days could be sexed by staining techniques[35]. Gonads can be fixed, sectioned and stained, either by a periodic acid-Schiff-hematoxylin technique[222], or by Delafield's hematoxylin and counterstained with eosin[92].

Lush reported that the sex of rabbits at birth could be determined by visual examination of the gonads[143]. Neonatal testes could be distinguished by a very conspicuous epididymis wrapped around one end of the testis and the testis was usually attached to a very thick gubernaculum. The neonatal ovary was generally attached to a less conspicuous ligament and had no structure resembling the epididymis. The location of the gonad within the abdominal cavity did not correlate very well with the sex of the offspring[143].

Comparison of the 2 methods by me showed 100% agreement in the sex of 51 offspring determined by evaluation of gross morphology and by microscopic examination of stained sections of the gonads.

b. <u>Chromosomes.</u> Vickers describes a method for the direct measurement of the sex ratio in mouse blastocysts by looking at chromosomes of the cells of the blastocysts which have been arrested in the metaphase stage of mitosis with colchicine[234].

Two methods which might be of assistance in gaining access to the cells of rabbit blastocysts have been reported. Edwards describes a method

for removing the zona pellucida of 1- and 2-celled rabbit eggs IN VITRO[74].

Mastroianni and Ehteshamzadeh discuss the role of fluid from rabbit oviducts in loosening the cells of the corona radiata of recently ovulated ova, pretreated with hyaluronidase[148].

c. Sex Chromatin. Vickers has found sex chromatin in 67-90% of ectodermal cell nuclei in the female mouse amnion and in 2-15% of such cells in the male[235]. She considers that criterion a reliable indicator of the sex of the fetus from the eleventh day of gestation onward[235].

Gardner and Edwards have been able to sex intact, living rabbit blastocysts by identifying sex chromatin in trophoblast cells approximately 5.75 days after mating[89].

The methods described by Vickers and by Gardner and Edwards would make available information on the sex ratio of offspring 3 weeks earlier than waiting for the full gestation period to elapse.

APPENDIX C

A New Artificial Vagina for Collecting
Rabbit Spermatozoa

The versatility of Teflon has been extended from facilitating the frying of eggs in the kitchen to simplifying the collection of spermatozoa with a Teflon artificial vagina. After the rabbit was selected as the animal to be used in this study of the biophysical properties of spermatozoa, the designs of artificial vaginas used by Gordon[92] and by Walton[239] were reviewed. With the assistance of Mr. John Begovitch, the artificial vagina shown in Figure 61 was constructed. A recent communication by Bredderman, Foote and Yassen[46] presents a number of considerations in the design and use of an artificial vagina for collecting rabbit semen.

The salient features of this artificial vagina are the Teflon shell connected to a removable base by a watertight junction. The base has inlet and outlet water conduits and an O-ring lined hole in its center, which holds a one dram shell vial (15 x 45 mm, No. 60930-L, Owens, Illinois, U.S.A.). The closed end of a tissue finger cot (Latex, 610 Large, Bitner Brand, U.S.A.) is incised and secured around the mouth of the one dram vial with masking tape. The open end of the finger cot is secured to the opening of the artificial vagina with a rubber band as shown in the photograph. The space around the rubber lumen and upper part of the shell vial is filled with water at a temperature of 40-42°C. Filling via one of the conduits connected by rubber tubing to a faucet facilitates control of both temperature and pressure in the shell. If desired, the rubber lumen and vial can be washed and autoclaved prior to insertion into the artificial vagina. The rubber lumen and vial are rinsed with sterile saline after assembly. Glycerol, KY Jelly, or another acceptable lubricant is spread over the mouth and into the artificial vagina with a small thermometer
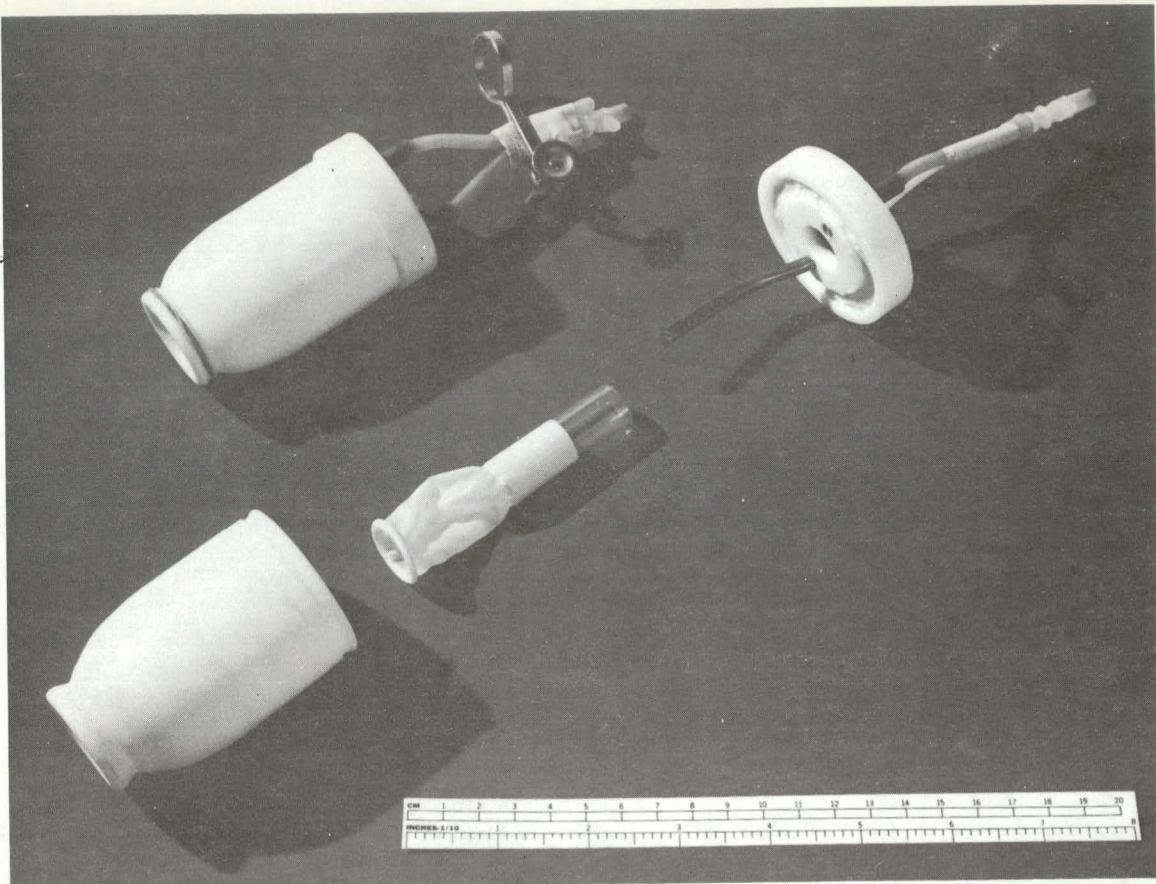
JHL-4935

Fig. 61. Photograph of two Teflon artificial vaginas showing one

assembled apparatus and the component parts of another.

which is also used to measure the temperature in the lumen.

Because Teflon is a good insulator, the temperature change in the artificial vagina at room temperature is 0.5°C/min for the first 8 minutes. This rate of cooling is compatible with collecting spermatozoa some distance from the source of warm water if necessary.

