STUDIES ON SPERMATOZOA

WITH SPECIAL REFERENCE

TO THE SEPARATION OF

X- AND Y-BEARING SPERM

by

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TABLE OF CONTENTS

CHAPTER TWO THE FLUORESCENT STAINING OF CHROMOSOMES AND SPERMATOZOA

CHAPTER THREE THE COUNTING OF SPERMATOZOA

CHAPTER FOUR SPERM SEPARATIONS USING BOVINE SERUM ALBUMIN

CHAPTER FIVE SPERM SEPARATIONS USING DENSITY GRADIENT CENTRIFUGATION

SUMMARY

Initially, the quinacrine staining technique for human sperm was studied and the use of both this stain and other acridine derivatives for the identification of the bovine Y sperm was investigated. During the experiments, human sperm counts were made using a haemocytometer, while bovine sperm were counted by using a haemocytometer and an electric counter. The two methods of counting bovine sperm were compared for accuracy and repeatability.

Using the quinacrine staining technique for human Y sperm as monitor, several separation procedures for human sperm and for bovine sperm were examined. In the first separation process investigated, columns of bovine serum albumin were used to separate sperm on the basis of differential motility, but no significant increase in the percentage of Y sperm recovered was noted. Secondly, density gradient techniques were applied to separate human and bovine ejaculates into populations according to differences in specific gravity or sedimentation rates. Three gradient media (Colloidal silica, sucrose and Metrizamide) were used in these experiments, but in no case was a significant increase produced in the percentage of Y sperm recovered.

The third method studied involved the use of a column of Sephadex G-50. The results of these experiments with human sperm were encouraging, as it was found that the numbers of Y sperm in the first fractions recovered after filtration were significantly lower than in the controls.

CHAPTER ONE

GENERAL INTRODUCTION

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1.1 INTRODUCTION

During the meiotic division of spermatogenesis, the sex chromosomes of the male divide, with half the sperm receiving the X chromosome and half receiving the Y chromosome. Thus, a normal ejaculate contains two populations of equal numbers of sperm, differing in sex chromosome content. At fertilisation, the sex of the resulting zygote depends on the sex chromosome borne by the fertilising sperm. The normal sex ratio of the offspring at birth is approximately unity, although in some species, notably man, there is a slight but constant excess of males at birth. Even under extreme environmental and physiological conditions, the variation is comparatively slight (Anon, 1974).

In addition to the differences in sperm because of the sex chromosome status, there are variations, in individual sperm, in age, biochemical and biophysical properties and probably in fertilising capacity (Lavon, Volcani and Danon, 1971). Also there is a proportion of dead or damaged sperm present and even in a normal ejaculate, diploid sperm have been recognised in a number of species (Beatty, 1970). If fertilisation by such a diploid sperm occurred, the resulting embryo would be an abnormal triploid form. It is possible that fertility after artificial insemination might be improved if dead, damaged or abnormal sperm could be removed from the

ejaculate without reducing the fertilising capacity of the remaining sperm. This would be desirable particularly in domestic species where artificial insemination is, or could be, practised on a commercial scale. Any fractionation of sperm might also lead to a partial or complete separation of X and Y sperm with resulting sex control of the offspring. Many attempts have been made to separate X and Y sperm and claims of success have been reported, (Beatty, 1974a), often with immense publicity, but substantiating evidence is lacking and a reliable reproducible technique for separation of X and Y sperm has yet to be devised.

Control of the sex ratio at conception would have advantages and disadvantages in many species, as well as raising moral and social issues if applied to humans. There are a number of deleterious X-linked recessive genes in man, e.g. haemophilia and the recessive X-linked form of muscular dystrophy, which are expressed, when present, in the single X chromosome of the male but which are suppressed in females possessing a second normal X chromosome. Sex predetermination would enable "carrier" mothers to produce only daughters, while an affected male parent might be enabled to produce only sons. The same end result could be achieved by amniocentesis during pregnancy and termination of the pregnancy if the foetus were of the "high risk" sex, but predetermination of sex before conception would avoid the need for abortion and the accompanying emotional distress (Anon, 1974).

In domestic species, the advantages of sex control would be mainly economic and would enable production of optimum proportions of males and females in particular husbandry situations to take advantage of phenotypic differences in sex-limited traits, such as milk and egg production and in sex-influenced traits, such

as rate of weight gain and body composition for meat carcases and better pelts in the fur trade. Sex control might also be advantageous in certain under-developed countries, such as India, where food shortages are acute, but the killing of male calves is against religious beliefs (Foote and Miller, 1971).

The development of embryo-transfer techniques on a commercial scale in cattle has led to some problems where twinning is desired. If twins of different sexes are born, the female may be a freemartin, (and consequently infertile), because of the influences of the adjacent male foetus during pregnancy. If the embryos implanted were of the same sex, such economic losses would be avoided. The development of sex control at insemination in this instance would be beneficial, since all the embryos produced in the donor female would be of the same sex.

However, grave disadvantages do exist. Firstly, the cost of any separation procedure must be economically feasible for wide-spread use. Sperm processing might damage genetic material. If widely practised, sex control might lead to increased in-breeding if only a small reservoir of males were left as sires. In both man and animals, many sperm may be chromosomally abnormal and would not fertilise under natural conditions: care would be needed to ensure that the sperm processing technique did not inadvertently select for these sperm.

In man, medical and social issues are raised over the possibility of sex control. Surveys have shown that most couples, if given a choice, would prefer their firstborn to be male, (Etzioni, 1968). In many cultures, boys provide an economic advantage (as work-horses) or a form of old-age insurance (where the State has

not established it), while girls may be a liability; a dowry, which may be a sizable economic burden, must be provided to marry them off. If sex control were widely practised, it has been predicted that this would lead to increases in prostitution, homosexuality, age of marriage of males and numbers of males who would never marry. However, if one or the other sex ever became heavily outnumbered, attitudes would change and, presumably, some restoration of the balance would follow. Most parents of more than one child seem to desire children of both sexes, so that deviation from the 1:1 ratio might be less than expected; also, the opportunity of choosing the sex of one's children might lead to smaller families and a reduction in the population. On the other hand, the birth order of the sexes in a family would change considerably, with most first-borns being male instead of a random sequence of males and females and as the position in birth order affects both physical and personality traits of the developing offspring, behavioural changes in the population might be expected.

The major problem in any plan to control the sex of the offspring at conception is the difficulty in recognising a sperm as being either X- or Y-bearing.

X- and Y-bearing sperm are known to differ in their chromosome status, the X chromosome being larger than the Y chromosome, in mammalian species, but apart from this, there are no known differences in size, mass, specific gravity, swimming rate or electrical charge, specifically between X and Y sperm. With the exception of the human male sperm cell, whose Y chromosome has been reported as being preferentially stained with a fluorochrome (Barlow and Vosa, 1970), there are no means yet of

distinguishing a male from a female sperm cell in mammals. Therefore, until recently, no measurements or determinations could be carried out on sperm individually identified as to sex. Researchers were handicapped in the past, by being forced to deal with a total mixed sperm population and applying to it measures which might cause separation of X and Y sperm, without being able to monitor their techniques directly, by counting the proportions of X and Y sperm in the different fractions.

In the human, dry mass measurements have been made on the autosomal pairs of chromosomes and considerable variation has been found (Bahr, 1971a) - from 436.1×10^{-13} gm for a full "heavy" set to 409.12×10^{-13} gm for a full "light" set - this difference is in the order of 6.2%. If the mass of a Y chromosome (9.70×10^{-13} gm) is added to the figures of the "heavy" set and the mass of an X chromosome (26.10×10^{-13} gm) added to those of the "light" set, it has been found that the "light" set plus the X chromosome are lighter by 2.4% than the "heavy" set plus Y chromosome. Under the assumption that at meiosis, all autosomes are randomly distributed, with regard to mass, Kiddy (quoted by Bahr, 1971a) has calculated the probability that 85.7% of Y sperm can be expected to be lighter than all X sperm and that 99.2% of Y sperm would be lighter than all but 0.006% of X sperm.

Using chromosomes from a male bovine, Baker, Salisbury and Fechheimer (1965) carried out measurements of mean area and calculated that the mean area (and therefore, probably the mass) of a haploid set of chromosomes, including an X chromosome would be 3.1% greater than that of a haploid set containing a Y chromosome. After measuring Feulgen-stained DNA in bovine sperm nuclei, using

an integrating microdensitometer, the authors indicated that it might be possible to detect a difference as small as 3.1% between two populations of sperm, to monitor any separation achieved.

Considering the sperm cell, where cytoplasm must also be taken into account, it has been suggested (Beatty, 1974b) that the Y sperm may contain a small amount of extra cytoplasm compared to the X sperm, to "balance" the difference in X and Y chromosomes. Consequently, the difference in mass between X and Y sperm might be actually less than that calculated from differences in the mass of the chromosomes.

1.2 VISUAL IDENTIFICATION OF X AND Y SPERM

Prior to the discovery that the human Y chromosome could be recognised within the sperm head, after staining with quinacrine dyes, several researchers had attempted a visual identification of X and Y sperm. If sperm were recognisable as being X- or Y-bearing, individual sperm might be studied further for other phenotypic differences (e.g. differences in specific gravity, antigenicity, sedimentation rate, etc.), in the hope of developing experimental techniques to produce effective separation of the two types of sperm. In addition, the identification method could be used, to monitor directly, the success of any separation technique, eliminating the need for time-consuming insemination and determination of the sex of the offspring, in field trials.

Shettles (1960 a + b, 1961) suggested that the methods of fixing and staining human sperm for bright light and electron

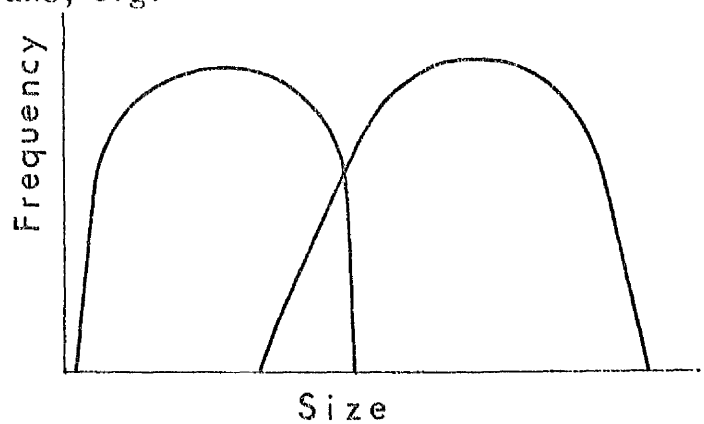
microscopy produced alteration and coalescence of the head contents into a homogeneous mass, which was misrepresentative of the normal state of the sperm head as it entered the egg at fertilisation. He carried out studies on the unfixed unstained sperm of more than 100 men, using phase contrast microscopy, and described the existence of two distinct populations - a smaller-headed type of sperm with a rounded central mass and a larger-headed type of sperm with an elongated central mass. In most specimens observed, although the ratio of the two types varied with the individuals, he found that the smaller-headed type predominated, but did not quote actual numbers of sperm counted. The central masses within the sperm heads were Feulgen-positive and were believed by Shettles to represent discrete chromosomes. He claimed that the smaller-headed sperm were Y-bearing and the larger-headed type, X-bearing, explaining the predominance of the "Y-bearing"sperm by the fact that in humans, there are more male births and therefore, probably a greater number of males conceived.

Rothschild (1960) stated that nuclear material is generally thought to be uniformly distributed within the mature sperm head. He was unable to explain what Shettles had actually observed, but commented that an unusual optical system had been used and suggested that the round and elongated masses within the sperm head may have been distorted vacuoles - known to occur in human sperm. Bishop (1960) also criticised the optical system used by Shettles and dismissed his observations on sperm as artefacts, produced by the non-uniform settling of sperm on the slide during the drying process.

In addition to the claims of Shettles, the possibility that X and Y sperm may differ in head size has been considered, (reviewed by Beatty, 1970). Both X and Y sperm may have their own size-frequency distribution and an essential part of the study has been to decide whether there is a significant difference between the mean values of each. Again, researchers have been confronted with the difficulty of dealing with the total mixed population of sperm in a semen sample. In mammals, no-one has reported the ideal situation of two completely separate non-overlapping sub-distributions and investigations have been concerned, therefore, only with overlapping sub-distributions which are classified into two types:

(a) Bimodal Distribution

If X and Y sperm sub-populations did exist and differed sufficiently in size, a bimodal size-frequency distribution might result, with two peaks, e.g.



An obvious bimodality would give evidence of the existence of two sub-populations, differing in size.

Beatty (1961) recorded results in which no bimodality was demonstrated and results which claimed a bimodal distribution of sperm. To these conflicting results and to new data (eight species of mammal were examined in all), he applied a mathematical formula to estimate the means of two underlying sub-populations and to assess

the significance of any difference between the two means. However, he obtained no significant evidence for the presence of two sub-populations of sperm. In support of Beatty's findings, no indication of a bimodal distribution of sperm was observed, using an electronic sizing method, in man (Segal and Laurence, 1962), rabbit (Fowler and Hellman, 1965), bull, boar or ram (O'Donnell, 1969).

(b) Platykurtic Distribution

If two sub-populations of sperm (in terms of size) did exist, the means of the two distributions might be so close that a bimodal frequency distribution curve was not obtained, but the compound curve would have the statistical characteristic of platykurtosis ("rounded shoulders and short tails"). When the mathematical formula used in (a) was applied to measurements of head length of 3,200 rabbit sperm, no difference between the means of two supposed sub-populations was found to exist (Beatty, 1961). He suggested that if a real difference was in fact obscured by sampling error, then the differences between the means was unlikely to be greater than 3%. In support of this finding, Van Duijn (1961) observed a unimodal size distribution of human sperm and calculated that the size differences in X and Y sperm were too small to be detected by any system of light microscopy.

Beatty (1972) has summarised his opinions by stating that there is no real evidence, in mammals, to support the existence of two sub-populations of sperm differing in head size and therefore, no real evidence for supposing, on observational grounds, that X and Y sperm differ in head size. If differences do exist, they are likely to be very small, with considerable overlap between the X and

Y distribution. This would mean that few individual sperm could be scored as either X- or Y-bearing, and even if the existence of two sub-populations could be demonstrated, there is no way of knowing from the data which population represents X sperm and which, Y sperm.

A breakthrough in the visual identification of X and Y sperm came unexpectedly, when Caspersson, Farber, Foley, Kudynowski, Modest, Simonsson, Wagh and Zech (1968) published observations on the appearance of mitotic chromosomes of various organisms, stained with quinacrine mustard and examined in ultra violet light. The chromosomes had varying quantities of fluorochrome bound along their length, the distribution of the fluorescence being constant for each particular pair of chromosomes. Later, Zech (1969), a member of the same team, made the important observation that, when human chromosomes were stained with quinacrine mustard, the Y chromosome could always be recognised easily, as the distal ends of its long arms showed a much more intense fluorescence than any other chromosome region.

This finding was soon verified by Pearson, Bobrow and Vosa (1970), using the more readily available and less expensive quinacrine dihydrochloride, and the field was extended when these workers showed that human interphase cells could be sexed, by staining with quinacrine for the presence of a small bright fluorescent body within the cell nucleus. Using buccal mucosa cells from normal volunteers and from 47/XYY volunteers, comparisons showed that no fluorescent body was present in cells from normal females, a single body was present in cells from normal males, and, in XYY males, around 30% of the cells showed two clearly separated

fluorescent bodies. The physical dimensions of this "male chromatin body" (0.25 μm diameter) in the interphase nucleus were similar to those of the highly fluorescent region of the Y chromosome seen in metaphase preparations and the authors inferred that the fluorescent body of male interphase nuclei represented the fluorescent region of the Y chromosome. Prior to this work, it had been impossible to identify individual chromosomes within the interphase nucleus.

Pearson and Bobrow (1970) continued their work by following the appearance of the fluorescent Y chromosome through the different stages of spermatogenesis and showed that the fluorescent body was clearly visible in most cells at all stages of meiosis. Barlow and Vosa (1970) extended their observations to human spermatozoa and showed, that when human sperm were stained with quinacrine (either the mustard or the dihydrochloride), and examined in ultra violet light, approximately 50% of sperm contained a fluorescent body (F-body) within the sperm head, which was much brighter than the rest of the sperm head. The position of the F-body within the sperm head was variable, but in most of the sperm, it was seen to lie at the boundary between dense and less dense regions of chromatin. They concluded that, because the frequency with which the F-body was seen in sperm approached that expected from the segregation of the Y chromosome at meiosis, (50% of sperm receive a Y chromosome, 50% an X chromosome), those sperm bearing an F-body carried a Y chromosome, while those sperm lacking an F-body, lacked a Y chromosome and might, therefore, be X-bearing sperm. Thus, the evidence that the F-body in the spermatozoa actually represented the fluorescent Y chromosome was largely circumstantial. Discrete chromosomes are not

normally visible in the mature sperm (Rothschild, 1960) so no direct method of confirmation was available, but an indirect approach to the problem was made, by Sumner, Robinson and Evans (1971).

The human Y chromosome is, on average, approximately one-third of the length of the X chromosome. Spermatozoa are haploid, each bearing 22 autosomes and one sex chromosome; those carrying the smaller Y chromosome have, theoretically, less chromosome material than the X-bearing sperm. This difference is very small - around 4%, calculated from the relative length of chromosomes seen in somatic cells. The difference in total chromatin length should be reflected by a similar level of difference in DNA content and DNA can be estimated by measurement of optical density after Feulgen-staining, the intensity of staining being proportional to the amount of DNA present.

The results of Sumner et al. (1971) showed that the mean relative Feulgen-DNA value for sperm lacking an F-body was higher than that for sperm with an F-body (putative Y sperm) and the conclusion was drawn that the fluorescent body in the sperm head did indeed represent a Y chromosome. Further evidence from the same workers showed that in sperm with two F-bodies, the Feulgen-DNA value was midway between the higher value for sperm lacking an F-body and the lower value for sperm with a single F-body, suggesting that these sperm contained two Y chromosomes.

Although Sumner et al. (1971) had been able to obtain satisfactory results, by measuring the Feulgen-DNA of sperm, they acknowledged that this staining process was known to cause some

variability in the measurement of DNA. Consequently, research was continued using the technique of integrating microinterferometry, by which the dry mass of the unstained sperm could be measured. Using this technique, Sumner and Robinson (1976) have shown that the dry mass of the sperm head is directly related to its DNA content and confirmed their earlier results, by showing that there is a highly significant difference of 2.13% in the dry mass (and therefore DNA content) of X- and Y-bearing human sperm.

Efforts to apply this fluorescent staining technique to the Y chromosomes and Y-bearing sperm of other species have failed, so far, (Pearson, Bobrow, Vosa and Barlow, 1971) but, at least, the development of the method for identification of human Y sperm has enabled researchers to monitor experiments to separate X and Y sperm directly. However, before the visual identification of human Y sperm became possible, many experimental approaches to the control of primary sex ratio in mammals had been made, with insemination of any separated sperm fractions and subsequent sexing of offspring produced as the only means of monitoring the success of the method. The eight experimental approaches reviewed now have often been claimed to be successful, but equally often, the claims have been disputed by other workers.

1.3 PARTHENOGENESIS

This means literally "reproduction without sexual union". Theoretically, if female organisms could be stimulated to parthenogenesis, they should produce all-female offspring. Parthenogenesis of spontaneous origin has been demonstrated in the eggs of several

mammalian species and has been induced experimentally in eggs of rabbit and mouse, by using various physical and chemical stimuli (Pincus, 1930; Tarkowski, Witkowska and Nowicka, 1970; Graham, 1970) but unfortunately, all parthenogenetic embryos appear to die and there seems no fully established case of development to birth. Inviability may be possibly due to high homozygosity and the offspring may die because they are suffering from the equivalent of extreme in-breeding depression. There seems to be some obstacle to the survival of parthenogenetic young and Beatty (1972) has speculated that the sperm may carry some non-nucleic component essential for normal embryogenesis.