Among the advantages of this artificial vagina are the following: (1) Ejaculation is close to the mouth of the shell vial reducing the loss of spermatozoa by adhesion to the lumen. In addition, the lumen can be washed with a small volume of diluent to enhance the recovery of spermatozoa. (2) Because the vial is kept at approximately the same temperature as the water in the chamber, the spermatozoa are not subjected to cold shock during collection. (3) Between collections, the Teflon shell can be washed with alcohol or hot water to minimize the chance of passing infection from one rabbit to another. (4) The shell vials and tissue finger cots are inexpensive and can be discarded after using once. (5) Many lumens (vial + finger cot) can be prepared in advance, thereby facilitating the collection of large numbers of ejaculates consecutively. Using this artificial vagina, one individual can collect ejaculates from rabbits at a rate of one every 5 minutes or less without assistance.

APPENDIX D

Evaluation of Media

Contents

1. Introduction

The ability to preserve the fertilizing capacity of spermatozoa for
long periods of time has been of great benefit in the field of mammalian
reproduction and genetics. In poultry husbandry, it is a much desired,
but at present relatively unavailable, tool. Consequently, the voluminous
literature on the subject attests to the tremendous effort that has been
devoted to the evaluation of media for the long-term storage of both
mammalian and fowl spermatozoa.

In regard to mammalian spermatozoa, both natural and artificial media have been evaluated. Napier showed that adding seminal fluid to the suspending medium enhanced the preservation of rabbit spermatozoa. He obtained better fertilization results with a medium consisting of 50% saline and 50% plasma than with saline alone[169]. Even though blood serum usually is an excellent medium for the IN VITRO culture of many animal cells, including rabbit ova, Chang found a spermocidal factor in fresh human, bovine, rabbit, guinea pig and rat sera[52]. In addition, Bedford observed a great tendency for agglutination of rabbit spermatozoa in media containing serum[27].

Gulyas reported excellent maintenance of motility of rabbit spermatozoa at room temperature for up to 6 days using Norman-Johnson Solution No. 2, a completely artificial medium. During this period, the percentage of live spermatozoa decreased from 89% to 69%, the percentage of motility from 91% to 72% and the quality of motility from 4.9 to 1.8 on a scale of 5[95].

Foote and coworkers found that sperm survival in all buffers was improved by the addition of egg yolk[83]. Bhattacharya describes an egg yolk-glycine-citrate medium that maintained the fertilizing capacity of rabbit spermatozoa at temperatures near 0°C for over 24 hours. He put the spermatozoa in a glucose vitalizing medium at room temperature for approximately 2 hours prior to insemination[35]. Other reports on the use of additives include those by Davis, Bratton and Foote concerning the successful use of tris-buffered and citrate-buffered yolk-glycerol media for the preservation of bovine spermatozoa[60,61], by Sawada and Chang on the use of dimethyl sulfoxide in the storage of rabbit spermatozoa at temperatures below 0°C[203], and by Roussel and Austin on the preservation of primate spermatozoa during storage in an egg yolk-sodium glutamate-

glycerol medium at or near -79°C[199].

Another common class of additives is antibiotics even though Sharma found only a slight benefit with them[211]. Adams reports that adding 1,000 units/ml of penicillin to a diluent consisting of physiologic saline or Ringer's solution is not detrimental with sperm dilutions up to $1:10^1$. However, a high concentration of antibiotics is harmful to the survival of spermatozoa[233]. Bhannasiri, Bogart and Krueger studied the effects of antibiotics on semen production in rabbits and concluded that any effects noted during the treatment of the disease were probably due to the disease and not to the antibiotics. They evaluated oxytetracycline (Terramycin), Chlortetracycline, Aureomycin and streptomycin[34].

In regard to the storage of high concentrations of fowl spermatozoa, Rowell and Cooper observed reductions in fertilizing capacity from 61.3% to 32.1% and from 31.1% to 12.5% with the insemination of equal volumes of undiluted and 1:3 dilutions of fowl spermatozoa in seminal plasma and in a solution of glycine and distilled water, respectively[200]. Van Wambeke observed almost no loss of fertilizing capacity of fowl spermatozoa diluted with equal volumes of either of 2 diluents containing skimmed milk and stored at 2-5°C for 24 hours. Fertilization results were based upon eggs collected from days 2-8 after insemination[231]. Polge diluted cock semen 1:3 with either of 2 diluents containing egg white or milk and observed average fertility rates of 89% and 53% after storage at 5°C for 1 and 24 hours, respectively, based upon eggs laid from days 2-7 following insemination[140].

Lorenz, in his review, mentions work by others at room temperature showing that (1) 1:3 dilution with seminal plasma gave a slight decrease in fertility but no additional loss occurred with dilution of up to 1:63

provided that the same number of spermatozoa was inseminated; (2) dilution of up to 1:50 in an artificial medium resulted in only a slight loss of fertility with insemination of at least 10^8 spermatozoa; poor results were obtained with greater dilutions or with insemination of fewer spermatozoa and (3) 1:1,250 dilution in saline or in Tyrode's or Baker's solutions produced immediate irreversible immobilization[140].

Recent studies have analyzed the fluids secreted in the female genital tract in fowl and epididymal fluid in mammals[238], organ systems in which the motility and fertilizing capacity of spermatozoa are preserved for long periods of time naturally.

The purpose of this appendix is to consolidate all of my observations on the evaluation of media where the information is of a supplemental nature. In addition, this appendix is a directory to the presentation of additional information on, or the evaluation of, media when it is an integral part of the experiments presented in the main body. Requirements peculiar to special experimental systems are discussed in the appropriate parts of the text.

2. Results and Discussion

a. "High" Ionic Strength Media.

(1) Egg Yolk Medium (EYM). This medium is described in Chapter VI A in conjunction with an evaluation of the preservation of the fertilizing capacity of fowl spermatozoa by "high" ionic strength media.

In regard to the preservation of rabbit spermatozoa in EYM, there was essentially no loss of motility after storage for 48 hours at 2°C in concentrations ranging from 1.5×10^6-88×10^6/ml in one study. The results of another study using an egg yolk and glycine-citrate medium in 25%-75% proportions, respectively, are shown in Table XXXIX. It is seen (Column 6) that there was no change in the quality of the spermatozoa after storage

Table XXXIX.   Preservation of Motility of Rabbit Spermatozoa in Modified Baker's Medium (MBM), Egg Yolk Medium (EYM)[a] and Varying Proportions of the Two Media at 2°C.

(1)	(2)	(3)	(4)	(5)	(6)
Storage Time (hours)	100% MBM	90% MBM - 10% EYM	75% MBM - 25% EYM	50% MBM - 50% EYM	100% EYM
2	15% FM, 70% TW 15% immotile	> 75% FM	> 75% FM	> 75% FM	> 75% FM
17	0% FM, 2-3% TW	14% FM, 60% TW 26% immotile	25% FM, 35% TW 40% immotile	40% FM, 30% TW Bad Clumping	> 75% FM
40	0% FM, 0% TW	2-3% TW	2-3% FM, 5-6% TW	2-3% FM, 5-6% TW	∿50% FM of high degree

a. 25% Egg Yolk - 75% Glycine-Citrate Medium by Volume.

for 17 hours and only a slight change after 40 hours. The difference between these results and those of the previous study suggests that 40% egg yolk is better than 25% egg yolk in the egg yolk-glycine-citrate medium.

Despite this excellent preservation of motility, the EYM has many undesirable features which militate against its use in the Staflo apparatus. They are: (1) It is an opaque medium and observations are impossible. (2) It coats the entire apparatus with an opaque film and makes cleaning difficult. (3) It precipitates with almost every medium mentioned in this report except the glycine-citrate buffer. (4) The method of making an optically clear egg yolk medium described by Rikmenspoel[194] involves a tremendous amount of work and is not justified for routine Staflo experiments. (5) The use of EYM for the initial cooling requires additional centrifugation (washing) to get the spermatozoa out of EYM before starting the experiment.

The EYM has a very important use after Staflo or centrifugation experiments. If the spermatozoa are reconcentrated and suspended in EYM, they can be left for many hours before insemination, thereby allowing greater flexibility in the conduct of the experiment and artificial insemination thereafter.

(2) Modified Baker's Medium (MBM). This modification of Baker's medium consists of 22.0 g $Na_2HPO_4 \cdot 12H_2O$, 2.0 g NaCl, 2.0 g $NaHCO_3$, and 0.155 g KH_2PO_4 diluted to 1,000 ml with distilled water. pH adjustments are made with concentrated HCl.

Table XXXIX compares the effectiveness of modified Baker's medium, 25% egg yolk-glycine-citrate medium and mixtures of these 2 media in maintaining the motility of spermatozoa over extended time periods at 2°C and, at a concentration of 6 x 10^6/ml. After 2 hours at 2°C in MBM, an aliquot was warmed to room temperature and evaluated. The marked decrease in motility

was much greater than observed in the "low" ionic strength glycine-citrate and glycine-phosphate buffers as reported in Chapter VI. As shown in Table XXXIX, the addition of a small amount of EYM to the MBM greatly enhanced the preservation of motility. No difference was observed among the media containing egg yolk at 2 hours; however, at 17 hours, the spermatozoa stored in the 100% egg yolk-glycine-citrate medium had a much higher percentage and degree of motility than those in any of the mixtures of MBM and EYM.

(3) Norman-Johnson Solutions (NJ-1, NJ-2). Evaluation of Norman-Johnson Solutions 1 and 2 (NJ-1,2)[95] showed that they were not so good for preserving motility at room temperature as the egg yolk-glycine-citrate medium. Both NJ-1 and NJ-2 solutions were inferior to the egg yolk-glycine-citrate and to the "low" ionic strength glycine-phosphate buffer for preservation of spermatozoan motility at temperatures of 2-5°C.

(4) Vitalizing Medium. This medium is described and evaluated in Chapter VI in regard to use with fowl spermatozoa.

b. "Low" Ionic Strength Media.

(1) Glycine-Ring Z^d Solution. The glycine-Ring Z^d solution used by Schröder[206] and Gordon[92] was evaluated for use in Staflo-electrophoresis experiments. Equivalent portions of the same ejaculates were diluted simultaneously in normal saline and in glycine-Ring Z^d solution at room temperature. After 30 minutes, spermatozoa in the saline solution had an average of 45% motility and in the glycine-Ring Z^d solution, only 1.5% motility. Using a nigrosin-eosin vital staining technique, the percentage of viable spermatozoa agreed with the observed motility in the saline solution. The percentage of viable spermatozoa in the glycine-Ring Z^d solution was approximately 1/3 that in the saline solution, but was still higher than the percentage of motility noted above. It should be noted

that because the spermatozoa sample had a fairly low percentage of motility initially, it could be expected to deteriorate more rapidly than a good specimen.

(2) <u>5% Dextrose in Distilled Water</u>. 5% dextrose and glycine-Ring Z^d solutions were compared at room temperature in regard to the preservation of motility using a normal saline control. Whereas there was essentially no change after 50 minutes in the control sample from the initial values of 50% forward motion and 65% viability (nigrosin-eosin stain), all spermatozoa were immotile in less than 15 minutes in the glycine-Ring Z^d solution. After 20 minutes in 5% dextrose, there was only 30% forward motion. After 22 minutes, the percentages of viable (unstained) spermatozoa in the glycine-Ring Z^d and 5% dextrose solutions were 21% and 29%, respectively. Thus, neither of these 2 "low" ionic strength media preserved motility as well as normal saline at room temperature.

(3) <u>Glycine-Citrate Buffer (GCB)</u>. This "low" ionic strength buffer is evaluated in Chapter VI A.

(4) <u>Glycine-Phosphate Buffer (GPB)</u>. The properties of this "low" ionic strength buffer are evaluated in Chapter V and its ability to preserve the motility and fertilizing capacity of fowl and rabbit spermatozoa are discussed in Chapters VI and VIII, respectively.

The addition of 1 part of egg yolk medium to 9 parts by volume of the "low" ionic strength glycine-phosphate buffer (GPB) preserved a high degree of motility for 24 hours at 2°C at spermatozoa concentrations ranging from 10^5-10^6/ml. After storage at such low concentrations in GPB alone for several hours, the spermatozoa would have lost all motility.

(5) <u>Lower Ionic Strength Glycine-Phosphate Buffers</u>. Fowl spermatozoa of high initial motility were used to evaluate the effect of varying

the amount of modified Baker's medium (MBM) mixed with an isotonic glycine-dextrose buffer. The spermatozoa were diluted 1:10 in duplicate vials containing the following percentages of MBM: 80%, 10%, 6%, 3% and 0%. After storage for 6 and 1/2 hours at 2°C in these media, the spermatozoa were warmed to room temperatures for evaluation. The spermatozoa in the medium containing 80% MBM had very poor motility, while those in the solutions containing 10% and 6% MBM had both a high percentage and degree of motility, comparable to their initial values. The spermatozoa in the 3% and 0% MBM solutions may have had a slightly lower percentage of motile spermatozoa but the degree of motility was as great as in the 6% and 10% solutions. In conclusion, the preservation of the motility of fowl spermatozoa was excellent in solutions containing from 0-10% MBM after 6 1/2 hours at 2°C. In contrast, the preservation of motility in the 80% MBM solutions was poor.

c. Density Gradient Materials.

(1) Staflo Density Gradient Materials. Dextrose, fructose and sucrose were evaluated for use as Staflo density gradient constituents by comparing the preservation of motility in 1% solutions of each sugar in normal saline at concentrations of 8×10^6 and 80×10^6 spermatozoa/ml. After 4 hours at room temperature, there was no difference in motility among the spermatozoa at high concentration in all 3 solutions. At the lower concentration, the degree of motility was greater in the fructose solution than in either of the other solutions.

Dextran (MW 73,000) was also evaluated for use in Staflo density gradients. In one study, with an initial sample having greater than 50% forward motion and 65% viability by vital staining, there was no difference in the motility of spermatozoa stored in saline or saline + 6% dextran after 50 minutes. In Chapter IV, it was shown that a high percentage and

degree of motility is preserved in Staflo sedimentation experiments using a glycine-dextran medium. As shown in Chapter VI, 2% dextran did not affect the fertilizing capacity of fowl spermatozoa.