1.4 EXPERIMENTALLY SEX-REVERSED FEMALES

If females (XX) could be sex-reversed to become functional XX males, i.e. produce X sperm only and were then mated to normal females, all offspring would be female. However, functional sex reversal has not yet been accomplished in any mammal and McLaren (1972) and Short (1972) have provided evidence that XX germ cells located in a testis cannot give rise to spermatozoa.

Any possibility of sex control resulting from either of the two above methods would seem remote. Therefore, greater attention has been paid to methods of separation of X- and Y-bearing spermatozoa.

1.5 ELECTROPHORETIC SEPARATION

Electrophoresis is the process by which ions or charged particles in aqueous buffered solutions move towards either the anode

or the cathode when a direct electric current is applied.

The study of sperm by electrophoretic techniques dates back to the 1920's when Mudd and Mudd (1929) found that sperm from a number of mammalian species were all negatively charged, and migrated, tail first, to the anode, in an electric field. Since then, most electrophoresis studies in mammalian sperm have been concerned with the possibility of separating male-producing from female-producing sperm as a means of sex control of the offspring. There seems to be no reason why X- and Y-bearing sperm should be expected to differ in surface charge, but electrophoretic methods might be regarded empirically as being one of several techniques that separate sperm into fractions, (in this instance, anode- and cathode-migrating), which might contain differing proportions of X and Y sperm.

Galvanic separation is a term used by some workers when an electric potential is applied to motile sperm. The electric current acts to orientate the sperm, while the tail propels the sperm in the direction in which the head is pointing. The velocity of the sperm depends upon the resultant of two velocity vectors:

(1) motility of the sperm

(2) force of electrophoretic migration.

Many workers have studied electrophoretic separation of X and Y sperm, with conflicting results. Koltzoff and Schröder (1933) and Schröder (1934 and 1941) claimed a successful separation of sperm into anodic and cathodic fractions in a buffer of pH 7.1. Subsequent insemination with the anodic fractions produced predominantly female litters, while the cathodic fractions produced predominantly male litters. Lewin (1956) and Gordon (1957 and 1958)

reported similar observations of a two-way migration with rabbit and human sperm and found that Schröder's results were qualitatively reproducible.

More recently, Sevinç (1968) published the results of four experiments using rabbit sperm. In the first experiment, a two-way migration was observed and insemination with the anodic fraction produced an excess of males in the litter, while the cathodic fraction yielded a predominance of females - the reverse of Schröder's findings. However, three subsequent experiments failed to repeat these results - the sex ratio of offspring born after insemination was not significantly different from that normally expected.

Kordts (1952) and Pilz (1952) found no evidence to confirm electrophoretic separation of sperm bearing X and Y chromosomes, while MacPherson and Vesselinovitch (1959) using bull sperm, reported a unidirectional migration towards the cathode, and the results of insemination showed, if anything, that a central fraction (neither anodic nor cathodic) might perhaps yield an excess of female offspring.

Nevo, Michaeli and Schindler (1961) conducted their experiments with bovine and rabbit sperm, immobilised either by low temperature or by carbon dioxide, and found only an anodic migration of sperm at pHs between 2.5 and 8.5. They also found that the isoelectric point of both rabbit and bovine sperm was around 3.5, much lower than that suggested by Schröder. Bangham (1962), using rabbit and ram sperm, observed two types of sperm when an electric field was applied:

(a) Tail-anode sperm, with a net negative charge on the tails, swimming at variable speed tail-first towards the anode. When the current was switched off, these sperm accelerated away from the anode.

(b) Head-anode sperm, with a net negative charge on the head, swimming very rapidly towards the anode and decelerating when the current was switched off.

The proportions of the two types varied with pH and ionic concentration. If the sperm were completely immobilised, at acid pHs, they were observed to show a uniform tail-first anodic migration.

In a study on galvanic separation, Hafs and Boyd (1971) accumulated sex ratio data after insemination with fractions of rabbit and bull sperm separated in three types of electrophoretic apparatus. Visual observations of sperm behaviour in a fourth cell revealed a two-way migration, with sperm becoming orientated as "head-anode" or "tail-anode" within 5 seconds. With bovine sperm, the sex ratio of the calves born after insemination with fractionated sperm was not significantly different from that expected normally, while the rabbit data suggested a slight deviation in sex ratio of the young born from anode- and cathode-migrating sperm, with cathodic fractions producing more males in the litters. These workers stressed that temperature, ionic strength of the buffer and electric potential applied were three very important variables which needed to be balanced to achieve a visual galvanic separation and they also pointed out that electrophoresis worked to reduce sperm motility and that the immobilised sperm were pushed towards the anode.

The most recent galvanic separation results have been those of Shisito, Shirai and Matsuda (1974) and Shisito, Shirai and Sasaki (1975), using human sperm, with the fluorescent staining technique for the Y-bearing sperm as a monitor. These findings, contrary to those of Hafs and Boyd (1971), were that Y sperm migrated predominantly to the anode and X sperm to the cathode, with sperm from infertile

patients showing the same trend as sperm from normal volunteers. The effects of galvanisation disappeared 15 minutes after the electric current was switched off, with the proportions of X and Y sperm reverting to pre-galvanisation levels. They observed also that sperm migrating to the cathode showed a reduced motility, while sperm migrating to the anode showed an increasing motility - but these changes were transitory.

From the results obtained using electrophoresis, it is obvious that the evidence is conflicting, with a lack of confirmation by different workers, or even by the same worker. Failure to repeat first experiments may be owing to inability to reproduce exact experimental conditions or it may be that the first result was merely chance fluctuation. However, when two workers obtain statistically significant but conflicting results, the difficulty occurs in deciding whether a real phenomenon exists or whether the experimental parameters are not yet fully understood.

1.6 ANTIGENIC SEPARATION

This involves an entirely different approach to the control of sex of the offspring. The question of whether immunisation of females against sperm would affect their breeding performance was raised many years ago when the ability of animals to form antibodies against sperm was demonstrated first by Metchnikoff (1899 and 1900). He injected guinea pigs with semen or with macerated testes from a variety of species and found subsequently that the serum of the guinea pigs immobilised the sperm of humans, bulls, rabbits and guinea pigs. This production of antibodies against sperm would undoubtedly be a

useful method of fertility control, but although there have been many experiments using materials from the male reproductive tracts of a number of species, no satisfactory answer has been obtained, as yet. Tyler (1961) has reviewed 150 reports of immunisation of females with sperm or with testis tissue, and has concluded that no reliable method for the immunological control of fertility has been demonstrated in any species.

From this work, the possibility arose that antibodies might be produced specifically against X- or Y-bearing sperm, with subsequent control of the sex of the offspring. The work began when Eichwald and Silmser (1955) reported that the behaviour of skin isografts among an inbred strain of mice, (C57BL), deviated from the expectation that all grafts would survive, the individuals of an inbred strain being genetically identical. Grafts from male donors were rejected by female recipients, while there were almost no graft failures in all other sex combinations. These male-to-female effects could be explained by the existence of a sex-linked histo-compatibility antigen, whose gene locus was most probably on the Y chromosome.

Feldman (1958) showed that the Y-linked histocompatibility antigen existed in other strains of mice and also, in male tissues, other than skin; male-to-female somatic tissue grafts were also rejected by an immune reaction. Both he and Sachs and Heller (1958) independently suggested that serum from in-bred females, immunised by grafts from males of the same strain, might selectively impair the fertilising capacity of sperm carrying the Y chromosome in a mixed population of X and Y sperm. The serum of females would, presumably, contain antibodies specifically directed against

the H-Y antigen expressed on the sperm cell surface. This agglutination and immobilisation of the Y sperm, leaving X-bearing sperm unaffected, would be an elegant method of sex control, resulting in the production of female offspring only. Immunised females might be mated naturally, but even if the circulating antibodies did not reach the sperm, the technique could be applied in vitro by mixing antiserum with the sperm prior to artificial insemination.

In order to test this theory, Beatty (1960) injected rabbit does either subcutaneously or intravenously with rabbit sperm, but following mating and conception, the sex ratio of the offspring was not found to be significantly different from that in untreated does. McLaren (1964, 1965), in her studies on mice, also failed to show any alteration in the sex ratio of the offspring after immunising mothers against the Y antigen, using skin grafts. Edwards (quoted by Tyler and Bishop, 1963), took two inbred strains of mice and backcrossed males of one inbred strain (A) with females of another strain (B) i.e. individuals were prepared in which the Y chromosome of strain A were associated with a background largely of B autosomal genes. The sperm of these males were used to immunise strain B females, or to produce antisera in rabbits. However, following mating of the immunised females or insemination of normal females with sperm treated with rabbit antisera, no significant difference in the sex ratio of the offspring was noted.

A positive claim came from Burkov (1968), who assumed that the Z chromosomes of birds were homologous to the Y chromosomes of mammals, (in birds, females are the heterogametic sex, ZW, males being homogametic, ZZ). After immunising female rabbits

with cock semen, and then inseminating them with rabbit semen, he recorded a deficiency of male offspring.

More recently, in 1973, Bennett and Boyse pointed out that some of the previous experiments had involved determining the sex ratio of the progeny of female mice that had rejected skin grafts from males of the same strain. These results had often been negative or equivocal. Consequently, these authors used a direct method for assaying H-Y antibody in serum, by a complement-dependent cytotoxicity test using sperm or male epidermal cells. They examined the effects of artificially inseminating mice with sperm that had been exposed in vitro to H-Y antibody of known cytotoxic potency and a sex ratio of 45.4% males was observed using the treated sperm, compared to 53.4% males for natural matings and controls. From these results, they suggested that the Y-chromosome-bearing sperm may have more H-Y antigen on their surfaces, implying haploid gene expression. However, since there was a relatively small shift in sex ratio, these workers felt that this might indicate only a small difference in H-Y antigen between X- and Y-bearing sperm - this is not logical, surely, if H-Y antigen is specifically related to a gene locus on the Y chromosome. They mentioned also a second possibility that, because skin grafts were used to produce antisera, contamination with other histocompatibility antibodies, cytoxic to sperm, might occur, resulting in a reduction in sensitivity of the method.

From a survey of human records, Renkonen, Mäkelä and Lehtovaaha (1962) claimed that a woman who had given birth to one or more male children was less likely to produce further male offspring. They postulated that immunisation against Y antigen might

occur during gestation of a male foetus and decrease the chance of a subsequent male foetus being carried to term. Both Edwards (1963) and McLaren (1965) have criticised this claim. Edwards has quoted Renkonen's findings of 1956, when he stated that "there was a highly significant tendency to duplicate the sex of the preceding child" - the opposite to his later immunisation hypothesis and from her work on mice, McLaren (1965) concluded that it was unlikely that immunisation against the Y antigen played the part suggested for it by Renkonen in the antigenically complex situation of human pregnancy.

More recently, human histocompatability antigens (HL-A antigens) were demonstrated in human semen and spermatozoa (Fellous and Dausset, 1970) but their role in human reproduction has not been fully determined. These authors suggested that the expression of the HL-A antigens may be linked to the sex chromosome carried by the sperm; if so, one might speculate on the possibility of gametic selection based on the use of cytotoxic antibodies recognising these or other sex-linked antigens.

Thus, the question of whether X- and Y-bearing sperm have specific antigenic phenotypes determined by their different chromosomal contents remains unanswered as yet and it may not be answered conclusively, perhaps, until some separation procedure has successively separated X- and Y-bearing sperm into pure fractions, which could then be tested for antibody production.

1.7 SEDIMENTATION SEPARATIONS

As the mammalian X chromosome is larger than the Y

chromosome it is possible that more DNA is present in the X-bearing sperm. On this basis, Harvey (1946) proposed that X-bearing sperm might be heavier, slightly more dense and slightly larger than Y-bearing sperm. Together with a clear statement of the problems, he outlined two possible procedures for the separation of X- and Y-bearing sperm by centrifugation:

(a) Non-Equilibrium Sedimentation

In this technique, sperm are allowed to fall through a fluid, with or without the assistance of centrifugation, until they are dispersed in the fluid. The rate of fall depends on specific gravity and on size and shape, the most rapid sedimentation occurring with high specific gravity and large volume.

Unbeknown to Harvey, Lush (1925) had used this technique on rabbit semen; he diluted the semen with Ringer's solution and separated it into fractions by centrifugation. The top and bottom thirds of the centrifuged liquid were used for the artificial insemination of does, but no changes in the sex ratio of the offspring was produced.

Bhattacharya (1958, 1962) in two sedimentation experiments, using an egg yolk diluent, produced consistent results with rabbit sperm. The upper fractions produced a significant excess of male progeny while the lower fractions produced a significant excess of females. Bhattacharya (1964) also claimed that examination and measurement of sperm from the separated fractions revealed a bimodal size distribution, with sperm from the lower fractions having greatest head length, breadth and density. However, although Lovelock (1960) observed a wide density range of rabbit sperm from a single ejaculate on albumin columns, insemination of

"high density" and "low density" sperm did not produce a change in the sex ratio of the offspring.

Subsequently, Andersen and Rottensten (1962), as well as Bedford and Bibeau (1967) were unable to obtain sex ratios deviating significantly from 50:50 in the rabbit and thus were unable to verify Bhattacharya's findings. Schilling (1971) also repeated Bhattacharya's method exactly, using rabbit sperm, and obtained similar low fertility and small litter sizes, but was unable to confirm Bhattacharya's results regarding sex ratio of the offspring, although insemination of the upper fractions did produce 60% male progeny. After trying several media to find one suitable for rabbit sperm, he concluded that this was an unsuitable species for sedimentation separations as some of the sperm always sedimented rapidly. He thought that this might be explained by Oresnik's findings (1969) that 5-10% of the sperm were unusually heavy, beyond the usual specific gravity range for the rabbit.

Stambaugh and Buckley (1971a and b) used a sedimentation separation for rabbit sperm, linked to an assay for lactic dehydrogenase enzyme (LDH), the enzyme which catalyses the final step in glycolysis. In most tissues, a maximum of five isoenzymes of LDH are found - these can be separated by paper electrophoresis and other methods of fractionation. Each isoenzyme is considered to be tetrameric, formed from two kinds of subunit, the H subunit and the M subunit, and these subunits can be assorted into five possible molecular forms: M_4, M_3H, M_2H_2, MH_3 and H_4 - these are the isoenzymes referred to as LDH_5, LDH_4, LDH_3, LDH_2 and LDH_1, respectively. Relatively recent evidence, (Appella and Zito, cited by Stambaugh and Buckley, 1971b) has

indicated that each subunit might consist of two polypeptide chains, thus:

H subunit < R polypeptide chain
A or anionic polypeptide chain

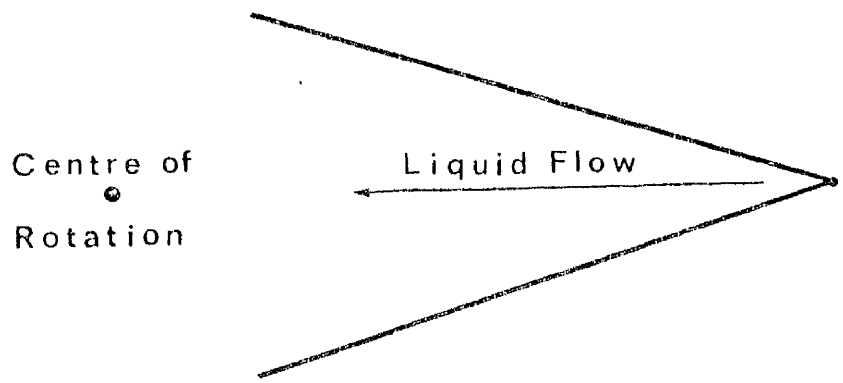
M subunit < M polypeptide chain
C or cationic polypeptide chain

A unique LDH isoenzyme, called the X_4 isoenzyme, has been found in large quantities in mature testis tissue, in spermatozoa and in seminal plasma. A survey of rabbit tissues also revealed that X_4 isoenzyme occurred in tissues such as heart and kidney, characterised by their deficiency of the M polypeptide. Stambaugh and Buckley (1967) proposed that this X_4 isoenzyme was a hybrid, formed from the R and C polypeptide chains found in H and M subunits. Since large quantities of the X_4 isoenzyme occur only in spermatozoa, which are haploid, and in mature testis tissue, which contains haploid spermatids and secondary spermatocytes (Goldberg and Hawtrey, 1967), the question was raised whether the gene for synthesis or control of synthesis of the M polypeptide might be sex-linked. Females lack a Y chromosome, but still produce the M polypeptide and the hypothesis is, then, that the gene locus for the M polypeptide might be located on the X chromosome. Thus the X_4 isoenzyme might represent haploid expression of the gene in male-producing (i.e. Y-bearing) sperm, due to the absence of the X chromosome carrying the M polypeptide locus.

To test this theory, rabbit sperm were fractionated on discontinuous dextran gradients (not centrifuged to equilibrium) and the supernatant layers were harvested, with half the sperm on these layers being used for an LDH assay. Subsequently, only

those supernatants with a definite predominance of R polypeptide chain were used for intrauterine insemination of the does. It was found that sperm from these supernatant layers produced a predominance of male foetuses, lending support to their hypothesis that the X_4 isoenzyme might be characteristic of Y-bearing sperm. This finding also introduced the possibility that the X_4 isoenzyme could be used as an assay for determining the percentage of male- and female-producing sperm in fractionated semen specimens.

Sedimentation separations have been applied also to bovine sperm. Lindahl (1948) described the principle of counter-streaming centrifugation as a means of separating particles and later applied this principle to the separation of bovine sperm. A simple explanation of this principle is that a conical tube is mounted in a rotor with its apex towards the periphery and its base towards the centre of rotation. Liquid, containing the particles to be separated, is streamed from the periphery towards the centre of rotation:



When centrifugation begins, the centrifugal force is applied to the particles in a direction opposite to the centripetal flow of liquid. For particles of a certain size, the centrifugal and centripetal forces are balanced at a certain distance from the centre of rotation. Smaller particles with lower sedimentation rates reach this point of dynamic equilibrium nearer the centre of rotation, while larger

particles with higher sedimentation rates reach equilibrium at points nearer the periphery. Very small particles, with very low sedimentation rates, are transported out of the tube. In this way, the conical tube acts as a sieve, separating particles according to the size and the limit between two fractions of particles, one retained and the other released from the tube, is determined by the speed of centrifugation, velocity of the liquid stream and the dimensions of the conical tube.

Lindahl's working hypothesis was that X sperm would sediment faster than Y sperm, and would be retained in the counter-streaming centrifuge. Cattle were chosen for the separation experiments, because of the relatively large size difference between X and Y chromosomes in this species. The results of Lindahl's experimental series in 1956 showed that insemination of the retained "heavier" sperm, produced all male progeny although the fertility of the sperm was reduced. However, attempts to confirm this result failed in 1958 (a, b), when the sex ratios of the offspring produced did not differ significantly from those considered normal for cattle. The result of all male progeny from the retained "heavier" sperm was not consistent with Lindahl's hypothesis that the "heavier" sperm would produce female progeny. Lindahl (1971) explained this by pointing out that higher centrifugal forces were used in the 1956 experiments, compared to those carried out later. This would tend to cause retention of more of the lighter sperm, but because an increased liquid velocity was used also in the early experiments, there would have been a tendency to carry "light" sperm from the separation chamber.

Sedimentation separations of bovine sperm have been carried

out extensively by Schilling (1965, 1966, 1971) and by Schilling, Jazbec and Schmid (1967) and consistent results from these experiments have been reported. A skim milk - glycerol - egg yolk diluent was used and insemination of the top and bottom fractions gave a preponderance of males and females respectively, i.e. "lighter" sperm produced male offspring, in contrast to Lindahl's results. Schilling has reported also that sperm from the lower fractions contained 3.7% more DNA, more arginine and other basic amino acids than sperm from the upper fractions, as well as differing in head size, specific gravity and sedimentation velocity. Knaack (1968), as reported by Schilling (1971), produced independently an egg yolk - milk medium for sedimentation of bovine sperm and reported similar results to those of Schilling (1965, 1966) and Schilling et al. (1967).

However, Bhattacharya, Bangham, Cro, Keynes and Rowson (1966) were unable to repeat the success of the earlier rabbit sperm separations, using bovine sperm on the same egg yolk medium, and found no change in the sex ratio of the offspring after insemination of upper and lower fractions. This finding was supported by Lavon et al. (1971), although Krzanowski (1970) found a significant increase in the proportion of female offspring from lower fractions when bovine sperm were allowed to sediment through skim-milk egg yolk media. However, the view was expressed that more rapid sedimentation of X-bearing sperm might not be because of their supposedly greater weight, but possibly because of their tendency to form agglomerations, which sediment much faster than individual sperm. The difference in ability between X and Y sperm to form agglomerations might depend on the surface properties of the sperm.

Since it has become possible to identify the human Y sperm by fluorescent staining, details of several techniques for the separation of human X and Y sperm have been published. In 1973, Ericsson, Langevin and Nishino described a procedure for the isolation of fractions rich in human Y sperm. Washed human sperm, resuspended in Tyrode's solution, were layered on top of bovine serum albumin solutions. After a time, the layers of albumin were harvested and the sperm recovered by washing and centrifugation. They found that the lower albumin layers contained more motile sperm and subsequent quinacrine staining of these sperm showed that a large percentage of them were Y-bearing. No results from insemination of these sperm have been published to date.

Sedimentation separations of human sperm, with fluorescent staining of the Y chromosome as monitor, have been reported also by Quinlivan and Sullivan (1974). Attempted separation of X and Y sperm under gravity was unsuccessful, although when sedimentation was aided by centrifugation, for 10 minutes at 120 g., there was a slightly higher percentage of X sperm (without an F-body) in the deposit than in either the original sample or in the supernatant.

Sedimentation separations of human sperm have been carried out recently by Rohde, Portsmann, Prehn and Dörner (1975). These workers found that, when washed human sperm were centrifuged on discontinuous sucrose gradients, a characteristic four band pattern was produced within the gradient and a precipitate formed at the bottom of the tube. The banding pattern was reproducible with centrifugal forces between 77,500 g. and 12,000 g. for centrifugation times of 30 mins. or 60 mins. After harvesting, the sperm were stained with quinacrine and the Y-bearing sperm

were counted. The results showed that the two uppermost bands in the gradient contained a greater proportion of Y-bearing sperm than either the lower bands, precipitate or control.

(b) Equilibrium Sedimentation

This describes the technique where spermatozoa are centrifuged through a density gradient and come to rest at the "isopycnic point" where the specific gravity of the individual sperm is equal to that of the surrounding medium. Thus, measurement of the specific gravity of individual sperm may be made, in addition to the separation of a mixed population into sub-populations with different specific gravities. The density of a cell reflects its average chemical composition rather than its surface characteristics or size, so that the equilibrium point is not dependent on cell size and shape, but on specific gravity alone.

Benedict, Schumaker and Davies (1967) after testing a variety of gradient media, used the methyl glucamine salt (MGU) of umbradilic acid for the separation of rabbit sperm and bovine sperm. They found that bovine sperm separated in a low-density band and a high-density band. Rabbit sperm behaved in a similar manner on the linear gradients, and the two bands were used for artificial insemination of does. However, no effect on the sex ratio of the offspring was reported. Beatty (1964 and 1969), provided supporting evidence for these findings after insemination of rabbit sperm fractionated into different specific gravity classes on a dextran density gradient.

The results of sedimentation separations are conflicting, with lack of confirmation by different workers and even by the same workers, and it is impossible to reach any general conclusion about

the efficacy of the method. However, the consistent results obtained by Schilling and co-workers, over several years, must be borne in mind and the trend of their results is supported by most other workers.

More recently, Beatty and Fechheimer (1972) have obtained a modest degree of separation of diploid and haploid rabbit sperm, by non-equilibrium sedimentation. Diploid (44-chromosome) rabbit sperm are twice the size of normal (22-chromosome) haploid sperm and can be recognised easily. Because of this great size difference, it was expected that the rate of sedimentation of the two types of sperm would differ and, indeed, the results showed that the lower fraction of the gradient contained a higher percentage of diploid sperm.

1.8 FROTH FLOTATION SEPARATION

Conflicting results from many workers using sedimentation and electrophoresis to separate X and Y sperm led to the hypothesis by More O'Ferrall, Meacham and Foreman (1968) that there might be differences in surface chemistry of the two types of sperm, depending on the sex chromosome carried. Froth flotation, which has been used to separate both minerals and microorganisms on the basis of surface differences, was applied to rabbit semen, with insemination of the separated sperm fractions. Air was allowed to bubble through diluted rabbit semen on a flotation cell. Some sperm attached to the air bubbles and were collected with the froth - this fraction was termed the "float", while the fraction remaining was referred to as the "sink". The sex ratio of the offspring born after

insemination with the float and sink fractions did not differ significantly from normal, although the fact that a separation did occur might suggest that some surface difference does exist between sperm. However, the authors found that the proportions of sperm in float and sink fractions could be varied by altering the pH of the diluted semen, which indicated that the surface difference, if it did exist, was not determined by the sex chromosome carried in the sperm.

1.9 SEPARATION OF HUMAN X AND Y SPERM, USING A GEL FILTRATION TECHNIQUE

Steeno, Adimoelja and Steeno (1975b) have described a gel filtration method for the separation of human X and Y sperm, using Sephadex G-50. When human sperm were applied to a 12 cm. column of the gel, in Locke's fluid, the fractions recovered first from the column contained motile sperm and, when these sperm were stained with quinacrine dihydrochloride, they appeared to be mainly X-bearing, i.e. lacking an F-body. The gel was washed, to free sperm which had been adsorbed, and fluorescent staining of these sperm showed that a high percentage of them contained an F-body, i.e. these sperm were Y-bearing. No explanation has been suggested by the authors for this phenomenon, except that perhaps the electrolyte balance of Locke's fluid might be important. Final proof of the separation must come from insemination trials, but no results are available yet.

1.10 TREATMENT OF SEMEN WITH HORMONES

A number of Russian and Eastern European workers have

reported successful control of the sex ratio following treatment of semen with hormones. For example, Vladimirskaya (1966) reported an increased proportion of male offspring after inseminating boars with androgen-treated semen, while oestrogen treatment yielded an increased proportion of females. No explanation of this has been advanced and substantiating evidence from other workers is lacking, although taken at face value, these reports present a case for easy and successful control of sex ratio. Recently, Beck, Herschel, Hungershöfer and Schwinger (1976) have reported that oestrogens, and to a lesser extent testosterone, accelerate sperm motility, but no specific differential effects on the migration and distribution of human X and Y sperm were observed.

In addition to the experimental approaches described, some authors have reported that environmental and biological factors may exert an influence on the sex ratio of offspring produced. These factors include:

1.11 CHANGES IN ATMOSPHERIC PRESSURE

Various aspects of environment have been modified by researchers to study reproductive phenomena, but little evidence is available regarding the influence of atmospheric pressure on mammalian reproductive activity. Unconfirmed reports suggested that bulls, moved from near sea level to high altitudes in the mountains of South America, tended to sire a higher proportion of male calves soon after being moved and later established a more equal sex ratio. These reports led to investigations by Foote and Quevedo (1971) on the influence of atmospheric pressure on sex ratio. Bull semen was collected at 300 m. above sea level and used to

inseminate a group of cows at 2,750 m. elevation. 68% of the 38 calves born to this group were male. In addition, rabbit semen and bull semen were subjected, in the laboratory, to 15 cm., 30 cm. or 60 cm. vacuum for 10 or 15 minutes before insemination. Of 13 Hereford calves born, 11 were male. For rabbits, the sex ratio of the offspring was not significantly different from normal, although the sex ratio modification was in the same direction as for cattle. From these results, the authors suggested that subjecting semen to a negative pressure of 15 - 30 cm. increased the proportion of males born, but a further decrease in pressure had no effect. Lindahl (1958b) suggested, from his centrifugation experiments, that female-producing sperm might be more sensitive to mechanical stress than the male-producing sperm, and Foote and Quevedo (1971) felt that this might apply in the case of atmospheric pressure changes, as their results had indicated that sperm bearing the X chromosome seemed to be particularly affected by a decrease in atmospheric pressure. They went on to suggest that X-bearing sperm might be more susceptible to "mild" stress and that if treatment became more severe, X and Y sperm would be affected about equally. In their experiments only the air pressure was changed, and it is not known whether variation in physical pressure or in availability of specific gases were the contributing factors.

Bahr (1971b) and Rajammanan (1971) both commented on interesting, although unconfirmed reports that Swedish and British deep sea divers had a preponderance of female children. However, as far as is known, no scientific research has been carried out. Scanlon (1971) also lent support to these comments by quoting from a Canadian underwater club newsletter, which reported a disproportionate number of female children among the offspring of

Scuba divers. However, when Kiddy and Bailey (1972) tested the effects of both increased and decreased atmospheric pressure on rabbit semen, their results did not indicate a significant change in the sex ratio of embryos from either regime.

1.12 MALE BLOOD pH

McWhirter (1960) published a short review on the relationship between male blood pH and sex ratio performance. He established the following sequence of propositions:

(a) Sex ratio performances were subject to significant variations.

(b) A large part of this variation could be traced to individual males.

(c) Circumstances affecting the blood pH of the male were connected with variations in sex ratio performance.

His own observations on 32 bulls, used for artificial insemination in Scotland, were that 5 of these bulls changed their sex ratio performances significantly from year to year, indicating that environmental factors might be involved. He also analysed the earlier data of Moore and Price (1948) on rats subjected to altitude stress. This data reported that after two months at altitudes of 7,500 ft. and 9,600 ft., an increased number of female offspring were produced, while at altitudes of 14,260 ft., a pro-male switch occurred after a delay of one month. At a control altitude, the sex ratio was 50%. In addition, McWhirter (1960) quoted the work of Heath (1954) who reported that athletic types of men tended to produce an excess

of daughters. Criteria for athleticism included pulse rate, speed of recovery from exertion and college records. These scientifically assessed data coincided with one of the oldest of the myths of human sex ratio, that the sex of the offspring was determined by the relative physical or mental attributes of the parents, the sex of the offspring being opposite to that of the superior parent.

McWhirter (1960) advocated that both these reports might be explained by the pH theory advanced by Weir (1955). This theory, induced from experiments with two inbred strains of mice, claimed that sires with a low blood pH tended to produce an excess of females. With regard to the results of Moore and Price (1948), altitude is known to produce variations in blood pH, but they conducted no experiments to relate the variations in sex ratio to either the male or female parent. With regard to Heath's data, McWhirter (1960) pointed out that athletic exertion could cause a transient acidosis and hypothesised that continued athleticism might induce a slight permanent acidosis. The question of whether acidosis or athleticism comes first remains unanswered.

Weir (1971) reported, in some detail, the results of his investigations on the sex ratio of mice as a response to the blood pH of the male. Over a number of years, his experiments with two inbred strains of mice showed that sires of the pHH strain (with high blood pH) produced more male offspring, while sires of the pHL strain (with low blood pH) produced more female offspring. Since his experiments showed that the blood pH of the male parent influenced the sex ratio of the offspring, he has endeavoured to relate this effect to an influence of the Y chromosome. He also found that feeding of acidic or basic diets to male mice could cause a change in serum pH, but these induced changes did not produce a

change in the sex ratio of the offspring. In view of these negative results, it is unlikely that control of blood pH would be a feasible means of sex control, although if blood pH values could be correlated with sex ratio performance of males of domestic species, a useful partial control of sex ratio might be achieved.

A possible explanation for the male blood pH-sex ratio relationship was advanced by McWhirter (1960) who thought that haploid selection of sperms might take place during maturation in the male reproductive tract. He favoured this explanation in preference to the other more dramatic possibility that meiosis might be disturbed by minute alterations of blood pH, with unequal numbers of X- and Y-bearing sperm being produced.

1.13 TIMING OF INSEMINATION

Sporadic interest has been shown in the possibility that the sex of human offspring might be related to the day of the menstrual cycle on which insemination occurred. It has been demonstrated in some lower animals that delayed fertilisation may lead to a disturbance of the sex ratio, e.g. Crew (1927) cited data suggesting that late insemination was related to a large excess of male offspring in the frog. In the case of humans, a number of early reviews have been published, e.g. Asdell (1927), but often the validity of the data was questioned. Siegel (1916), among others, produced data suggesting that insemination early in the cycle was more likely to result in conception of males. However, this early German data fell into disrepute, mainly following suggestions that the sexes differed in the time interval from insemination to confinement. James (1971) has

examined this literature extensively.

Experiments with laboratory animals by Hart and Moody (1949) and Hammond (1934) and limited early work with cattle by Pearl and Parshley (1913) from breeding early and late in oestrus, gave some indication that late breeding produced more males, but these results were not always consistent from one set of experiments to the next, or even within individual reports. Some of this variation may have been owing to the small numbers of observations in some investigations.

Van Demark and Malven (1960) pointed out that, if insemination late in oestrus in cattle did affect sex ratio, this effect would be expected to be revealed in artificial insemination records, since the average time of breeding artificially is later than natural breeding. Gardner (1950) found practically the same percentage (51.4%) males in 1,783 calves from artificial insemination as Johansson (1932) found in 125,000 calves from natural service (51.5%). Foote and Hall (1954) also failed to find an increase in the percentage of male calves from nearly 50,000 artificial inseminations. Thus, there would seem to be no substantial data to indicate the production of more male calves as a result of time of breeding in routine artificial insemination.

The effect of the timing of insemination or coitus on the sex ratio of the offspring may be related to the pH changes which occur in the fluids of the female reproductive tract throughout the cycle. Lamar, Shettles and Delfs (1940) postulated that the penetration of cervical mucus by sperm was facilitated by high pH, while at other times in the cycle, when pH was low, sperm penetration was poor. They claimed that the use of acidic or alkaline douches could influence the penetrability of cervical mucus by X or Y sperm. However,

many attempts to repeat these controversial acid/alkali sex control experiments produced negative results.

Subsequently, Shettles (1970) reported that X and Y sperm could be selected for on the basis of differential migration rates in acidic and basic media. Because the pH of human cervical mucus changed during the menstrual cycle, he suggested that specific timing of coitus might be used to select for either sex prior to conception. His hypothesis was that X-bearing sperm migrated further than Y-bearing sperm in an acidic medium and might therefore be more resistant than the Y sperm to the deleterious effects of low pH and that although alkaline pH was hospitable to both X- and Y-bearing sperm, the smaller Y sperm migrated faster and would reach the ovum first.

Kleegman (1954) offered clinical support to Shettles's theory, through artificial insemination studies, while Moghissi (1966) showed that human cervical mucus was alkaline around mid-cycle and that pH was highest (between 7.5 and 8.0) around ovulation. Inseminations performed at the time of ovulation produced a preponderance of male offspring, while inseminations 2 days before ovulation, when pH was lower, produced a high percentage of females. However, Cohen (1967), also using artificial insemination data, could not confirm Kleegman's findings.

When the identification of the human Y-bearing sperm became possible, by fluorescent staining, Diasio and Glass (1971) re-examined the influence of pH on the migration of human X- and Y-bearing sperm. Human sperm were allowed to migrate into capillary tubes containing media of varying pH. They found, contrary to Shettles, that the percentage of Y-bearing sperm migrating into the capillary tubes was not influenced by the pH of the media.

More recently, Rohde, Porstmann and Dörner (1973), using cervical mucus obtained from women shortly before mid cycle, observed a highly significant increase in numbers of Y-bearing sperm, identified by quinacrine staining, in the frontal zone of sperm migrating through the mucus. The absolute number of sperm in this frontal zone was very small - only an average of 22 cells, so this method would appear to be unsuitable for sex control. However, these findings were supported by Kaiser, Broer, Citoler and Leister (1974) who found that with preovulatory cervical mucus, there was a statistically significant increase of Y-bearing sperm, up to a distance of 3 cms. of mucus, after incubation under physiological conditions. From their results, they suggested that, under artificial conditions at least, some selection did take place in the human cervical mucus.

1.14 AGE OF SEMEN

It has been claimed that X- and Y-bearing sperm show different mortality rates and that delayed fertilisation can result in a change in the sex ratio because of this. Blandau and Young (1939) showed that the ageing of the sperm and eggs of laboratory animals resulted in a higher embryonic death rate, especially when the ageing occurred at body temperature, while Salisbury, Bratton and Foote (1952) showed an apparent slight increase in the embryonic death rate in cattle when semen was stored prior to breeding.

A preliminary study by Baier (1957) analysed the sexes of calves from artificial insemination in Germany and found a significant increase in the percentage of females after storage of the semen.

The work continued and when data from 1,600 births was analysed, this trend was reversed and Baier and Haeger (1958) reported no deviation in sex ratio from semen stored for up to 4 days.

Van Demark and Malven (1960) made a study in Illinois of the sex of calves produced from artificial insemination following the storage of diluted semen at room temperature for up to 1 week. Statistical analysis of 525 single births and 10 sets of twins showed no significant change in the sex ratio with the ageing of semen, although the overall sex ratio of 48 males per 100 calves was slightly lower than the expected normal number of males.

More recently, Mathai and Namboodiripad (1971) recorded the sexes of calves born after artificial insemination with either Jersey or Red Sindhi semen, 0-20, 21-40 or 41-60 hours after semen collection. They found that the percentage of males (51.8%) from the 21-40 hour stored semen was significantly greater than for the other groups (43.2% and 47.2% respectively). However, they did not record any analysis comparing this figure to the sex ratio for either natural service or usual artificial insemination practice.

From the foregoing review of the literature, it may be concluded that confusion exists in the field of primary sex control. Some techniques, such as electrophoresis and froth flotation, have been used empirically, because they are capable of splitting an ejaculate into two or more fractions, not because they exert an influence on any known properties of X- or Y-bearing sperm. Often, the conditions of experimentation were poorly defined - perhaps part of the reason why subsequent attempts to repeat previously successful experiments have failed. However, this empirical approach must not be dismissed carelessly without considering the problem confronting the researcher - does identification or separation of X and Y

sperm come first? Until identified, the separation of X and Y sperm could not be approached logically; until separated, they could not be identified. At least, this was the situation until fairly recently.

In the early 1970's, one technique of great importance emerged - the means of identifying the human Y chromosome within the sperm head, by quinacrine staining (Barlow and Vosa, 1970). This technique reduced greatly the task of monitoring sperm separations, allowing estimates of the numbers of Y sperm in separated fractions to be made directly, by counting. Unfortunately, attempts to apply a similar technique to the sperm of other species have not been successful, although recent evidence from Bhattacharya (1976) has claimed that the quinacrine staining technique may be modified and applied to the Y-bearing sperm of cattle. Preliminary results have been published, but until full details of the technique are made known, further comment is not possible.

1.15 OUTLINE OF THE STUDY

In this thesis, consideration will be given to the possibility of identifying the bovine Y chromosome in mitotic preparations, using a variety of fluorescent dyes. Established methods of separating sperm will be applied, in parallel, to human and to bovine semen, enabling the recognised quinacrine staining technique for the identification of the human Y sperm to be used to monitor the separations.

CHAPTER TWO

THE FLUORESCENT STAINING OF CHROMOSOMES AND SPERMATOZOA

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THE FLUORESCENT STAINING OF CHROMOSOMES AND SPERMATOZOA

2.1 INTRODUCTION

Zech (1969) showed that the distal ends of the long arms of the human Y chromosome fluoresced more brilliantly than any of the other chromosomes, when stained with quinacrine mustard and examined in ultra violet light. Vosa (1970) reported that a similar staining of the human Y chromosome was produced with quinacrine dihydrochloride (Atebrin). The field was extended when Barlow and Vosa (1970) showed that, when stained with quinacrine dyes, the human Y-bearing sperm could be identified by the presence of a fluorescent spot or F-body within the sperm head.