(2) Equilibrium Centrifugation Density Gradient Materials. Among the materials evaluated for use in equilibrium density gradient centrifugation of spermatozoa were dextran (Sigma Chemical Company), Ficoll, bovine serum albumin (BSA), and the radiographic contrast materials listed in Table XL. The high viscosity of large weight % solutions of dextran, Ficoll and bovine serum albumin is an important disadvantage in the use of those materials. Radiographic contrast materials can be used to prepare solutions of high specific gravity and low viscosity. Another advantage of the contrast media is that when used in a clear solution such as saline or phosphate buffer, they add no color, thereby enhancing observation and photography. The disadvantage of these compounds is the hyperosmolarity of high concentrations due to their small size compared to dextran, Ficoll and BSA. The knowledge that the contrast media could be used in humans without deleterious effects gave encouragement for their use with spermatozoa.

The initial evaluation of the effect of contrast media on spermatozoa consisted of exposure of rabbit spermatozoa to 4.5% Diodrast in egg yolk medium for approximately 4 hours, 2 hours of which were at 4°C. After this period, 5 female rabbits were inseminated with approximately 15×10^6 sperm each. These inseminations resulted in 4 litters with 22 offspring. There was no change in the motility or staining characteristics of the spermatozoa after 6 hours in the egg yolk-Diodrast medium.

A longer term study of the effect of Conray 400 was conducted by exposing spermatozoa to 7.5% and 3.5% Conray 400 in a 25% egg yolk-glycine-

Table XL.   Radiographic Contrast Solutions Evaluated for Use as Density Gradient Constituents in Equilibrium Centrifugation Studies of Spermatozoa.

Brand Name	Chemical Name	Percent Solution / Iodine by Weight (%)	Specific Gravity	Molecular Weight
Angio-Conray	Na 5-acetamido-2,4,6-triiodo-N-methyliso-phthalamic acid	80 / 59.87	1.508	614
Conray-400	"	66.8 / 59.07	1.422	614
Cholografin meglumine	bis N-meglumine salt of N,N' adipyl-bis (3-amino-2,4,6-trii-odobenzoic acid)	52 / 64.3		1552
Diodrast	3,5-diiodo 4-pyridone N-acetic acid diethanolamine	35 / 49.8		305
Hypaque	Na 3,5-diacetamido-2,4,6-triiodobenzoate	50 / 59.87		636
Renografin 60 or 76	Methylglucamine 3,5-diacetamido-2,4,6-triiodobenzoic acid	60 or 76 / 47		797
Cardiografin	"	85 / 47		797

citrate medium at 2°C. Table XLI summarizes these results. It is seen that 3.5% Conray 400 in the egg yolk medium had only a slight effect on the survival of spermatozoa over a period of 48 hours. The initial quality of the spermatozoa used in this evaluation was relatively poor. However, because poor spermatozoa generally tolerate stress less well than high quality spermatozoa, the observation that poor spermatozoa tolerated exposure to low concentrations of Conray 400 for extended periods of time was encouraging.

The effect of high concentrations of contrast media on fowl spermatozoa was evaluated as follows: 0.1 ml of pooled cock semen, which had been diluted and cooled in egg yolk medium to a temperature of 3°C, was placed in each of four 1 ml vials containing 30% Angio-Conray, 45% Cholografin, 30% Diodrast or 40% Renografin, all in normal saline. At 3°C, the spermatozoa had a moderate degree of motility in the egg yolk medium but lost this motility in less than 2 minutes in all of the media except Cholografin, in which the motility was lost within 5 minutes. After 10 minutes, at 3°C, the 1.1 ml suspension was placed in 5 ml of a saline-glucose medium and centrifuged to reconcentrate the spermatozoa. The supernatant was removed and the spermatozoa were resuspended in egg yolk medium at 3°C. All of the spermatozoa which had been exposed to the contrast media showed a percentage and degree of motility that was comparable to that in an egg yolk medium control sample. This suggested that there was no detrimental effect on the motility of fowl spermatozoa for exposures of up to 32 minutes in any of the contrast media. The fertilizing capacity of the spermatozoa was not evaluated in this study.

These studies showed that the motility of fowl spermatozoa is maintained during exposure to high concentrations of radiographic contrast materials for 30 minutes or less and that the fertilizing capacity of rabbit sperma-

Table XLI. Effect of Sodium Iothalamate (SI) in Egg Yolk
Medium (EYM) on the Motility of Rabbit Spermatozoa.

Storage Time (Hours)	Egg Yolk Medium[a]	3.5% SI in EYM	7.0% SI in EYM
Sample # 1			
48	Fair FM, High % TW·	Same as 25% Egg Yolk	Few % FM and TW
68	30-40% FM, 30% TW	5% FM, 20% TW	1% TW
144	3% FM, ∼50% TW	2% FM, 20% TW	1 motile sperm
Sample # 2			
28	20% FM, 50% TW	5% FM, 10% TW	5% FM, 5% TW
96	2-3% FM, 50% TW	2% FM, 15% TW	< 1% TW

a.25% Egg Yolk, 75% glycine-citrate medium by volume.

tozoa is preserved after exposure to low concentrations of Diodrast
for at least 4 hours. Thus, these materials can be used in either Staflo
or equilibrium centrifugation density gradients.

APPENDIX E

Expressions for Minimum, Maximum and Average Migration
Distances for Particles in Staflo Collection Fractions

The expressions presented below were derived by using Figures 13 and

14 for Staflo sedimentation and sedimentation-downward-electrophoresis

(SDE) experiments and Figures 20 and 21 for Staflo sedimentation-upward

electrophoresis (SUE) experiments. The expressions could be shortened.

However, this would eliminate the ability to compare parts of the expres-

sions with the respective parts of the range and distribution diagrams

from which they were derived. As the equations are written, the migra-

tion distances are given in millimeters.

The restriction that the bottom of the sample stream at the inlet

coincide with the bottom of that layer, as discussed in the text, results

in restrictions in the use of the equations for sedimentation and SDE

experiments when the top of the migration pattern remains in the inlet

layer. From Figures 13 and 14, it can be seen that in this case, there

is only one range of values for the minimum migration distance, using

x' in Cases 3 and 4.

In the expressions, n is the number of layers from and including

the inlet layer to the layer of exit for the particles that have migrated

the greatest distance.

1. **Two-Layer Migration Pattern in Sedimentation and Sedimentation-
   Downward-Electrophoresis Experiments.**

a. **Possible Distributions:**

Case 1: $([n-3] \times 1.25 + h) < x < (n-2) \times 1.25;$

$(n-2) \times 1.25 < y < ([n-2] \times 1.25 + h)$

Case 2: $([n-3] \times 1.25 + h) < x < (n-2) \times 1.25;$

$([n-2] \times 1.25 + h) < y' < (n-1) \times 1.25$

Case 3: $(n-2) \times 1.25 < x' < y$;

$x' < y < ([n-2] \times 1.25 + h)$

Case 4: $(n-2) \times 1.25 < x' < ([n-2] \times 1.25 + h)$;

$([n-2] \times 1.25 + h) < y' < (n-1) \times 1.25$

b. **Restrictions:** For $n = 2$ or $n = 3$ use Cases 3 and 4 only.

c. **Case 1:**

$\lambda_{(n-1)_{min}} = x$ mm $\qquad\qquad \lambda_{(n-1)_{max}} = y$ mm

$\lambda_{n_{min}} = (n-2) \times 1.25$ mm $\qquad \lambda_{h_{max}} = y$ mm

$\lambda_{(n-1)_{ave}} = \dfrac{h\ ([n-2] \times 1.25 - x)([n-2] \times 1.25 + x)}{2\ h\ ([n-2] \times 1.25 - x)}$ x

$\qquad\qquad + \dfrac{(y - [n-2] \times 1.25)([n-2] \times 1.25 + 2h - y)}{+\ (y - [n-2] \times 1.25)}$ x

$\qquad\qquad \dfrac{\frac{1}{3}\ (y + [n-1] \times 1.25)}{([n-2] \times 1.25 + 2\ h - y)}$ mm

$\lambda_{N_{ave}} = (n-2) \times 1.25 + \dfrac{2}{3}\ (y - [n-2] \times 1.25)$ mm

d. **Case 2:**

$\lambda_{(n-1)_{min}} = x$ mm $\qquad\qquad \lambda_{(n-1)_{max}} = (n-2) \times 1.25 + h$ mm

$\lambda_{n_{min}} = (n-2) \times 1.25$ mm $\qquad \lambda_{n_{max}} = y'$ mm

$\lambda_{(n-1)_{ave}} = \dfrac{([n-2] \times 1.25 - x)([n-2] \times 1.25 + x)}{2\ ([n-2] \times 1.25 - x)}$ x

$\qquad\qquad + \dfrac{h\ ([n-2] \times 1.25 + \frac{h}{3})}{+\ h}$ mm

$\lambda_{n_{ave}} = \dfrac{h\ ([n-2] \times 1.25 + \frac{2h}{3}}{h}$ x

$\qquad\qquad + \dfrac{(y' - [n-2] \times 1.25 - h)(y' + [n-2]\ 1.25 + h)}{+\ 2\ (y' - [n-2] \times 1.25 - h)}$ mm

e. **Case 3:**

$\lambda_{(n-1)_{min}} = x'$ mm $\qquad\qquad \lambda_{(n-1)_{max}} = y$ mm

$\lambda_{n_{min}} = x'$ mm $\qquad\qquad \lambda_{n_{max}} = y$ mm

$\lambda_{(n-1)_{ave}} = x' + \dfrac{1}{3}\ (y - x')$ mm

$$\lambda_{n_{ave}} = x' + \frac{2}{3} \ (y - x') \ mm$$

f. <u>Case 4</u>:

$$\lambda_{(n-1)_{min}} = x' \ mm \qquad\qquad \lambda_{(n-1)_{max}} = (n-2) \times 1.25 + h \ mm$$

$$\lambda_{n_{min}} = x' \ mm \qquad\qquad \lambda_{n_{max}} = y' \ mm$$

$$\lambda_{(n-1)_{ave}} = x' + \frac{1}{3} \ ([n-2] \times 1.25 + h - x') \ mm$$

$$\lambda_{n_{ave}} = \frac{(h + [n-2] \times 1.25 - x')(h - [n-2] \times 1.25 + x')}{(h + [n-2] \times 1.25 - x')} \ x$$

$$\frac{(x' + \frac{2}{3} [h + (n-2) \times 1.25 - x'])}{(h - [n-2] \times 1.25 + x')} \ x$$

$$\frac{+ h \ (y' - [n-2] \times 1.25 - h)(y' + [n-2] \times 1.25 + h)}{+ 2 h \ (y' - [n-2] \times 1.25 - h)} \ mm$$

2. <u>Three-Layer Migration Pattern in Sedimentation and Sedimentation-Downward-Electrophoresis Experiments.</u>

a. <u>Possible Distributions</u>:

Case 1: $([n-4] \times 1.25 + h) < x < (n-3) \times 1.25$

$(n-2) \times 1.25 < y < ([n-2] \times 1.25 + h)$

Case 2: $([n-4] \times 1.25 + h) < x < (n-3) \times 1.25$

$(n-2) \times 1.25 + h) < y' < (n-1) \times 1.25$

Case 3: $([n-3] \times 1.25 < x' < ([n-3] \times 1.25 + h)$

$(n-2) \times 1.25 < y < ([n-2] \times 1.25 + h)$

Case 4: $([n-3] \times 1.25 < x' < ([n-3] \times 1.25 + h)$

$([n-2] \times 1.25 + h) < y' < (n-1) \times 1.25$

b. <u>Restrictions</u>: For n = 3, use Cases 3 and 4 only.

c. <u>Case 1</u>:

$$\lambda_{(n-2)_{min}} = x \ mm \qquad\qquad \lambda_{(n-2)_{max}} = (n-3) \times 1.25 + h \ mm$$

$$\lambda_{(n-1)_{min}} = (n-3) \times 1.25 mm \qquad\qquad \lambda_{(n-1)_{max}} = y \ mm$$

$$\lambda_{n_{min}} = (n-2) \times 1.25 \ mm \qquad\qquad \lambda_{n_{max}} = y \ mm$$

$$\lambda_{(n-2)_{ave}} = \frac{([n-3] \times 1.25 - x)([n-3] \times 1.25 + x)}{2 \ ([n-3] \times 1.25 - x)([n-3] \times 1.25 + x)} \ x$$

$$\frac{+ h \ ([n-3] \times 1.25 + \frac{h}{3} \ )}{+h} \ mm$$

$$\lambda_{(n-1)_{ave}} = \frac{h^2\left([n-3] \times 1.25 + \frac{2h}{3}\right)}{h^2} \quad x$$

$$\frac{+\ h\ (1.25 - h)([2\ n-5] \times 1.25 + h)}{+\ 2\ h\ (1.25 - h)} \quad x$$

$$\frac{+\ (y - [n-2] \times 1.25) \times ([n-2] \times 1.25 + 2\ h - y)}{+\ (y - [n-2] \times 1.25)} \quad x$$

$$\frac{([n-2] \times 1.25 + \frac{1}{3}\ (y - [n-2] \times 1.25)\ )}{([n-2] \times 1.25 + 2\ h - y)} \quad mm$$

$$\lambda_{n_{ave}} = (n-2) \times 1.25 + \frac{2}{3}\ (y - [n-2] \times 1.25)\ mm$$

d. <u>Case 2</u>:

$$\lambda_{(n-2)_{min}} = x\ mm \qquad\qquad \lambda_{(n-2)_{max}} = (n-3) \times 1.25 + h\ mm$$

$$\lambda_{(n-1)_{min}} = (n-3) \times 1.25mm \qquad\qquad \lambda_{(n-1)_{max}} = (n-2) \times 1.25 + h\ mm$$

$$\lambda_{n_{min}} = (n-2) \times 1.25mm \qquad\qquad \lambda_{n_{max}} = y'\ mm$$

$$\lambda_{(n-2)_{ave}} = \frac{([n-3] \times 1.25 - x)([n-3] \times 1.25 + x)}{2\ ([n-3] \times 1.25 - x)([n-3] \times 1.25 + x)} \quad x$$

$$\frac{+\ h\ ([n-3] \times 1.25 + \frac{h}{3}}{+\ h} \quad mm$$

$$\lambda_{(n-1)_{ave}} = \frac{1}{2}\ ([2\ n-5] \times 1.25 + h)\ mm$$

$$\lambda_{n_{ave}} = \frac{h\ ([n-2] \times 1.25 + \frac{2h}{3}\ )}{2\ y'} \quad x$$