Attempts to apply this quinacrine staining method to the Y chromosomes of 26 other species, including the domestic cow, pig, horse and cat, have not proved successful, (Pearson, Bobrow, Vosa and Barlow, 1971), although Hansen (1972) has claimed that the long arms of the bovine Y chromosome fluoresce brightly when stained with quinacrine mustard.

It was decided to examine the staining of human and bovine chromosomes with the quinacrine dyes and with other acridine derivatives, to see if intense fluorescence of the Y

chromosome of either species was produced. In addition, the staining of human spermatozoa with quinacrine dihydrochloride, for identification of the F-body, was examined.

2.2 MATERIALS AND METHODS

2.2.1 Human Chromosome Preparations

Peripheral blood samples were taken aseptically from healthy adult males. 0.5 ml whole blood was added to 4.5 ml Ham's F10 culture medium, containing glutamine, (Gibco-Biocult Ltd., Paisley, Scotland) and penicillin and streptomycin and 0.1 ml phytohaemagglutinin (Reagent grade, Wellcome Research Laboratories, Beckenham, Kent, were added. The culture was incubated at 37°C for 72 hours, then 0.2 ml colcemid, of concentration 80 µg/ml (Ciba Laboratories Ltd., Horsham, Sussex) was added, and the incubation continued for a further 2 hours. After this time, the cells were harvested by centrifugation and the supernatant removed. The cell pellet was resuspended in hypotonic potassium chloride solution (0.075M) and incubated at 37°C for $4\frac{1}{2}$ minutes. The cells were recovered by centrifugation and, after discarding the supernatant, were resuspended in 5-10 ml of cold, freshly prepared fixative (3:1, methanol:acetic acid) and kept at 4°C for 30 minutes. The cells were recovered by centrifugation, resuspended in fresh fixative and centrifuged again. Several drops of fresh fixative, (enough to make a milky suspension), were added to the cell deposit and smears were prepared on clean, wet slides and dried rapidly on a hot plate.

2.2.2 Bovine Chromosome Preparations

Blood was drawn, aseptically, from the jugular veins of mature bulls, into irradiated, sterile, heparinised tubes. 1-2 ml whole blood were added to 10 ml Weymouth's MB 752/1 culture

medium (Flow Laboratories, Irvine, Scotland), with added foetal calf serum, penicillin, streptomycin, glutamine and 5 ml phyto-haemagglutinin and the culture was incubated at 37°C for 46-48 hours. After this time, 0.10-0.15 ml 1% (w/v) colchicine was added and the culture returned to incubate for a further 90 minutes. The cells were harvested by centrifugation, the supernatant removed, and the cell deposit resuspended in fixative (3:1, methanol:acetic acid) at 4°C for 15 minutes. Then, the cells were recovered by centrifugation and resuspended in fresh fixative. After a further 30 minutes at 4°C, the cells were harvested by centrifugation and resuspended in 1 ml fixative. Smears were prepared on clean wet slides and dried rapidly on a hot plate.

2.2.3 Collection of Human Semen

Initially, human semen, collected by masturbation, was obtained from a hospital clinic, but subsequently, an arrangement was made for semen to be donated anonymously by 13 adult men.

On arrival at the laboratory, all semen samples were examined on a heated stage, under a light microscope, and assessed for percentage motility and degree of progressive motility, according to the method of Ulstein (1972).

2.2.4 Preparation of Human Sperm for Fluorescent Staining

Human semen was diluted with approximately twice its volume of Krebs-Hensleit-Ringer solution, prepared according to Mann (1964) and centrifuged at 4°C for 10 minutes at 110 g. The supernatant was removed and the cell deposit resuspended in fresh diluent and centrifuged again. This procedure was repeated until

three washes had been completed, then the supernatant was discarded and the cell deposit resuspended in a few drops of fixative (3:1, methanol : acetic acid), to produce a milky suspension. This suspension was allowed to stand for 20 - 30 minutes, to complete fixation, then smears were prepared, by dropping the suspension on to clean wet slides, held at an angle of 45°, to give a thin, even smear. The smears were dried rapidly on a hot plate.

2.2.5 Fluorescent Staining

All dye solutions were prepared immediately before use.

1. Quinacrine Mustard (Sigma Chemical Co. Ltd., St. Louis, USA)

0.25 gm quinacrine mustard dihydrochloride was dissolved in 100 ml distilled water. Human and bovine chromosome preparations were stained in this solution for 10 - 15 minutes, then washed briefly in running tap water and in distilled water. After drying rapidly on a hot plate, the preparations were mounted in distilled water and the coverslips sealed with rubber solution.

2. Quinacrine Dihydrochloride ("Atebrin", Gurr, Searle Diagnostic, High Wycombe, Bucks.)

A 0.5% aqueous solution of quinacrine dihydrochloride was prepared. Chromosome preparations were stained for 5 - 6 minutes, followed by brief rinses in running tap water and in distilled water. After rapid drying, the preparations were mounted in distilled water and the coverslips sealed with rubber solution.

Human sperm preparations were stained in the solution for either 2, 5, 8, 11, 14, 17 or 20 minutes, followed by a brief rinse in running tap water and a brief rinse in distilled water. After rapid drying, the smears were mounted in distilled water, and the coverslips sealed, as before.

3. Acridine Orange (Gurr, Searle Diagnostic)

0.1% (w/v) and 0.5% w/v) solutions were prepared, in distilled water. Chromosome preparations were stained for 5 - 6 minutes, then rinsed briefly in running tap water and in distilled water. After rapid drying, they were mounted in distilled water and the coverslips sealed, as before.

4. Euchrysine 2GNX (Gurr, Searle Diagnostic)

0.1% (w/v) and 0.5% (w/v) solutions were prepared, in distilled water. The chromosome preparations were stained for 5 - 6 minutes, then rinsed briefly in running tap water and in distilled water. After rapid drying, the preparations were mounted in distilled water and the coverslips sealed, as before.

2.2.6 Conditions for Microscopic Examination

A Leitz Laborlux microscope, fitted with an HBO 200W/4 mercury vapour lamp, BG12 and BG38 exciter filters, and K530 suppression filter, was used. Chromosome preparations and human sperm smears were examined under oil, at x 1,000 magnification, using a dark field condenser.

2.3 RESULTS

Human mitotic chromosome preparations stained similarly with both quinacrine mustard dihydrochloride and quinacrine dihydrochloride. All the chromosomes fluoresced greenish-yellow in ultraviolet light but the distal ends of the long arms of the Y chromosomes fluoresced more brightly than any other area of the chromosomes. Plate 2.1 shows a human mitotic chromosome preparation stained with 0.5% quinacrine dihydrochloride. Note the bright fluorescence of the distal ends of the long arms of the Y chromosome.

Bovine mitotic chromosome preparations stained with both the quinacrine dyes fluoresced faintly, to a much lesser degree than human chromosomes and there was no outstanding brilliance of the bovine Y chromosome.

When human mitotic chromosome preparations were stained with both 0.1% and 0.5% acridine orange, they fluoresced bright orange in ultraviolet light but began to fade quite rapidly. No particular brilliance of the Y chromosome was seen, as shown in Plate 2.2. Bovine mitotic chromosome preparations also fluoresced orange when stained with acridine orange solutions but the fluorescence was much fainter than that shown with human chromosome preparations and there was no outstanding brilliance of the Y chromosome.

When human mitotic chromosome preparations were stained with either 0.1% or 0.5% euchrysine 2 GNX and examined in ultraviolet light, they fluoresced greenish-yellow but began to fade very rapidly. There was no particular brilliance of the Y chromosome, as may be seen in Plate 2.3. Bovine mitotic chromosome

Plate 2.1 Human Chromosomes stained with 0.5% Quinacrine Dihydrochloride (Atebrin)

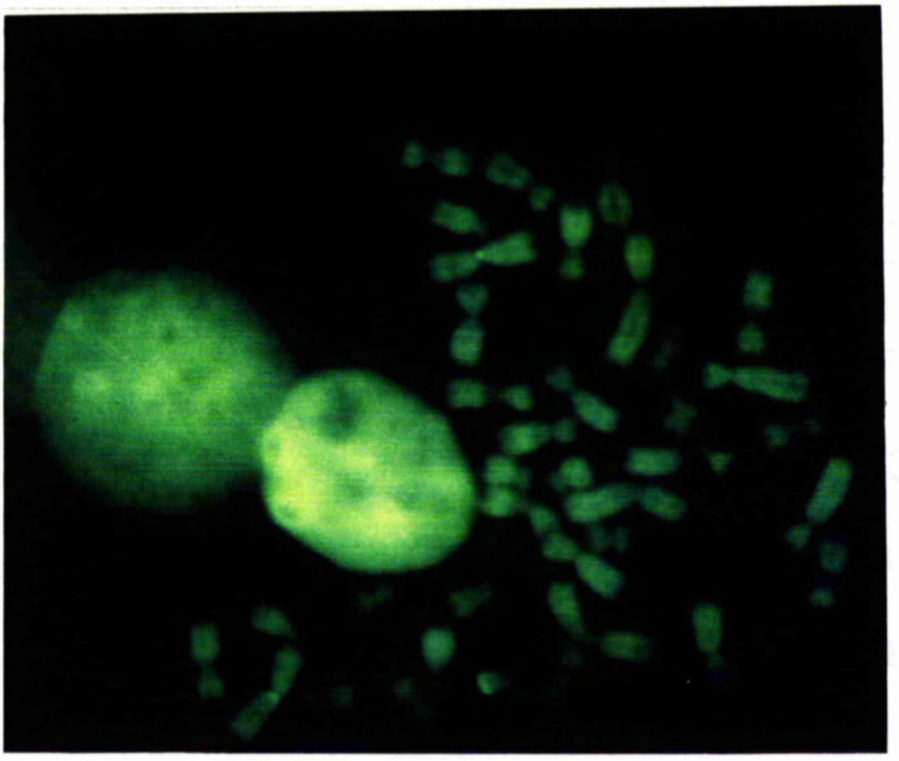
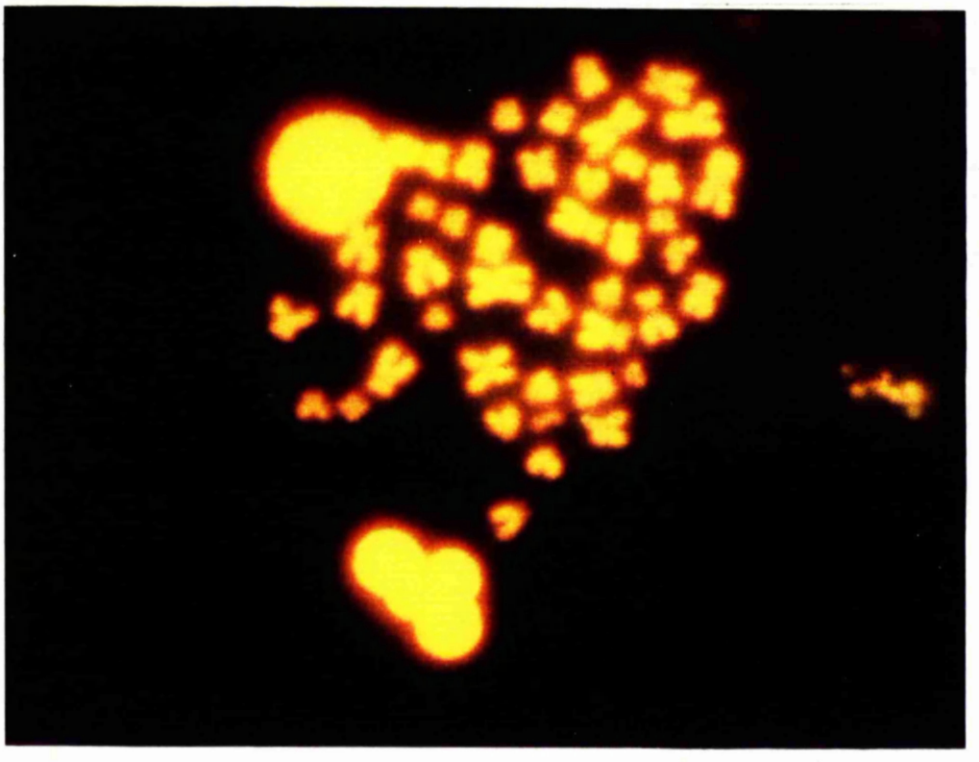


Plate 2.2 Human Chromosomes stained with 0.5% Acridine Orange



preparations, stained also with the euchrysine solutions, fluoresced faintly yellow-green in ultraviolet light and again fading occurred rapidly. No particular brilliance of the bovine Y chromosome was observed.

Both quinacrine mustard and quinacrine dihydrochloride stain the human Y chromosome in the same manner, (Barlow and Vosa, 1970) and for subsequent examination of the human Y-bearing sperm, the more readily available quinacrine dihydrochloride was used. Plate 2.4 shows a human sperm stained with quinacrine dihydrochloride to identify the F-body within the sperm head.

To investigate the relationship between length of staining time and the numbers of Y-bearing sperm identified, 15 smears of washed fixed human sperm were prepared from a single ejaculate. Two smears were stained with quinacrine dihydrochloride for each of the following times: 2, 5, 11, 14, 17 and 20 minutes, with three smears being stained for 8 minutes. The smears were examined "blind" in random order, (except for two of the smears stained for 8 minutes, which were examined consecutively), under the same microscopic conditions described previously. The results of this staining trial are shown in Table 2.1; there was no significant difference in the numbers of Y-bearing sperm scored for 2 - 17 minutes staining, but the numbers of Y sperm scored for 20 minutes staining were significantly lower, ($p = \lessdot 0.05$). To examine the repeatability of the staining technique, duplicate counts were compared. No significant difference was found between these, ($p = \lessdot 0.05$), except when a staining time of 14 minutes was used, and in this case, significantly different counts were obtained.

Plate 2.3 Human Chromosomes stained with 0.5% Euchrysine 2GNX

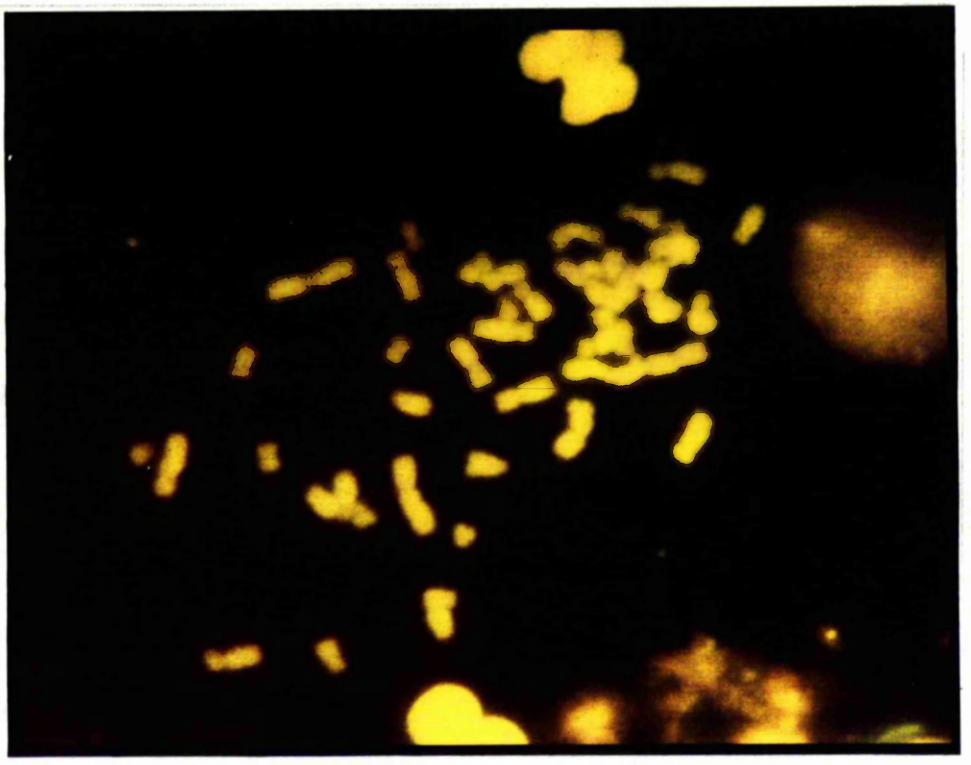


Plate 2.4 Human Sperm stained with 0.5% Quinacrine Dihydrochloride (Atebrin)

TABLE 2.1 Results of Quinacrine Staining of Human Spermatozoa using different lengths of staining time

Length of Staining Time (mins)	Total Sperm Counted	Number of Y Sperm Counted
2 2	300) 302) 602	81) 73) 154
5 5	302) 300) 602	76) 76) 152
8 8 8	300) 312) 915 303)	87) 71) 229 71)
11 11	300) 303) 603	80) 66) 146
14 14	300) 300) 600	85) 58) 143
17 17	300) 303) 603	81) 63) 144
20 20	300) 303) 603	57) 52) 109

2.4 DISCUSSION

Human semen was obtained initially from a hospital clinic but there were two disadvantages: firstly, many of the samples showed poor sperm density and motility and secondly, the time between ejaculation and arrival of the sample at the laboratory was very variable. Therefore an arrangement was made for volunteers to donate semen anonymously for use within 3 - 4 hours of ejaculation.

The fluorescent dyes used in these experiments to stain the mitotic chromosome preparations were all acridine derivatives. When human chromosomes were stained with the quinacrine compounds, there was intense fluorescence of the distal ends of the long arms of the Y chromosome, as described previously by Zech (1969) and by Vosa (1970). Staining of bovine chromosomes with the quinacrine compounds produced a faint fluorescence of the autosomes and no specific fluorescence of the Y chromosome, similar to the findings of Pearson et al. (1971) but contrary to those of Hansen (1972). No intense fluorescence of either the human Y chromosome or the bovine Y chromosome was produced by staining with either acridine orange or euchrysine 2GNX solutions.

The quinacrine compounds are thought to stain chromosomes by binding to the bases on the DNA but the exact mechanism of staining has not been determined. Caspersson and Zech (1972) suggested that quinacrine mustard binds to the guanine base of DNA and that the distal ends of the long arms of the human Y chromosome might contain repetitious DNA sequences, but this view was not supported by Evans (1972). He pointed out that there was no

reason why quinacrine dihydrochloride, which gave the same fluorescent specificity as the mustard derivative, should react with guanine and suggested that the fluorescence might be a property of the physical state of DNA rather than its chemical composition. Pearson et al. (1971) showed also that the long arms of the Y chromosome of the lowland gorilla (Gorilla gorilla) fluoresced intensely when stained with quinacrine compounds. However, for reasons as yet undetermined, it would appear that the bovine Y chromosome does not show a particular affinity for the quinacrine compounds. It would appear also that neither of the other two acridine derivatives used in the present experiments stained the human Y chromosome in a similar manner to the quinacrine dyes.

It was reported first by Barlow and Vosa, (1970), that when human sperm were stained with quinacrine dyes, the Y-bearing sperm could be identified by the presence of an F-body within the sperm head. They reported that, in 60% of sperm, the F-body lay at the boundary between dense and less dense regions of chromatin, while in the other sperm, the F-body lay with equal frequency in either the dense or less dense regions of the sperm head (see Plate 2.4). On examination of the literature on the staining of human sperm with 0.5% aqueous quinacrine dihydrochloride, to identify the F-body, a very wide variety of staining times was noticed. For example, Barlow and Vosa (1970) stained human sperm for 20 minutes, Sumner Robinson and Evans, (1971), for 8 minutes, and Pawlowitzki and Bosse, (1971), for 5 minutes. From this wide variation, it appeared that the staining time was not critical but in order to determine if there was an optimal length of time of staining, a trial was carried out to investigate the relationship

between length of staining time and the numbers of Y sperm identified.

As may be seen from Table 2.1, there appeared to be no optimal staining time, although it was concluded that when staining time exceeded 20 minutes, there was a decrease in the numbers of Y sperm identified, possibly because of the interference of background fluorescence. Pearson (1973) also reported that time of staining and concentration of the stain were not critical. From Table 2.1, it may be seen also that consistently low percentages of Y sperm, (approximately 25%), were identified. This may be attributed partly to lack of experience in the identification of Y-bearing sperm. However, subsequent comparison of the counts of Y-bearing sperm in five smears of human semen was made with an experienced independent observer and an average 4% difference between the counts was noted (lower counts being reported from this laboratory).

CHAPTER THREE

THE COUNTING OF SPERMATOZOA

CHAPTER THREE

THE COUNTING OF SPERMATOZOA

3.1 INTRODUCTION

One of the factors used in assessing the quality of a semen sample is the sperm concentration. Estimates of sperm concentration are usually made, using a haemocytometer, from the numbers of sperm counted in a known volume of diluted semen. This method was used for estimating the sperm concentration in human semen samples, but the process was found to be slow, tedious, and liable to error. Bane (1952) reported that this error may be up to 10% of the true number of sperm.

It was expected that large numbers of bovine sperm counts would be carried out during this research, and consequently the use of an electronic counter was investigated. The main advantage of the electronic counter (model FN, Coulter Electronics Ltd.), is its ability to count very large particles automatically, in a very short time. Counts are made by drawing particles in suspension through a circular aperture. As each particle passes through, a volume of electrolyte proportional to the particle volume is displaced, producing a drop in voltage in the circuit and generating a count pulse. Thus, the number of particles in a known volume of suspension passing through the aperture may be determined. The threshold setting of the counter may be adjusted so that particles

below a chosen size are excluded from the count. The shape of the particle is irrelevant, so that even biological cells of asymmetrical shape may be counted and a Coulter counter has been used successfully for counting the spermatozoa of rabbits, [Fowler and Hellman (1965), Kihlström and Fjellström (1967)], guinea pigs [Laurence and Carpuk (1963)], bulls [Glover and Phipps (1962), Iversen (1964 and 1965), O'Donnell (1969)], and humans [Segal and Laurence (1962), Gordon, Moore, Thorslund and Paulsen (1965), Brotherton and Barnard (1974)].

3.2 THEORY

For the Coulter counter, the general expression for calibration is:

$$V = K_v.t \times F \times B \quad - \quad \text{Equation (1)}$$

where V = volume of the sphere equivalent to the volume of the particle counted, in cubic microns.

K_v = Calibration factor, in terms of volume, for the counter.

t = threshold setting

B = attenuation setting

F = scale expansion factor, determined from known values of D, the aperture current setting and R, the aperture resistance in Kilohms. This factor may be obtained from Table 7, appendix of instructions.

From Equation (1)

$$K_v = \frac{V}{t \times F \times B}$$

Also,

$$V = \frac{\pi}{6} d^3$$

where d = the diameter of the sphere of equivalent volume of the particle counted in microns.

Therefore, the calibration constant, Kd, in terms of diameter is now expressed as:

$$Kd = \frac{d}{\sqrt[3]{t \times F \times B}} \quad - \quad \text{Equation (2)}$$

The above Equation was used for subsequent calibration of the counter.

3.3 ABSOLUTE CALIBRATION OF THE COUNTER

A Coulter counter (model FN), fitted with a 50 μm orifice tube and set to count 0.1 ml suspension, was used. The suspending medium for particles was a filtered Eagle's medium (Isoton, Coulter Electronics Ltd.), and standard fungal spores and calibration latex spheres (Coulter), of known diameter, were used. The latex spheres were supplied in a self-dispersing suspension and one drop of suspension was added to 100 ml Isoton. Fungal spores were difficult to disperse evenly and a suspension was prepared by adding 1 - 2 mgs of these to 100 ml Isoton, containing 2 drops of Triton X30 wetting agent. All particles chosen for calibration were within 2% - 40% of the aperture diameter. All particle suspensions were prepared immediately before use.

The half-count calibration method was carried out with each suspension of particles, as described by Brotherton (1969a), and the threshold setting for each particle was determined. In each instance, the value of Kd was calculated from Equation (2), and these results are shown in Table 3.1. From these results the average value of Kd = 3.1802.

Brotherton (1969a) suggested a more accurate graphical method of solving Equation (2), by plotting $\sqrt[3]{\frac{d}{t}}$ against $\sqrt[3]{F \times B}$, Kd being given by the slope of the line. Using the values given in Table 3.1, the regression coefficient (Kd) was found to be 3.188 and this value was used in all subsequent calculations.

Table 3.1 The t Values and Kd Values for the Monosized Particle Suspensions

Particle Diameter d, in um	Threshold Value, t	Attenuation Setting, B	Aperture Current F Factor	Kd
2.03	31.8	0.500	0.0167	3.158
4.84	20.0	0.707	0.254	3.160
4.97	22.0	0.707	0.254	3.145
8.06	30.0	2	0.254	3.251
12.50	30.0	4	0.503	3.187

Average value of Kd = 3.1802

3.4 PREPARATION OF BOVINE SEMEN FOR COUNTING

All semen samples were used within eight hours of collection. A 1 : 10,000 dilution of semen with Isoton was made in two stages : 0.2 ml semen, measured with an opsonic pipette, was added to 20 ml Isoton and mixed well, then 0.2 ml of this suspension was added to a further 20 ml Isoton. Two drops of Zapoglobin (Coulter) were added to this final dilution, to lyse seminal debris, and the suspension was mixed well and allowed to stand for three minutes before counting .

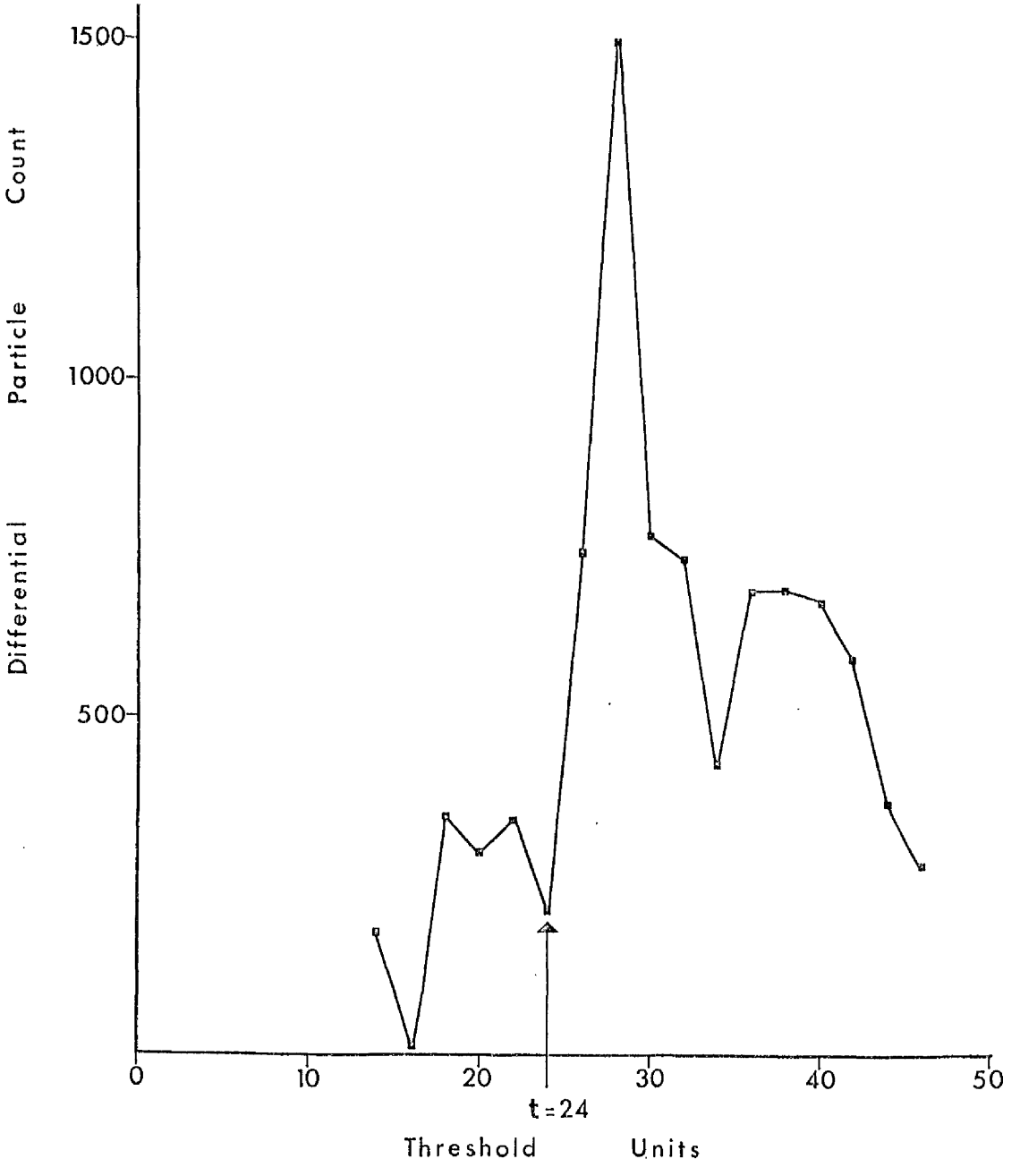
3.5 SIZE DISTRIBUTION OF BOVINE SPERMATOZOA

By carrying out a succession of counts, on equal volumes of suspension, at different threshold settings, a cumulative size distribution curve was obtained for the semen sample. Fig. 3.1 shows a typical size distribution curve and it may be noted that:

1. The size distribution is wider than would be expected from microscopic examination .
2. The curve is skewed.
3. There is a small peak immediately before the largest peak on the graph.

Size distribution curves were plotted for sperm from 16 ejaculates, from four bulls. All the curves corresponded to the general pattern described by Glover and Phipps (1962) and by O'Donnell (1969). O'Donnell (1969) has claimed that the smaller first peak of the size distribution curve (see Fig. 3.1) represents free cytoplasmic droplets present in the suspension and argued that a setting for minimal size of sperm should be taken beyond this point .

Fig. 3.1 A typical size distribution curve for bovine sperm obtained by using a Coulter counter.


Differential Particle Count
1500
1000
500
0
10
20
30
40
50
t=24
Threshold Units

Following O'Donnell's theory, from the size distribution curves of 4 bulls, a threshold setting of 24, with values of B = .707 and F = 0.0327, was chosen for subsequent counts. Using these settings sperm counts were carried out on 19 ejaculates and compared with corresponding results from haemocytometer counts, (Table 3.2). The correlation coefficient was calculated and found to be 0.97. Fig. 3.2 shows the relationship between electronic counts and haemocytometer counts for these ejaculates.

From the size distribution curves, minimum, modal and maximum threshold values were obtained and using Equation (2), the minimal, modal and maximal diameters of bovine sperm were calculated. The results from eight ejaculates are shown in Table 3.3.

From these results the average diameter range for bovine sperm was found to be 2.6 - 3.3μ, with an average modal value of 2.73μ.

3.6 ARBITRARY SETTINGS FOR THE ELECTRONIC COUNTER

For comparison, an empirical approach to obtaining settings for bovine counts was made. For one ejaculate, duplicate haemocytometer counts were carried out and the average value calculated. Then the settings of the electronic counter were adjusted until the count approximated to that obtained using the haemocytometer, and these settings were noted.

Using the settings t = 32, B = .500 and F = 0.0167, a total of twenty-two ejaculates were counted (Table 3.2). Fig. 3.3 shows the relationship between electronic counts and haemocytometer

Table 3.2 Results of Haemocytometer Counts and Electronic Counts on Bovine Ejaculates

BULL	Haemocytometer Count (x 10^6/ml) Average of two Counts	Coulter Count (x 10^6/ml) Average of four Counts Settings: B = 0.707 D = 16 (F = 0.0327) t = 24	Coulter Count (x 10^6/ml) Average of four Counts Settings: B = 0.500 D = 8 (F = 0.0167) t = 32
Number 1	1,318.75	1,320.9	1,313.6
" 1	1,208.75	1,106.5	1,236.8
" 1	653.1	609.8	677.9
" 1	875.63	874.57	933.9
" 1	717.5	692.6	778.5
" 1	1,525.0	1,365.9	-
" 1	1,218.75	-	1,280.35
" 1	1,927.5	-	1,988.15
" 1	886.88	-	1,037.05
" 2	2,173.13	2,054.1	2,610.4
" 3	653.75	634.0	682.2
" 4	813.75	882.9	-
" 5	1,980.0	1,772.0	-
" 6	866.25	847.1	-
" 6	891.25	-	1,258.0
" 6	1,055.0	-	1,142.0
" 7	1,290.0	-	1,385.56
" 7	416.88	-	477.05
" 8	1,207.5	1,200.2	1,188.7
" 8	857.5	851.4	868.65
" 9	1,835.0	1,658.4	1,688.8
" 10	1,785.0	1,182.7	1,639.8
" 11	2,683.13	2,405.2	2,525.6
" 12	1,451.7	1,381.3	1,426.7
" 13	1,589.38	1,469.4	1,552.15
" 13	745.0	643.4	702.57

Fig. 3.2 The relationship between haemocytometer and electronic counts of bovine sperm when t = 24.

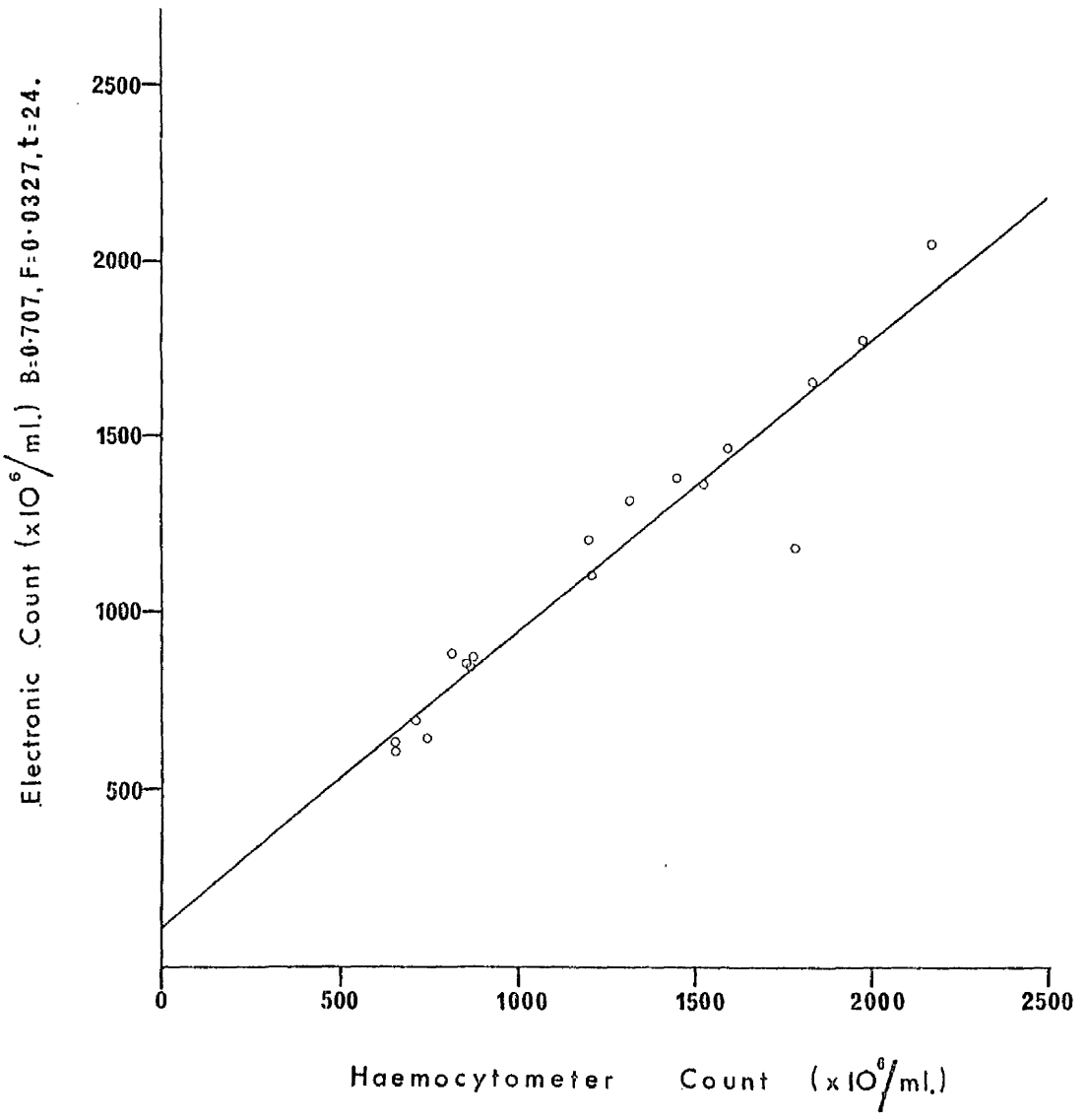

Electronic Count (x10⁶/ml.) B=0·707, F=0·0327, t=24.
2500
2000
1500
1000
500
0
500
1000
1500
2000
2500
Haemocytometer Count (x10⁶/ml.)

Table 3.3 The Diameter Range of Bovine Sperm, obtained from Size Distribution Curves

Bull	Ejaculate	Minimum Diameter um	Modal Diameter um	Maximum Diameter um
Number 1	1	2.62	2.76	3.25
	2	2.50	2.62	3.50
Number 2	1	2.62	2.76	3.11
	2	2.62	2.80	3.60
Number 5	1	2.69	2.76	3.21
	2	2.55	2.76	3.25
Number 6	1	2.62	2.69	3.16
	2	2.62	2.76	3.16

counts for these ejaculates. The correlation coefficient was calculated and found to be 0.96.

3.7 THE EFFECT OF THE ADDITION OF ZAPOGLOBIN TO THE FINAL SPERM SUSPENSION

The size distribution curve obtained for one ejaculate, after the addition of Zapoglobin to the final sperm suspension, was compared to the size distribution curve obtained for the same ejaculate when Zapoglobin was omitted from the final dilution. These curves are shown in Fig. 3.4, where it may be noted that when Zapoglobin was not added to the final sperm suspension, the size distribution curve obtained was more irregular, with a greater number of small particles present.

Counts were carried out on these two sperm suspensions and these results are shown in Table 3.4, where it may be seen that the count obtained when Zapoglobin was added to the final sperm suspension was within 5% of the haemocytometer count.

Smears were prepared from these two sperm suspensions and were stained with nigrosin/eosin, according to the method of Campbell, Hancock and Rothschild, (1953).

The smears were examined under oil immersion at x 1,000 magnification. Sperm from both smears were found to be markedly eosinophilic, with head and tail attached and structure apparently normal, but less cellular debris was present in the smear where Zapoglobin had been added.

Fig. 3.3 The relationship between haemocytometer and electronic counts of bovine sperm when t = 32.