$$\frac{+\ (y' - [n-2] \times 1.25 - h)(y' + [n-2] \times 1.25 - h)}{-\ 2\ (n-2) \times 1.25 - h} \quad mm$$

e. <u>Case 3</u>:

$$\lambda_{(n-2)_{min}} = x'mm \qquad\qquad \lambda_{(n-2)_{max}} = (n-3) \times 1.25 + h\ mm$$

$$\lambda_{(n-1)_{min}} = x'mm \qquad\qquad \lambda_{(n-1)_{max}} = y\ mm$$

$$\lambda_{n_{min}} = (n-2) \times 1.25mm \qquad\qquad \lambda_{n_{max}} = y\ mm$$

$$\lambda_{(n-2)_{ave}} = x' \times \frac{1}{3}\ ([n-3] \times 1.25 + h - x')\ mm$$

$$\lambda_{(n-1)_{ave}} = \frac{(h + [n-3] \times 1.25 - x')(h - [n-3] \times 1.25 + x')}{(\ h + [n-3] \times 1.25 - x')} \quad x$$

$$\frac{(x' + \frac{2}{3}\ ([n-3] \times 1.25 + h - x')\ )}{(h - [n-3] \times 1.25 + x'} \quad x \quad \text{(continued)}$$

$$\frac{+ \ h \ (1.25 - h) \ x \ ([2 \ n-5] \ x \ 1.25 + h)}{+ \ 2 \ h \ (1.25 - h)} \ x$$

$$\frac{+ \ (y - [n-2] \ x \ 1.25)(2 \ h - y + [n-2] \ x \ 1.25)}{+ \ (y - [n-2] \ x \ 1.25)} \ x$$

$$\frac{([n-2] \ x \ 1.25 + \frac{1}{3} \ (y - [n-2] \ x \ 1.25) \ )}{(2 \ h - y + [n-2] \ x \ 1.25)} \ mm$$

$$\lambda_{n_{ave}} = (n-2) \ x \ 1.25 + \frac{2}{3} \ (y - [n-2] \ x \ 1.25) \ mm$$

f. **Case 4:**

$$\lambda_{(n-2)_{min}} = x' \ mm \qquad\qquad \lambda_{(n-2)_{max}} = (n-3) \ x \ 1.25 + h \ mm$$

$$\lambda_{(n-1)_{min}} = x' \ mm \qquad\qquad \lambda_{(n-1)_{max}} = (n-2) \ x \ 1.25 + h \ mm$$

$$\lambda_{n_{min}} = (n-2) \ x \ 1.25 mm \qquad\qquad \lambda_{n_{max}} = y' \ mm$$

$$\lambda_{(n-2)_{ave}} = x' + \frac{1}{3} \ ([n-3] \ x \ 1.25 + h - x') \ mm$$

$$\lambda_{(n-1)_{ave}} = \frac{(h + [n-3] \ x \ 1.25 - x')(h - [n-3] \ x \ 1.25 + x')}{(h + [n-3] \ x \ 1.25 - x')} \ x$$

$$\frac{(x' + \frac{2}{3} \ ([n-3] \ x \ 1.25 + h - x') \ )}{(h - [n-3] \ x \ 1.25 + x')} \ x$$

$$\frac{+ \ h \ (1.25 - h)([2 \ n-5] \ x \ 1.25 + h)}{+ \ 2 \ h \ (1.25 - h)} \ x$$

$$\frac{+ \ h^2 \ ([n-2] \ x \ 1.25 + \frac{h}{3} \ )}{+ \ h^2} \ mm$$

$$\lambda_{n_{ave}} = \frac{h \ ([n-2] \ x \ 1.25 + \frac{2h}{3} \ )}{2 \ y' - 2 \ (n-2) \ x \ 1.25} \ x$$

$$\frac{+ \ (y' - [n-2] \ x \ 1.25 - h)(y' + [n-2] \ x \ 1.25 - h)}{+ \ h} \ mm$$

3. **Two-Layer Migration Pattern in Sedimentation-Upward-Electrophoresis Experiments.**

   a. **Possible Distributions:**

   Case 1: $(n-2) \ x \ 1.25 < x < ([n-1] \ x \ 1.25 - h)$;
   $([n-1] \ x \ 1.25 - h) < y < ([n-1] \ x \ 1.25)$

   Case 2: $(n-2) \ x \ 1.25 < x < ([n-1] \ x \ 1.25 - h)$;
   $(n-1) \ x \ 1.25 < y' < (n \ x \ 1.25 - h)$

Case 3: $([n-1] \times 1.25 - h) < x' < y$;
           $x' < y < ([n-1] \times 1.25)$

Case 4: $([n-1] \, 1.25 - h) < x' < (n-1) \times 1.25$;
           $(n-1) \, 1.25 < y' < (n \times 1.25 - h)$

b.   <u>Restrictions</u>: None.

c.   <u>Case 1</u>:

$$\lambda_{(n-1)_{min}} = x \text{ mm} \qquad\qquad \lambda_{(n-1)_{max}} = y \text{ mm}$$

$$\lambda_{n_{min}} = (n-1) \times 1.25 - h \text{ mm} \qquad \lambda_{n_{max}} = y \text{ mm}$$

$$\lambda_{(n-1)_{ave}} = \frac{h \, ([n-1] \times 1.25 - h - x)([n-1] \times 1.25 - h + x)}{2h \, ([n-1] \times 1.25 - h - x)} \; x$$

$$+ \frac{(y - [n-1] \times 1.25 + h)([n-1] \times 1.25 + h - y)}{+ (y - [n-1] \times 1.25 + h)} \; x$$

$$\frac{\left(\frac{2}{3} \, ([n-1] \times 1.25 - h) + \frac{y}{3} \right)}{([n-1] \times 1.25 + h - y)} \; \text{mm}$$

$$\lambda_{n_{ave}} = \frac{1}{3} \, ([n-1] \times 1.25 - h + 2y) \text{ mm}$$

d.   <u>Case 2</u>:

$$\lambda_{(n-1)_{min}} = x \text{ mm} \qquad\qquad \lambda_{(n-1)_{max}} = (n-1) \times 1.25 \text{ mm}$$

$$\lambda_{n_{min}} = (n-1) \times 1.25 - h \text{ mm} \qquad \lambda_{n_{max}} = y' \text{ mm}$$

$$\lambda_{(n-1)_{ave}} = \frac{([n-1] \times 1.25 - h - x)([n-1] \times 1.25 - h + x)}{n \times 1.25} \; x$$

$$\frac{+ \, h \, ([n-1] \times 1.25 - \frac{2h}{3})}{- h - 2x} \; \text{mm}$$

$$\lambda_{n_{ave}} = \frac{h \, ([n-1] \times 1.25 - \frac{h}{3})}{h} \; x$$

$$\frac{+ \, (y' - [n-1] \times 1.25)(y' + [n-1] \times 1.25)}{+ 2y' - n \times 1.25} \; \text{mm}$$

e.   <u>Case 3</u>:

$$\lambda_{(n-1)_{min}} = x' \text{ mm} \qquad\qquad \lambda_{(n-1)_{max}} = y \text{ mm}$$

$$\lambda_{n_{min}} = x' \text{ mm} \qquad\qquad \lambda_{n_{max}} = y \text{ mm}$$

$$\lambda_{(n-1)_{ave}} = x' + \frac{1}{3} (y - x') \text{ mm}$$

$$\lambda_{n_{ave}} = x' + \frac{2}{3} (y - x') \text{ mm}$$

f. <u>Case 4</u>:

$$\lambda_{(n-1)_{min}} = x' \text{ mm} \qquad\qquad \lambda_{(n-1)_{max}} = (n-1) \times 1.25 \text{ mm}$$

$$\lambda_{n_{min}} = x' \text{ mm} \qquad\qquad \lambda_{n_{max}} = y' \text{ mm}$$

$$\lambda_{(n-1)_{ave}} = x' + \frac{1}{3} ([n-1] \times 1.25 - x') \text{ mm}$$

$$\lambda_{n_{ave}} = \frac{([n-1] \times 1.25 - x')(2 h + x' - [n-1] \times 1.25)}{([n-1] \times 1.25 - x')} \times$$

$$\frac{(x' + \frac{2}{3} [(n-1) \times 1.25 - x'])}{(2 h + x' - [n-1] \times 1.25)} \times$$

$$\frac{+ h (y' - [n-1] \times 1.25)(y' + [n-1] \times 1.25)}{+ 2 h (y' - [n-1] \times 1.25)} \text{ mm}$$

4. <u>Three-Layer Migration Pattern in Sedimentation-Upward-Electrophoresis</u> <u>Experiments</u>.

a. <u>Possible Distributions:</u>

Case 1: $(n-3) \times 1.25 < x < ([n-2] \times 1.25 - h)$
$([n-1] \times 1.25 - h) < y < (n-1) \times 1.25$

Case 2: $(n-3) \times 1.25 < x < ([n-2] \times 1.25 - h)$
$(n-1) \times 1.25 < y' < (n \times 1.25 - h)$

Case 3: $([n-2] \times 1.25 - h) < x' < (n-2) \times 1.25$
$([n-1] \times 1.25 - h) < y < (n-1) \times 1.25$

Case 4: $([n-2] \times 1.25 - h) < x' < (n-2) \times 1.25$
$(n-1) \times 1.25 < y' < (n \times 1.25 - h)$

b. <u>Restrictions</u>: None.

c. <u>Case 1</u>:

$$\lambda_{(n-2)_{min}} = x \text{ mm} \qquad\qquad \lambda_{(n-2)_{max}} = (n-2) \times 1.25 \text{ mm}$$

$$\lambda_{(n-1)_{min}} = (n-2) \times 1.25 - h \text{ mm} \qquad \lambda_{(n-1)_{max}} = y \text{ mm}$$

$$\lambda_{n_{min}} = (n-1) \times 1.25 - h \text{ mm} \qquad \lambda_{n_{max}} = y \text{ mm}$$

$$\lambda_{(n-2)_{ave}} = \frac{([n-2] \times 1.25 - h - x)([n-2] \times 1.25 - h + x)}{n \times 1.25} \times \text{(continued)}$$

$$\frac{+ h \left([n-2] \times 1.25 - \frac{2h}{3}\right)}{- h - 2 x} \text{ mm}$$

$$\lambda_{(n-1)_{ave}} = h^2 \left([n-2] \times 1.25 - \frac{h}{3}\right) + h (1.25 - h)([2 n-3] \times 1.25 - h) \over 2.50 h - h^2 \quad x$$

$$\frac{+ (y - [n-1] \times 1.25 + h)(h + [n-1] \times 1.25 - y)}{+ (y - [n-1] \times 1.25 + h)} \text{ x}$$

$$\frac{\left(\frac{2}{3} [(n-1) \times 1.25 - h] + \frac{y}{3}\right)}{(h + [n-1] \times 1.25 - y)} \text{ mm}$$

$$\lambda_{n_{ave}} = \frac{1}{3} ([n-1] \times 1.25 - h + 2 y) \text{ mm}$$

d. <u>Case 2</u>:

$$\lambda_{(n-2)_{min}} = x \text{ mm} \qquad \lambda_{(n-2)_{max}} = (n-2) \times 1.25 \text{ mm}$$

$$\lambda_{(n-1)_{min}} = (n-2) \times 1.25 - h \text{ mm} \qquad \lambda_{(n-1)_{max}} = (n-1) \times 1.25 \text{ mm}$$

$$\lambda_{n_{min}} = (n-1) \times 1.25 - h \text{ mm} \qquad \lambda_{n_{max}} = y' \text{ mm}$$

$$\lambda_{(n-2)_{ave}} = \frac{([n-2] \times 1.25 - h - x)([n-2] \times 1.25 - h + x)}{n \times 1.25} \text{ x}$$

$$\frac{+ h \left([n-2] \times 1.25 - \frac{2h}{3}\right)}{- h - 2 x} \text{ mm}$$

$$\lambda_{(n-1)_{ave}} = \frac{1}{2} ([2 n-3] \times 1.25 - h) \text{ mm}$$

$$\lambda_{n_{ave}} = \frac{h \left([n-1] \times 1.25 - \frac{h}{3}\right)}{h} \text{ x}$$

$$\frac{+ (y' - [n-1] \times 1.25)(y' + [n-1] \times 1.25)}{+ 2 y' - n \times 1.25} \text{ mm}$$

e. <u>Case 3</u>:

$$\lambda_{(n-2)_{min}} = x' \text{ mm} \qquad \lambda_{(n-2)_{max}} = (n-2) \times 1.25 \text{ mm}$$

$$\lambda_{(n-1)_{min}} = x' \text{ mm} \qquad \lambda_{(n-1)_{max}} = y \text{ mm}$$

$$\lambda_{n_{min}} = (n-1) \times 1.25 - h \text{ mm} \qquad \lambda_{n_{max}} = y \text{ mm}$$

$$\lambda_{(n-2)_{ave}} = x' + \frac{1}{3} ([N-2] \times 1.25 - x') \text{ mm}$$

$$\lambda_{(n-1)_{ave}} = \frac{([n-2] \times 1.25 - x')(x' - [n-2] \times 1.25 + 2h)}{([n-2] \times 1.25 - x')} \times$$

$$\frac{(\frac{x'}{3} + \frac{2}{3} [n-2] \times 1.25) + h (1.25 - h)([2 n-3] \times 1.25 - h)}{(x' - [n-2] \times 1.25 + 2 h) + (1.25 - h) \cdot 2h} \times$$

$$+ \frac{(y - [n-1] \times 1.25 + h)(h + [n-1] \times 1.25 - y)}{+ (y - [n-1] \times 1.25 + h)} \times$$

$$\frac{(\frac{2}{3} [(n-1) \times 1.25 - h] + \frac{y}{3})}{(h + [n-1] \times 1.25 - y)} \quad mm$$

$$\lambda_{n_{ave}} = \frac{1}{3} ([n-1] \times 1.25 - h + 2 y) \ mm$$

f. **Case 4:**

$$\lambda_{(n-2)_{min}} = x' \ mm \qquad\qquad \lambda_{(n-2)_{max}} = (n-2) \times 1.25 \ mm$$