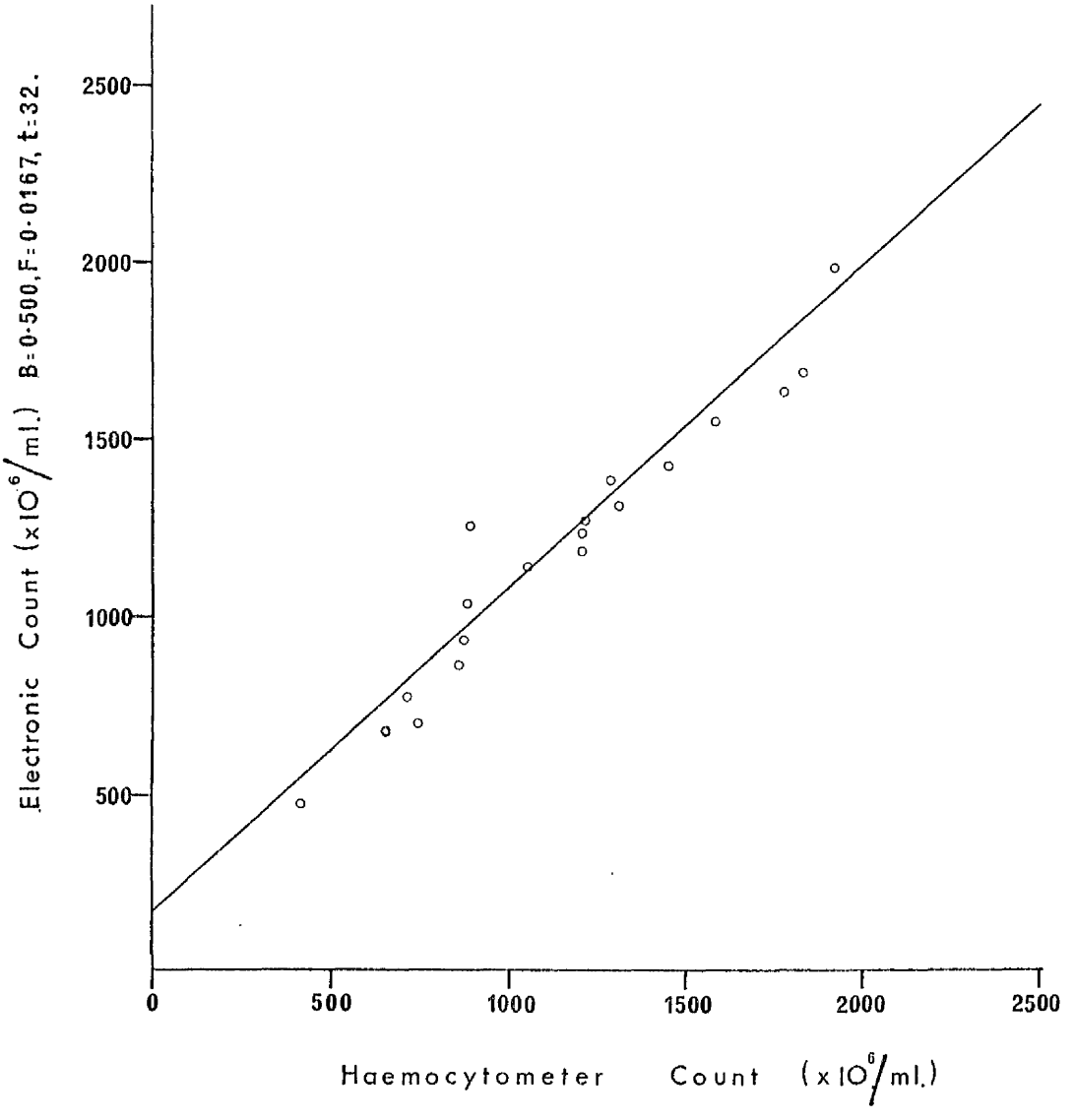

Electronic Count (x10⁶/ml.) B=0·500, F=0·0167, t=32.
2500
2000
1500
1000
500
0
500
1000
1500
2000
2500
Haemocytometer Count (x10⁶/ml.)

Fig. 3.4 Size distribution curves for bovine sperm, with and without added Zapoglobin.

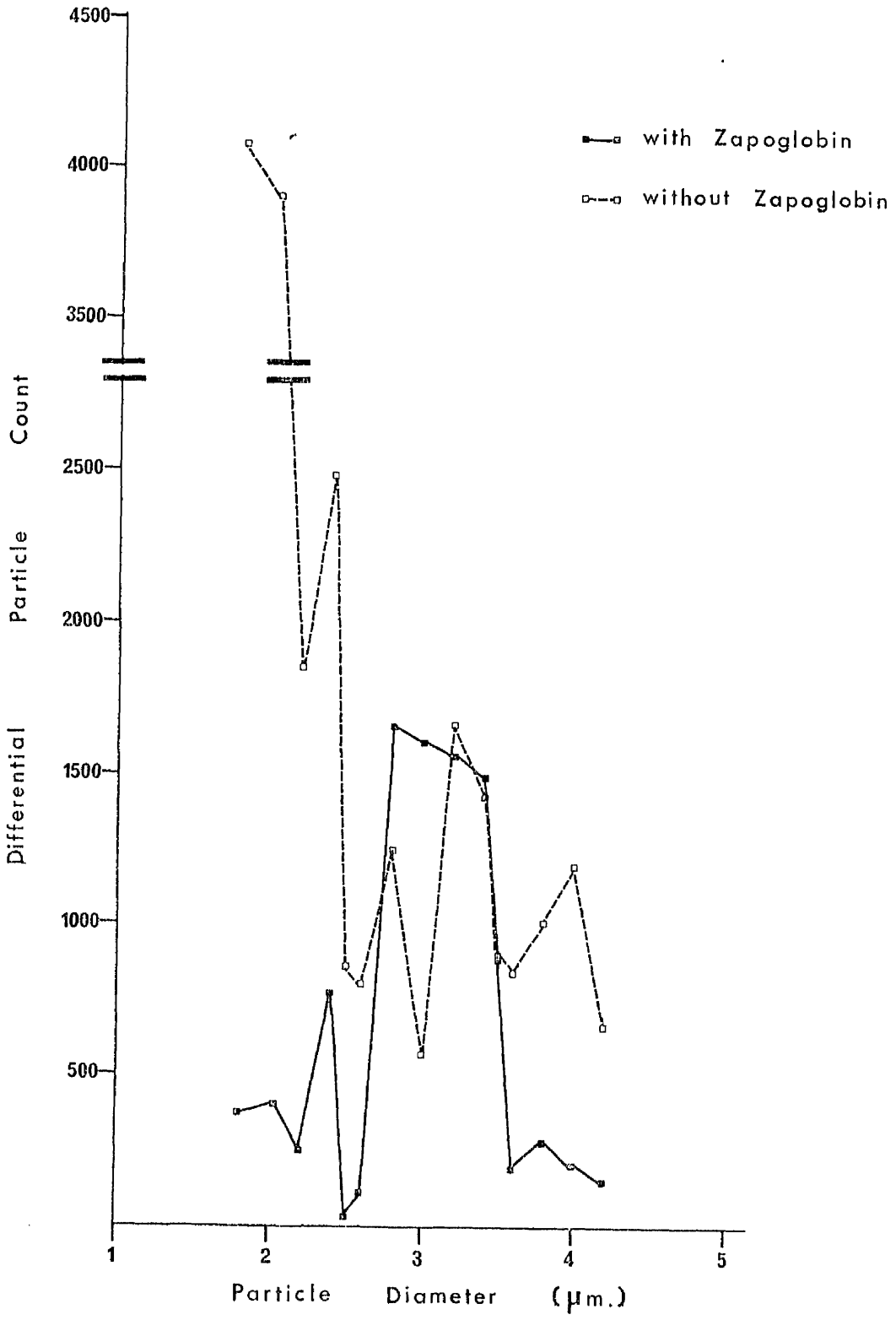

4500
4000
3500
2500
2000
1500
1000
500
Differential Particle Count
with Zapoglobin
without Zapoglobin
1
2
3
4
5
Particle Diameter (µm.)

Table 3.4 Comparison of Sperm Counts obtained with and without added Zapoglobin

Haemocytometer Count ($\times 10^6$/ml)	Electronic Count ($\times 10^6$/ml)
733.8	(a) with added Zapoglobin 762.1 (b) without added Zapoglobin 882.1

3.8 DISCUSSION

Modifications of the haemocytometric methods of counting blood cells have long been used to determine directly the sperm concentration in semen samples, but the procedure is laborious and time-consuming. The ejaculate must be diluted before counting, and inaccuracy in the pipetting and mixing of the sample and in filling of the counting chamber may give rise to error. Therefore, indirect methods of estimating sperm concentration have been developed in an attempt to produce repeatable results rapidly.

The principle of the Coulter counter, i.e. displacement of a volume of electrolyte equal to the volume of the particle, enables counts to be made irrespective of the shape of the particle. Once a threshold setting has been chosen, all particles (both relevant and irrelevant), above a certain size are included in the count. When estimating the sperm concentration in a semen sample, the "relevant particles" are the intact spermatozoa, while detached sperm heads, tail - midpieces, cytoplasmic droplets and epithelial debris should be excluded. O'Donnell (1969) has shown that cytoplasmic droplets, detached heads and tail - midpieces can generate count pulses, and the modal diameters obtained for these structures were 2.75 μm, 2.9 μm and 2.39 μm respectively, compared to the modal diameter range for intact sperm of 3.3 - 3.6 μm. Thus, if the threshold setting for a sperm count is not chosen carefully from size distribution curves to exclude cellular debris, false counts may arise. In addition, epithelial debris present in the suspension may cause blockage of the orifice of the counter. Therefore, some means of removing this debris is desirable, so that the remaining intact sperm can be counted accurately.

From size distribution curves obtained in this work, a modal diameter of 2.5 μm was calculated for cytoplasmic droplets and the minimum threshold setting chosen for bovine sperm counts was 2.62 μm. This setting was chosen to exclude free cytoplasmic droplets from the particle count and, from the calculations of O'Donnell (1969), would seem to exclude also the smaller detached tail - midpieces, although detached sperm heads might be included in the count. Zapoglobin (a blend of lytic agents and cyanide) was added to the sperm suspension shortly before counting commenced to lyse seminal debris. The size distribution curve plotted for one ejaculate after the addition of Zapoglobin to the final sperm suspension has been compared to the size distribution curve for the same ejaculate when Zapoglobin was not added (see Fig. 3.4). From Fig. 3.4, it may be seen that the addition of Zapoglobin reduced the number of small particles present and produced a more regular size distribution curve. The sperm count obtained after the addition of Zapoglobin was lower than when Zapoglobin was not added and was within 5% of the haemocytometer count. Microscopic examination of the bovine sperm suspension, after the addition of Zapoglobin, showed that the sperm tails appeared to be intact and were still attached to the sperm heads, but background droplets and debris were removed. This finding was similar to that of Brotherton and Barnard (1974), who found that the addition of Zaponin (also a lytic agent) to a suspension of human sperm, one minute before the start of the counting, gave better results than if an untreated suspension was used. The length of time of contact between Zapoglobin and the sperm suspension may be important; prolonged contact between the lytic agent and the sperm might result in unwanted lysis of the more resistant sperm cells. Richardson (1975)

has obtained results to show that a human sperm count may fall by 10 - 15%, one hour after the addition of Zapoglobin to the sperm suspension. In this work, Zapoglobin was allowed to mix with the sperm suspension for three minutes before counting commenced, in the hope that in this short period, only epithelial debris would be lysed. Both the histological evidence and the close correlation obtained between haemocytometer counts and electronic counts would seem to suggest that spermatozoa remained intact after the addition of the lytic agent.

The size distribution curves obtained for bovine sperm varied considerably in shape between individual bulls and between ejaculates, but there was no evidence of a bimodal size distribution which might be attributed to X and Y chromosome dimorphism (see Figs. 3.1 and 3.4). Even though the shape of the curve varied between individuals and ejaculates, there was relatively little variation in the diameter of particles at the modes of the curves (see Table 3.3). From the size distribution curves of eight ejaculates, the average range in diameter of bovine sperm was found to be 2.6 - 3.3 μm with an average modal diameter of 2.73 μm. These values were smaller than the value of 4.0 μm, calculated by Glover and Phipps (1962) to be the diameter of the sphere equivalent in volume to a bovine sperm, as well as being slightly smaller than the actual values measured by them (modal diameter of 3.2 μm) and by O'Donnell (1969) who found the diameter range to be 3.3 - 3.6 μm. However, the values are greater than the diameter of 2.18 μm obtained by Iversen (1964). Brotherton (1969b) has reported that results of particle size determinations using Coulter counters have tended to differ from those obtained by other standard methods. She suggested, from her work on the size

distribution of erythrocytes, that different aperture currents and levels of amplification might cause swelling or shrinkage of biological cells. This may explain why the sizes of bovine sperm obtained in this work differ from those obtained by other authors, using a variety of makes and models of counter. It may mean that an electronic counter is of more value for the determination of size of inert particles, rather than biological cells, but it should not preclude the use of an electronic counter whenever rapid counting of cells is required.

From the results of sperm counts carried out in these experiments, it may be seen that there is good correlation between the haemocytometer counts and the electronic counts, for both settings used. When the settings were determined from the size distribution curves, the limiting diameter of particles being counted was calculated, from Equation (2), as 2.62μm. Similarly, the limiting diameter of particles counted using the arbitrary settings was calculated to be 2.05μm. The results show that the correlation between haemocytometer counts and electronic counts using the settings derived from the size distribution curves is slightly better than that between haemocytometer counts and empirically derived settings. From previous discussion, it can be pointed out that a threshold setting of 2.05μm is sufficiently low to allow the inclusion of cytoplasmic droplets and probably detached sperm tail-midpieces in the count, explaining why higher counts were obtained for ejaculates using this setting. As Iversen (1964) has stated, the accuracy of any indirect method of counting sperm depends on the accuracy of the method used for calibration. Although acceptable sperm counts might be obtained, using the empirically-derived settings, the already large error of the haemocytometer count may

be magnified further in the transfer to the electronic counter. Therefore, it would seem that absolute calibration of the counter, using particles of known size, is the method of choice.

CHAPTER FOUR

SPERM SEPARATIONS USING BOVINE SERUM ALBUMIN

CHAPTER FOUR

SPERM SEPARATIONS USING BOVINE SERUM ALBUMIN

4.1 INTRODUCTION

Human Y-bearing sperm have been shown to possess approximately 3% less DNA than X sperm (Sumner, Robinson and Evans, 1971). It was suggested by Roberts (1972) that, because of their lower DNA content, Y sperm may have a smaller head volume than X sperm and would be expected to show superior swimming ability as a function of time, enabling them to penetrate further into a medium than X sperm. Ericsson, Langevin and Nishino (1973) have demonstrated that, when human sperm were applied to columns of albumin, progressively motile sperm moved into the albumin medium, while immotile or morphologically abnormal sperm were excluded. These authors, using the quinacrine staining technique to identify Y-bearing sperm, showed also that the majority of these progressively motile sperm were Y-bearing. This was the first reported separation of human X and Y sperm where the newly-developed fluorescent staining technique had been used as monitor and, because of its potentially great importance, was felt worthy of further investigation. If successful, it was hoped that the technique might be applied to bovine sperm.

4.2 MATERIALS AND METHODS

4.2.1 Diluent for Human Sperm

Tyrode's solution, of pH 7.4 - 7.6 was prepared. All chemicals used in its preparation were of the Analar grade, (BDH Chemicals Ltd., Poole, Dorset). The osmolarity of the solution was measured on a Knauer semi-micro osmometer (Shandon Scientific Co. Ltd., Willesden, London).

4.2.2 Preparation of Human Semen

The ejaculate was diluted 1 : 1, (v/v), with Tyrode's solution and centrifuged at 20°C for 15 minutes at 2,500 g. The supernatant was removed and the cell pellet resuspended in Tyrode's solution to give a final sperm density of approximately 100×10^6 /ml.

4.2.3 Preparation of Albumin Columns

6%, 10%, 15% and 20% (w/v) solutions of bovine serum albumin (fraction V, BDH Chemicals Ltd.) were prepared in Tyrode's solution and the osmolarity of each solution measured. The glass columns each consisted of a Pasteur pipette, internal diameter 5 - 7 mm, broken off immediately below the point of tapering. To this broken end was attached a 30 mm length of soft polythene tubing, closed by a clip.

4.2.4 Recovery of Sperm from Albumin Fractions

All recovered albumin fractions were diluted 1 : 1 with Tyrode's solution and centrifuged at 20°C for 20 minutes at 2,500 g.

The supernatant was removed, the cell deposit resuspended in Tyrode's solution and centrifuged again. Most of the supernatant was discarded, leaving only a few drops to mix the sperm to a milky suspension. Smears were made by dropping the suspension on to clean, wet slides, which were dried rapidly and fixed for 30 minutes in methanol.

4.2.5 Experimental Controls

Prior to application to the column, smears were prepared from sperm, which had been washed and resuspended in Tyrode's solution. The smears were dried and fixed as above.

4.2.6 Staining of Sperm

All smears were stained in 0.5% (w/v) aqueous quinacrine dihydrochloride solution, for 8 minutes, followed by a brief rinse in running tap water and a brief rinse in distilled water. After rapid drying on a hot plate, the smears were mounted in distilled water and the coverslips sealed with rubber solution.

The smears were coded and examined "blind" immediately after mounting, under the microscopic conditions described before. Around 200 sperm were counted on each slide and the proportion of these having a fluorescent body within the sperm head was noted.

4.3 RESULTS

4.3.1 Osmolarity of Solutions

The osmolarities of Tyrode's solution and the bovine serum albumin solutions were measured by the depression of freezing point produced. The results are shown on Table 4.1, where it may be seen that with increasing concentration of albumin, the osmolarity of the solution rose. Measurement of the osmolarity of human semen gave an average value of 320 mOsmols.

4.3.2 Separation Procedures

The albumin colums used for the separation experiments are shown in Fig. 4.1. Three separations procedures were used:

(a) Single Layer

(b) Two Layer

(c) Three Layer

(a) Single Layer Technique

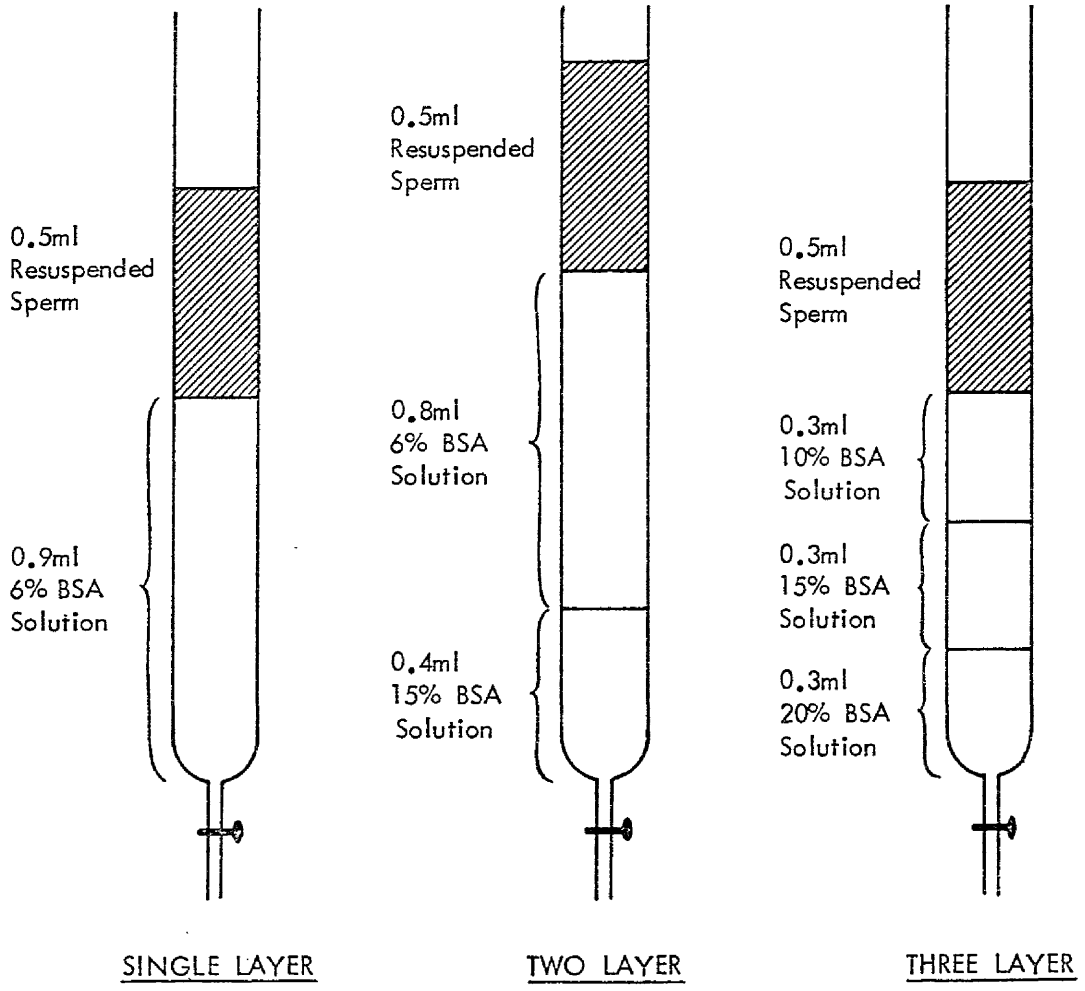
0.9 ml 6% BSA solution was measured into the glass column. 0.5 ml sperm suspension was applied carefully to the top of the albumin solution, using a Pasteur pipette, and left for one hour at room temperature. After this time, the top sperm layer was removed with a pipette and the albumin fraction (isolation fraction) was recovered by opening the clip at the base of the column. The sperm were recovered from the albumin fraction by centrifugation and fixed and stained with quinacrine dihydrochloride, as described before.