$$\lambda_{(n-1)_{min}} = x' \ mm \qquad\qquad \lambda_{(n-1)_{max}} = (n-1) \times 1.25 \ mm$$

$$\lambda_{n_{min}} = (n-1) \times 1.25 - h \ mm \qquad \lambda_{n_{max}} = y' \ mm$$

$$\lambda_{(n-2)_{ave}} = x' + \frac{1}{3} ([n-2] \times 1.25 - x') \ mm$$

$$\lambda_{(n-1)_{ave}} = \frac{([n-2] \times 1.25 - x')(x' - [n-2] \times 1.25 + 2 h)}{([n-2] \times 1.25 - x')} \times$$

$$\frac{(\frac{x'}{3} + \frac{2}{3} [n-2] \times 1.25) + h (1.25 - h)([2 n-3] \times 1.25 - h)}{(h + [n-2] \times 1.25 - x') + 2.50 h} \times$$

$$\frac{+ h^2 ([n-1] \times 1.25 - \frac{2h}{3})}{- h^2} \ mm$$

$$\lambda_{n_{ave}} = \frac{h ([n-1] \times 1.25 - \frac{h}{3})}{h} \times$$

$$+ \frac{(y' - [n-1] \times 1.25)(y' + [n-1] \times 1.25)}{2 y' - n \times 1.25} \ mm$$

APPENDIX F

Sample Calculation of Sedimentation Velocities and Rates for
Staflo Sedimentation Experiment 12-26-67

1. Summary of Measurements

$$h = 0.08 \text{ cm}; \quad x = 0 \text{ cm}; \quad y = 0.17 \text{ cm}$$

2. Sedimentation Migration Distances

The sedimentation migration distances (λ_S) are calculated in accordance with the discussion in Chapter III and the equations in Appendix E 2. They are as follows:

Collection Tube	Sperm Collected (%)	Sedimentation Distance (λ_S)		
		Minimum (cm)	Maximum (cm)	Average (cm)
8	23.1	0	0.080	0.027
9	61.1	0	0.170	0.10
10	15.3	0.125	0.170	0.155

3. Sedimentation Velocities and Rates

Using a minimum residence time (τ_{min}) of 28.7 min. (1,722 seconds) and the expression

$$\frac{dx}{dt} = \frac{\lambda_S}{\tau_{min}} \quad \frac{(cm)}{(sec)} \tag{18}$$

one gets minimum, maximum and average sedimentation velocities of 0, 0.465 x 10^{-4} and 0.157 x 10^{-4} cm/sec for collection fraction 8.

The sedimentation velocities can be converted to sedimentation coefficients by the expression

$$S = \frac{1}{g} \frac{dx}{dt} \quad (sec) \tag{19}$$

where $g = 980 \text{ cm/sec}^2$

Using the average sedimentation velocity of 0.157×10^{-4} cm/sec for

collection tube 8, one gets

$$S_{8_{ave}} = 0.161 \times 10^{-7} \text{ sec}$$

Converting to Svedberg units (1 Svedberg $= 10^{-13}$ sec) gives

$$S_{8_{ave}} = 0.161 \times 10^{6} \text{ Svedbergs}$$

The results of similar calculations for other sedimentation distances

and collection fractions are summarized in Table XXII.

4. Weighted Average Sedimentation Velocity

The weighted average sedimentation velocity ($\overline{\frac{dx}{dt} \text{ ave}}$) for spermatozoa

in Experiment 12-26-67 is determined as follows:

(1) Collection Fraction	(2) Fraction of Spermatozoa	(3) ($\frac{dx}{dt}$ ave) (cm/sec)	(4) (2) x (3)
8	0.231	0.157×10^{-4}	0.0362×10^{-4}
9	0.616	0.580×10^{-4}	0.3570×10^{-4}
10	0.153	0.90×10^{-4}	0.1380×10^{-4}
		$\Sigma =$	0.5312×10^{-4} cm/sec

The weighted average sedimentation velocity for rabbit spermatozoa is

0.53×10^{-4} cm/sec at 3°C in a 1% sucrose solution in glycine-phosphate

buffer.

APPENDIX G

Sample Calculation of Electrophoretic Mobilities for Staflo
Sedimentation-Upward-Electrophoresis (SUE) Experiment 7-29-67

1. Summary of Measurements

$$h = 0.07 \text{ cm}; \quad x = 0.190 \text{ cm}; \quad y = 0.305 \text{ cm}$$

2. SUE Migration Distances

The SUE migration distances (λ_{SUE}) are calculated in accordance

with the discussion in Chapter III and the equations in Appendix E 3.

They are as follows:

(1) Collection Tube	(2) Sperm Collected (%)	(3) SUE Migration Distances (λSUE) Minimum (cm)	(4) Maximum (cm)	(5) Average (cm)
6	58.6	0.190	0.305	0.259
7	41.0	0.190	0.250	0.210

3. Weighted Average SUE Migration Distance

The weighted average SUE migration distance $(\lambda_{SUE_{ave}})$ is the sum of

the products of columns (2) and (5) above divided by 100 as follows:

$$\overline{\lambda_{SUE_{ave}}} = \frac{58.6 \times 0.259 \times 41 \times 0.210}{100} = 0.238 \text{ cm}$$

4. Weighted Average Electrophoretic Migration Distance

The weighted average electrophoretic migration distance $(\overline{\lambda_{E_{ave}}})$

is given by equation (11) from Chapter III:

$$\overline{\lambda_{E_{ave}}} = \overline{\lambda_{SUE_{ave}}} + \overline{\lambda_{S_{ave}}} \qquad (11)$$

The value of $\overline{\lambda_{SUE_{ave}}}$ was determined in paragraph 3.

$\overline{\lambda_{S_{ave}}}$ is determined by correcting the value of the weighted average

sedimentation velocity of 0.53×10^{-4} cm/sec calculated in Appendix F

for 3°C and 1% sucrose to that expected at 5.4°C and 0.5% sucrose using

the formula of Bier[38]:

$$\eta_1 \ (\frac{dx}{dt})_1 \ = \ \eta_2 \ (\frac{dx}{dt})_2 \tag{20}$$

This gives $(\overline{\frac{dx}{dt}_{ave}})$ 5.4°C = 0.58×10^{-4} cm/sec. Then, using a minimum

residence time of 14.2 min. (852 sec.), one gets a weighted average sed-

imentation distance at 5.4°C of 0.050 cm.

Thus, $\overline{\lambda_{E_{ave}}}$ = 0.238 + 0.050 = 0.288 cm

5. Weighted Average Electrophoretic Mobility

In this experiment, the weighted average electrophoretic mobility

$(\overline{\mu_{ave}})$ is calculated using equation 13 from Chapter III with the fol-

lowing values: λ_E = 0.288 cm, τ_E = 569 sec, I = 0.088 amps, and R =

1,430 ohms. This gives $\overline{\mu_{ave}}$ = 0.94×10^{-4} cm/sec/V/cm at 5.4°C.

6. Normalization of $\overline{\mu_{ave}}$ to 3°C

The formula of Bier[38] is used to normalize the value of $\overline{\mu_{ave}}$ at

5.4°C to that expected in the same density gradient at 3°C. The corrected

value is 0.852×10^{-4} cm/sec/V/cm.

Similar calculations have been done for the other SUE experiments in

this series and are summarized in Table XXVII.

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