Five single layer separation experiments were carried out, using a different ejaculate for each experiment. The results of these experiments are shown in Table 4.2, where the percentage

Table 4.1 Osmolarity of Tyrode's Solution and Bovine Serum Albumin Solutions

Solution	Osmolarity (mOsmols)
Tyrode's Solution	300
6% BSA Solution	320
10% BSA Solution	330
15% BSA Solution	335
20% BSA Solution	340

Fig. 4.1 The single layer, two layer and three layer columns of Bovine Serum Albumin solutions.



SINGLE LAYER TWO LAYER THREE LAYER

of Y-bearing sperm recovered from the albumin layer is compared with the percentage of Y-bearing sperm in the control. There was no significant difference ($p \leq 0.05$) between the sperm recovered from the albumin layer and the sperm in the control.

(b) Two Layer Technique

0.4 ml 15% BSA solution was placed in the glass column and overlaid with 0.8 ml 6% BSA solution. 0.5 ml sperm suspension was applied, as before, and the column was left for one hour. Then, the top sperm layer was removed with a pipette and the column was allowed to stand for a further 30 minutes. After this time, the albumin layers were harvested separately, by withdrawing them slowly from the base of the column. The sperm were recovered from the albumin fractions by centrifugation and fixed and stained with quinacrine dihydrochloride as before.

Nine experiments were carried out on separate ejaculates, using the two layer technique and the results of fluorescent staining of the sperm recovered from the two albumin layers are compared with controls in Table 4.3.

The results of experiments 6, 7 and 9, in Table 4.3, show an increase in the percentage of Y-bearing sperm recovered from the albumin layers, compared to the controls. However, these increases were not significant, ($p \leq 0.05$). The rest of the results in Table 4.3 show a decreased percentage of Y-bearing sperm recovered from the albumin layers, compared to the controls, but only the result from the 15% BSA layer in experiment 13 and the results from experiment 14 were significant ($p \leq 0.05$).

(c) Three Layer Technique

The column was prepared by layering, successively,

Table 4.2 Results of the Single-Layer BSA Separation Technique

Experiment Number	Control (Washed Sperm)			Sperm Recovered from 6% BSA		
	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)
1	322	106	32.9	306	99	32.4
2	306	123	40.2	413	141	34.1
3	309	121	39.2	541	203	37.5
4	366	113	30.9	423	113	26.7
5	320	94	29.4	422	110	26.1

Table 4.3 Results of Two-Layer BSA Separation Technique

Experiment Number	Control (Washed Sperm)			Sperm from 6% BSA Layer			Sperm from 15% BSA Layer		
	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)
6	126	46	36.5	121	52	43.0	43	18	41.8
7	413	143	34.6	200	72	36.5	155	56	36.1
8	120	51	42.5	299	111	37.1	155	47	30.3
9	210	79	37.6	209	84	40.2	200	85	42.5
10	209	83	39.7	218	64	29.4	104	35	33.7
11	221	99	44.8	212	84	39.6	214	72	33.6
12	313	153	48.9	129	50	38.8	183	65	35.5
13	504	200	39.7	629	150	23.8	593	150	25.3
14	618	254	41.1	333	101	30.3	250	56	22.4

0.3 ml volumes of 20%, 15% and either 10% or 6% BSA solutions within the pipette. 0.5 ml sperm suspension was applied to the column, as before, and the column was left to stand for one hour. Then, the top sperm layer was removed and the column left for a further one hour. After this time, the three albumin layers were withdrawn separately from the base of the column. Sperm were recovered from the albumin layers by centrifugation and fixed and stained as described before.

Five experiments were carried out, on separate ejaculates, using the three layer separation technique. The results of fluorescent staining of sperm recovered from the albumin layers are shown in Table 4.4.

There was no significant increase in the numbers of Y sperm recovered from the more concentrated albumin layers in experiment 15. However, the decreased numbers of Y sperm recovered from the 15% BSA layers in experiments 16 and 19 and from the 10% BSA layer in experiment 18 were significant ($p = \lessdot 0.05$).

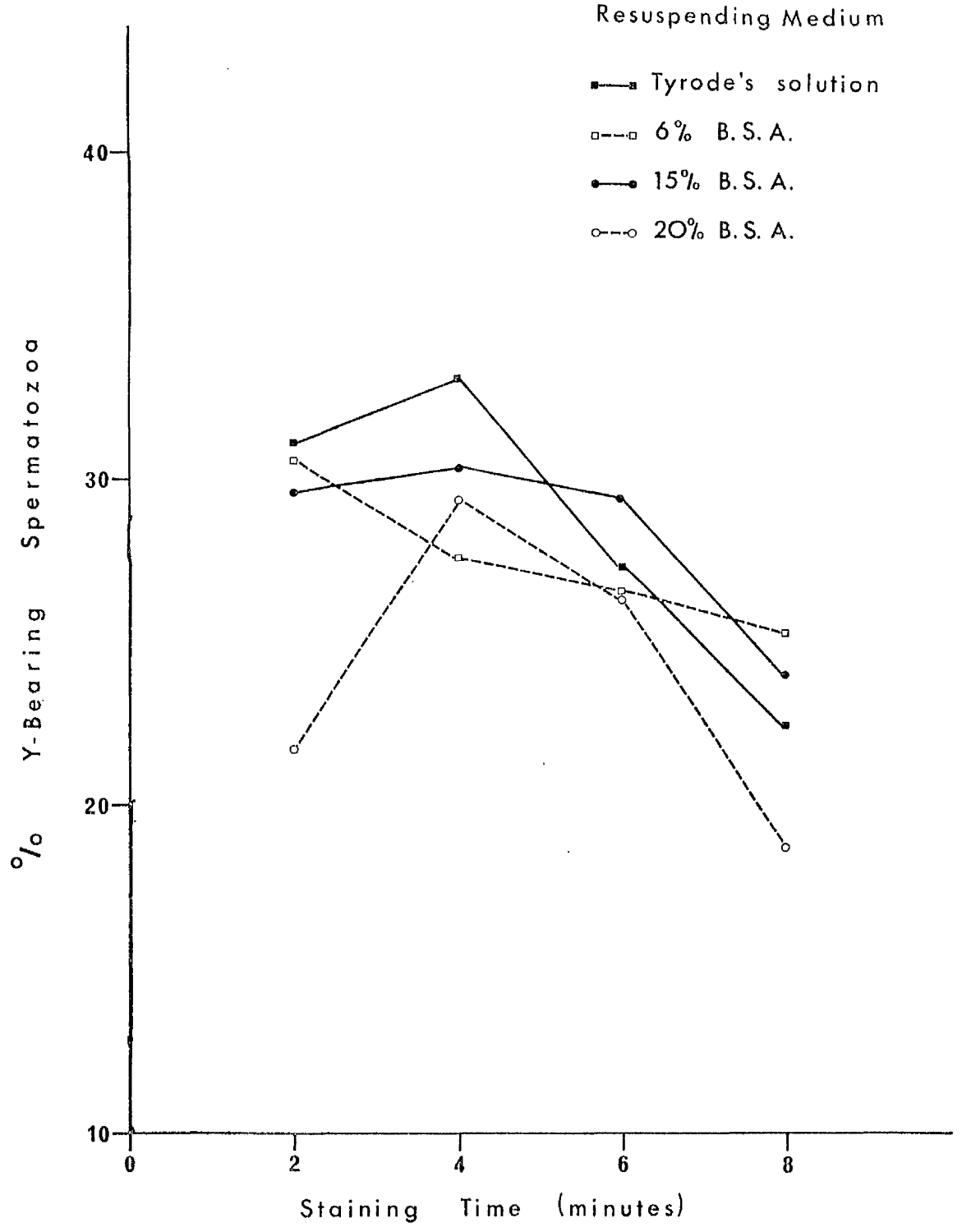
Because of the low numbers of Y sperm apparently recovered in these separation experiments, it was considered that perhaps the presence of albumin or the length of time of staining may have produced an effect on the numbers of sperm showing the presence of an F body. To investigate this possibility, washed sperm were resuspended in either Tyrode's solution, 6% BSA solution, 15% BSA solution or 20% BSA solution and smears were made of these preparations, as before. The smears were stained with quinacrine dihydrochloride for 2, 4, 6 or 8 minutes and examined in ultra-violet light.

Table 4.4 Results of the Three-Layer BSA Separation Technique

Experiment No.	Control (Washed Sperm)			Sperm from 6% BSA Fraction			Sperm from 10% BSA Fraction			Sperm from 15% BSA Fraction			Sperm from 20% BSA Fraction		
	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)	Total No. of Sperm Counted	No. of Y Sperm Counted	Y Sperm (%)
15	238	93	39.0	205	72	35.1	-	-	-	209	106	50.7	154	67	43.5
16	135	56	41.5	60	30	50.0	-	-	-	150	30	20.0	105	28	26.7
17	209	81	38.8	-	-	-	64	29	45.3	108	40	37.0	20	6	30.0
18	210	67	31.9	-	-	-	115	17	14.8	288	89	30.9	190	66	34.7
19	316	110	34.8	-	-	-	328	85	25.9	288	71	21.7	273	78	28.6

Approximately 700 sperm were counted in each smear and the number of Y-bearing sperm identified was noted. The results of this investigation are shown in Fig. 4.2, from which it would appear that 4 minutes staining was optimal for the identification of Y-bearing sperm. When a staining time of eight minutes was used, the percentage of Y-bearing sperm scored was reduced for all preparations. It appears that the presence of albumin in the smears reduced slightly the numbers of Y sperm identified, except in the case of a 20% solution, where, with staining times of two and eight minutes, there was a significant decrease in the numbers of Y sperm identified, ($p =< 0.05$).

Fig. 4.2 The quinacrine staining of human sperm, when resuspended in a variety of media.


Resuspending Medium
Tyrode's solution
6% B.S.A.
15% B.S.A.
20% B.S.A.
40
30
20
10
%
Y-Bearing Spermatozoa
0
2
4
6
8
Staining Time (minutes)

4.4 DISCUSSION

Roberts (1972) suggested that human Y sperm, because of their probable smaller mass compared to X sperm, might tend to swim upwards, in a medium of low viscosity, in greater numbers than X sperm. Ericsson et al. (1973) have adapted this idea, by creating a medium with barriers of increasing viscosity and have demonstrated that sperm showing progressive motility can surmount these barriers, leaving immotile or weakly motile sperm behind. Using the quinacrine staining technique, they showed also that the majority of these progressively motile sperm were Y-bearing.

The separation medium used in the experiments was bovine serum albumin, in Tyrode's solution, in concentrations of 6 - 20%, compared to concentrations of 6 - 25% used by Ericsson et al. (1973). The lower maximum concentration of BSA was used because the high viscosity of the more concentrated BSA solutions led to some difficulty in their preparation. The osmolarity of all solutions was determined by the depression of freezing point produced and, from Table 4.1, it may be seen that, with increasing albumin concentration, the osmolarity rose, to reach 340 mOsmols for the 20% BSA solution. This value is higher than both the measured osmolarity of human semen and the value of 301 mOsmols given by Mann (1964) for human semen. However, Mann (1964) has stated also that, in general, sperm may be immobilized more readily by hypotonic rather than by hypertonic diluents. Preliminary observations on the behaviour of human sperm in BSA solutions showed that the percentage of sperm exhibiting progressive motility was reduced in the 15% and 20% solutions, although in the 6% albumin solution, progressive motility was maintained to a greater

extent. This reduction in motility may have been a manifestation of the increased osmolarity.

Ericsson et al. (1973) suggested that the basis of separation was progressive motility of the sperm. In the experiments described here, the sperm were washed once with Tyrode's solution, for 15 minutes at 2,500 g before application to the albumin column. Examination of the sperm, after washing and removal of the seminal plasma, revealed that there was a decrease in the number of sperm showing progressive motility. Also, difficulty was experienced in preparing a homogeneous resuspension of sperm in Tyrode's solution after washing, as the sperm tended to aggregate. Some of these aggregations of sperm were so large that they could be seen to fall through the albumin columns under gravity. Further evidence was obtained by Ross, Robinson and Evans (1975) who confirmed that sperm motility was reduced after washing and centrifugation. No value for centrifugal force was given by Ericsson et al. (1973) but Ross et al. (1975) showed that 800 g was the maximum centrifugal force that could be used without adverse effect on the motility of sperm in Tyrode's solution. Undoubtedly from the foregoing evidence, the centrifugal force of 2,500 g used in these experiments must have produced an adverse effect on sperm motility, but some sperm showing progressive motility were present after resuspension. This large centrifugal force may have contributed also to the formation of sperm aggregates, although Krzanowski (1970) has reported that motile sperm tend to aggregate at room temperature when allowed to sediment through transparent liquids, such as dextran or milk plasma. Because of these adverse effects, the multiple layer techniques seemed preferable to the single layer procedure, since sperm were not

subjected to centrifugation and resuspension before being placed on subsequent albumin columns to complete the separation.

A staining time of eight minutes had been used successfully in previous experiments, to identify human Y-bearing sperm and this procedure was used to examine sperm recovered from the albumin layers. The results of fluorescent staining of these sperm are compared to controls in Tables 4.2, 4.3 and 4.4. From Table 4.2, it may be seen that there was no significant difference between the percentages of Y sperm recovered from the 6% albumin layer and the percentages of Y sperm in the controls. In Table 4.3, the results of three experiments showed an increased percentage of Y sperm, recovered from the albumin layers, compared to the controls, although the increases were not significant. The results of a further six experiments showed a decreased percentage of Y sperm recovered from the albumin layers, compared to the controls. In Table 4.4, the results of only one experiment showed an increased percentage of Y sperm recovered from the albumin layers, and this increase was not significant; the results of four successive experiments did not confirm this finding.

Therefore it was concluded from these experiments, that, contrary to the results of Ericsson et al. (1973), there was no evidence of a positive increase in the numbers of Y sperm recovered after albumin separation. This conclusion was supported by the findings of Ross et al. (1975).

From the results shown in Tables 4.2, 4.3 and 4.4, it might appear that, because of the decreased numbers of Y sperm recovered from the albumin layers, compared to controls, an actual increase in the numbers of X sperm recovered may have taken place.

However in these experiments, the assessment of the Y sperm was carried out very critically, in that if there was any doubt whatsoever of the presence of an F-body, it was assumed to be absent. Such rigorous assessment of the Y sperm would undoubtedly lower the percentage of Y sperm apparently present.

In a study of this nature, the staining procedure is of paramount importance. Consequently, an investigation of the effect of the presence of albumin and the length of staining time on the percentage of sperm showing the presence of an F-body was carried out. These results are shown in Fig. 4.2, where it appears that four minutes' staining resulted in the greatest number of sperm being identified, irrespective of the presence of albumin in the smear. When a staining time of eight minutes was used, the percentage of Y sperm scored in all preparations (but especially in the 20% BSA preparation) was reduced significantly, probably because of the greater background fluorescence encountered. In the separation experiments, sperm recovered from the albumin layers were washed at least once in Tyrode's solution, before smears were prepared for staining, so that the concentration of albumin present would certainly have been reduced, and would be unlikely to have interfered greatly with the identification of the Y sperm. Although overstaining may have caused the numbers of Y sperm identified in these experiments to be lower than expected, it would seem improbable that any increase in the numbers of Y sperm, of the magnitude reported by Ericsson et al. (1973) would have been overlooked.

CHAPTER FIVE

SPERM SEPARATIONS

USING

DENSITY GRADIENT CENTRIFUGATION

CHAPTER FIVE

SPERM SEPARATIONS USING DENSITY GRADIENT CENTRIFUGATION

5.1 INTRODUCTION

Density gradient centrifugation is a technique used extensively in the separation and purification of a wide variety of biological materials. The method involves the use of a supporting column of fluid, the density of which increases towards the bottom of the tube. When a mixture of cells or particles is placed on the gradient and centrifuged under suitable conditions, separation may take place. This separation may be rate zonal, where the cells or particles separate in the gradient by virtue of their different rates of sedimentation, or isopycnic, where the cells or particles sediment to the position in the gradient where their density equals that of the surrounding medium.

There is no ideal all-purpose density gradient material and many substances have been used for different types of separation, e.g. caesium chloride, sucrose, salts, polysaccharides. However, when living cells are to be separated, particular requirements must be fulfilled in order to maintain cell viability. Usually, aqueous systems are used and toxic substances and extremes of pH and osmotic pressure must be avoided.

Separation at 4°C is preferable also, to slow cell metabolism and improve the chance of survival during lengthy separation procedures.

Harvey (1946) calculated that sperm carrying a Y chromosome, and possessing less DNA than X-bearing sperm, might be expected to have a smaller specific gravity than X sperm and he suggested that it might be possible to separate these two types of sperm on a density gradient. Since then, using density gradient techniques, sperm of a number of species have been separated into populations (see General Introduction), but insemination of these separated populations has had no consistent effect on the sex ratio of offspring produced, suggesting that no real separation of X and Y sperm has been achieved. However, Beatty and Fechheimer (1972), using a Ficoll gradient, managed to separate diploid from haploid rabbit sperm. The difference in DNA content of these sperm is far greater than that between X and Y sperm, but the result provided some encouragement to hope that the development of a finer technique might perhaps produce, at least, a partial separation of X and Y sperm.

With the development of the quinacrine staining technique, it has become possible to monitor separations of human sperm directly, to see if any change in the ratio of X and Y sperm has been produced. Because of this, it was decided to study density gradient separations of both human sperm (as a monitor) and the more readily available bovine sperm. Three density gradient materials were chosen for this investigation.

Colloidal silica, the first material examined, has been used as a density gradient medium for the concentration and

purification of a herpes virus (Pertoft, 1970) and for the separation of blood cells (Pertoft and Laurent, 1969), while Beatty (1964) has described the use of a colloidal silica gradient for the separation of rabbit sperm into a number of populations.

Sucrose, the second gradient material examined in these experiments, has been used to separate both bovine and rabbit sperm into populations (Benedict, Schumaker and Davies, 1967). Insemination of the separated rabbit sperm fractions (to investigate any change in the ratio of X and Y sperm) was carried out, but no significant change in the sex ratio of the embryos recovered was reported. Recently, Rohde, Porstmann, Prehn and Dörner (1975) published details of a discontinuous sucrose gradient, capable of separating human sperm into populations with distinct percentages of X and Y sperm and this was the gradient examined in the present studies.

The third gradient material, Metrizamide, a tri-iodinated benzamido-derivative of glucose, is a relatively new density gradient material. It is an inert, non-cytotoxic compound, readily soluble in aqueous media, and even dense solutions have a relatively low viscosity and osmolarity. These properties should mean that Metrizamide is a useful gradient material for the isolation of cells, viruses and sub-cellular particles. Munthe-Kaas and Seglen (1974) have reported the isopycnic separation of rat liver cells on a Metrizamide gradient, while Rickwood, Hell and Birnie (1973) have used a Metrizamide gradient for the isopycnic separation of sheared chromatin. However, the use of a Metrizamide gradient for the separation of sperm has not been reported previously.

5.2 MATERIALS AND METHODS FOR COLLOIDAL SILICA GRADIENTS

5.2.1 Diluent for Sperm

Baker's medium, modified to pH 7.6 was prepared according to the method of Beatty (1964), as follows:

Glucose	3.362 g	anhydrous substances
NaCl	0.252 g	
Na_2HPO_4	0.252 g	
KH_2PO_4	0.0355 g	

Distilled water to 100 ml

This solution was taken as concentration 1.00 and, by dilution of this with distilled water, solutions of the following relative concentrations were prepared - 0.35, 0.50, 0.70, 0.90 and 0.93. In addition, solutions of 1.25 and 1.50 concentration were prepared.

5.2.2 Dialysis of Colloidal Silica

Colloidal silica (Syton X 30, Monsanto Ltd., London, U.K.) was supplied as an opalescent suspension with a pH of 9.6 - 10.0 at 20°C. The suspension was poured into transparent seamless tubing (Visking tubing) and dialysed against 0.93 Baker's medium for 24 - 48 hours.

5.2.3 Preparation of Gradients

A density gradient of 9.5 ml volume was prepared in a Perspex double chambered gradient maker, starting with equal volumes of 0.93 Baker's medium and dialysed colloidal silica.

The gradient was pumped by a peristaltic pump, (LKB-Produkter Ltd., Bromma 1, Sweden), through an ultra violet absorptiometer detector unit (LKB) into a cellulose nitrate centrifuge tube, $\frac{5}{8}$" diameter x $2\frac{1}{2}$".

5.2.4 Preparation of Semen

Each human and bovine ejaculate was diluted with an equal volume of 0.93 Baker's medium. Using a Pasteur pipette, 0.3 ml of the diluted semen was applied to the gradient. The remainder of the diluted semen was retained as experimental control.

5.2.5 Centrifugation of Gradients

After diluted semen had been applied to the gradients, the tubes were sealed with caps. The gradients were centrifuged in a Type 50 fixed angle aluminium rotor, at 20°C for two hours at 2,000 g, in an L2 - 65B ultra-centrifuge (Beckman Instruments Inc., Spinco Division, Palo Alto, California, U.S.A.).

5.2.6 Examination of Gradients

At the end of centrifugation, the gradients were removed from the rotor and examined in daylight, for the distribution of sperm. Each gradient was harvested by piercing the base of the tube with a 21 gauge needle, attached by polythene tubing to a peristaltic pump. The gradient was pumped through an ultra violet absorptiometer detector unit (LKB - Produkter, Ltd.) and collected in 0.5 ml fractions.

5.2.7 Recovery of Sperm from Harvested Fractions

Each fraction containing sperm was diluted with an approximately equal volume of 0.93 Baker's medium and centrifuged at 20°C for 20 minutes at 950 g. After centrifugation, the cell deposit was resuspended in 0.93 Baker's medium and centrifuged again. At the end of this time, the cell deposit was resuspended in sufficient fixative (3 : 1, methanol : acetic acid) to form a milky suspension and allowed to stand for 20 - 30 minutes, then smears were prepared on clean wet slides and dried rapidly.

5.2.8 Fluorescent Staining of Human Sperm

The smears were stained in 0.5% aqueous quinacrine dihydrochloride, for 5 - 6 minutes, rinsed briefly in running tap water and in distilled water and dried rapidly. Then, they were mounted in distilled water with sealed coverslips and examined immediately in ultra violet light, as described already.

5.3 RESULTS FOR COLLOIDAL SILICA GRADIENTS

5.3.1 Survival of Sperm in Baker's Medium

(a) Human Sperm

Eight test tubes were prepared so that each contained 1.0 ml of Baker's medium of a particular concentration. A few drops of fresh human semen were added to each solution, mixed gently and left to stand for two hours at room temperature. Then, a few drops of the mixture were removed from each tube and examined microscopically. Estimates were made, for each sample, of:

(a) the % sperm showing motility and

(b) the motility score of the sperm, according to the method of Ulstein (1972), where sperm is scored between 0 (immotile) and 4 (very rapid progressive motility).

These results are shown in Table 5.1, and it may be seen that human sperm survival appeared to be best in 0.93 Baker's medium.

(b) Bovine Sperm

A similar examination of bovine sperm survival in the range of concentrations of Baker's medium was made and these results are shown in Table 5.2. It may be seen that bovine sperm seemed also to survive best in 0.93 Baker's medium.

Therefore, in subsequent experiments with both human and bovine sperm, 0.93 Baker's medium was used.

Dialysis of colloidal silica with 0.93 Baker's medium reduced the pH of this material to around 7.6 . The dialysed colloidal silica was used immediately, because of its tendency to gel on standing.

Table 5.1 Survival of Human Sperm in Baker's Medium

Concentration of Baker's Medium	% Motile Sperm	Motility Score
0.35	0 - 5	0 - 1
0.50	20	2
0.75	50	3
0.90	50	3
0.93	60	3
1.00	55	3
1.25	40	2
1.50	30	2

Table 5.2 Survival of Bovine Sperm in Baker's Medium

Concentration of Baker's Medium	% Motile Sperm	Motility Score
0.35	0 - 5	0 - 1
0.50	30	1
0.75	40	1
0.90	40	2
0.93	65	2
1.00	50	1
1.25	40	1
1.50	40	1

5.3.2 Refractive Index and Specific Gravity of Dilutions of Colloidal Silica

The refractive indices of dilutions of dialysed colloidal silica with 0.93 Baker's medium were measured, using an Abbé 60 refractometer (Bellingham and Stanley Ltd., London, U.K.). The specific gravity of each dilution was measured directly, by weighing. The results are shown in Table 5.3. From these, the relationship between specific gravity and refractive index, for dilutions of colloidal silica with 0.93 Baker's medium, was plotted as shown in Fig. 5.1, where it may be seen that a direct linear relationship exists. Because of this direct linear relationship, it is possible to make a rapid assessment of the specific gravity of any colloidal silica dilution with 0.93 Baker's medium, by measuring its refractive index.

5.3.3 Examination of the Prepared Gradient for Linearity

Tracings were obtained from the ultra violet absorptiometer during gradient formation. A typical example of the trace obtained is shown in Fig. 5.2, and it may be seen that a smooth gradient was formed, apart from small flattened areas at the beginning and end of gradient formation. From refractive index measurements, the average specific gravity range of the gradient was found to be 1.01 - 1.17 gm/ml .

5.3.4 Behaviour of Human Sperm on the Gradients

When human sperm were centrifuged on a colloidal silica gradient, they "clumped" together in the middle of the gradient, at a specific gravity of 1.08 gm/ml, as shown in Fig. 5.3.

Table 5.3 Specific Gravity and Refractive Index of Dilutions of Colloidal Silica with 0.93 Baker's Medium

Dilution of Colloidal Silica with 0.93 Baker's Medium(v/v)	Specific Gravity gm/ml	Refractive Index
0.93 Baker's Medium (B.M.)	1.0010	1.3379
0.2 Colloidal Silica : 0.8 B.M.	1.0420	1.3415
0.4 Colloidal Silica : 0.6 B.M.	1.0770	1.3455
0.6 Colloidal Silica : 0.4 B.M.	1.1145	1.3499
0.8 Colloidal Silica : 0.2 B.M.	1.1520	1.3545
Colloidal Silica	1.1865	1.3587

Fig. 5.1 The relationship between specific gravity and refractive index of dilutions of colloidal silica with 0.93 Baker's medium.

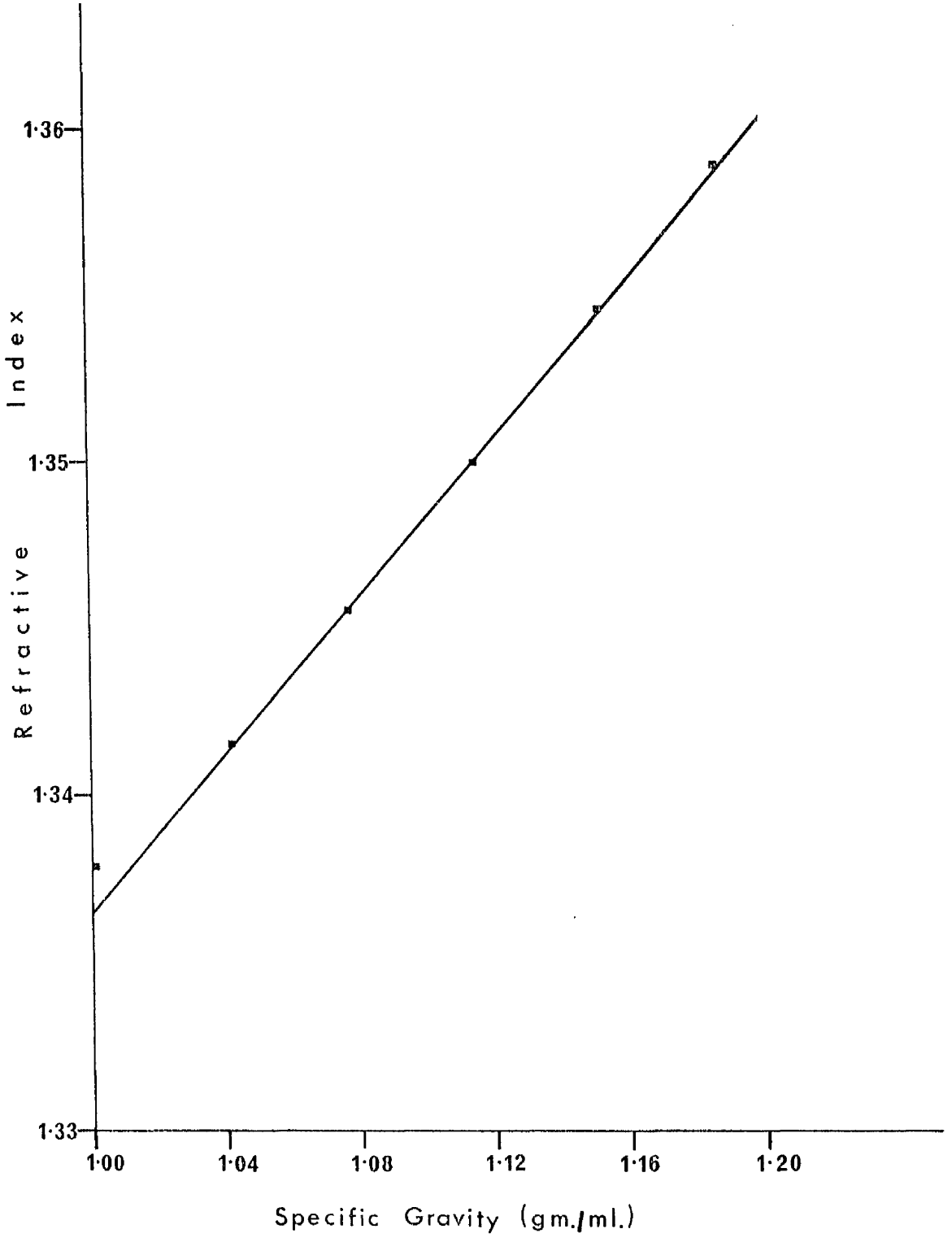

1·36
1·35
1·34
1·33
Refractive Index
1·00
1·04
1·08
1·12
1·16
1·20
Specific Gravity (gm./ml.)

Fig. 5.2 Example of the tracing obtained from the ultra violet absorptiometer during formation of a colloidal silica gradient.

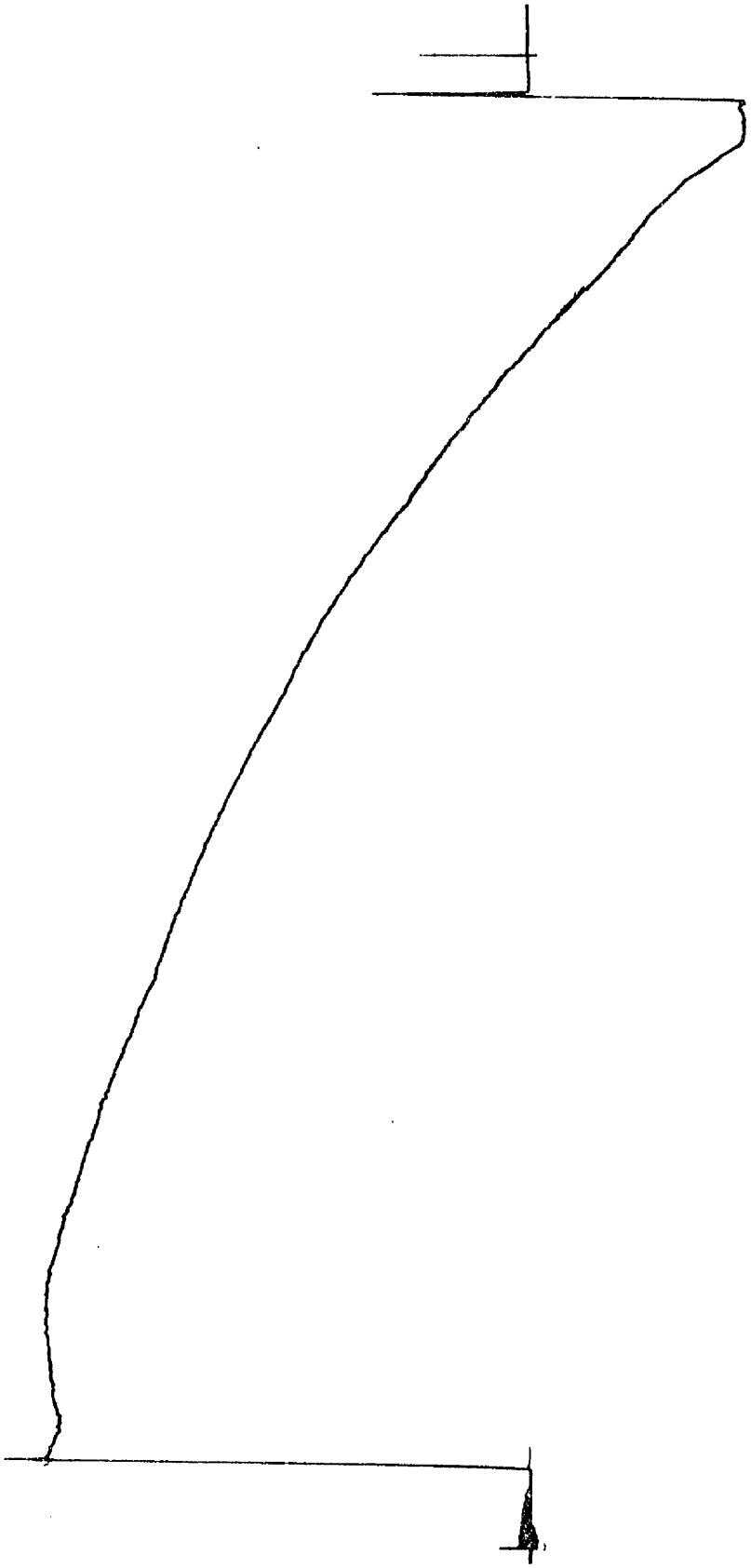
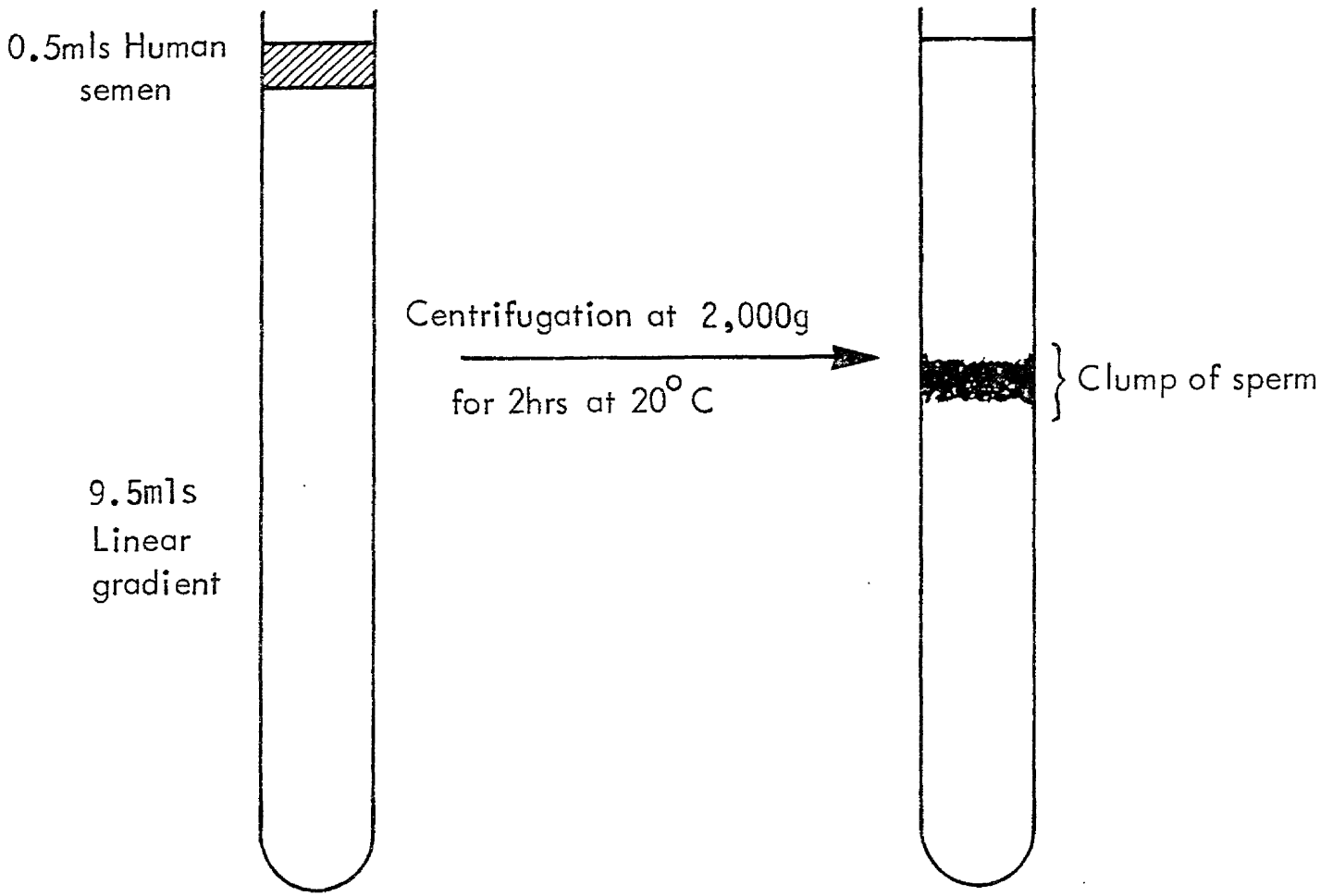


Fig. 5.3 Human sperm centrifuged on the colloidal silica gradient.


0.5mls Human semen
9.5mls Linear gradient
Centrifugation at 2,000g
for 2hrs at 20° C
Clump of sperm

Attempts to remove the seminal plasma, by washing the sperm in 0.93 Baker's medium prior to their application to the gradient, produced no change and reductions in the force and time of centrifugation did not produce further separation of the sperm.

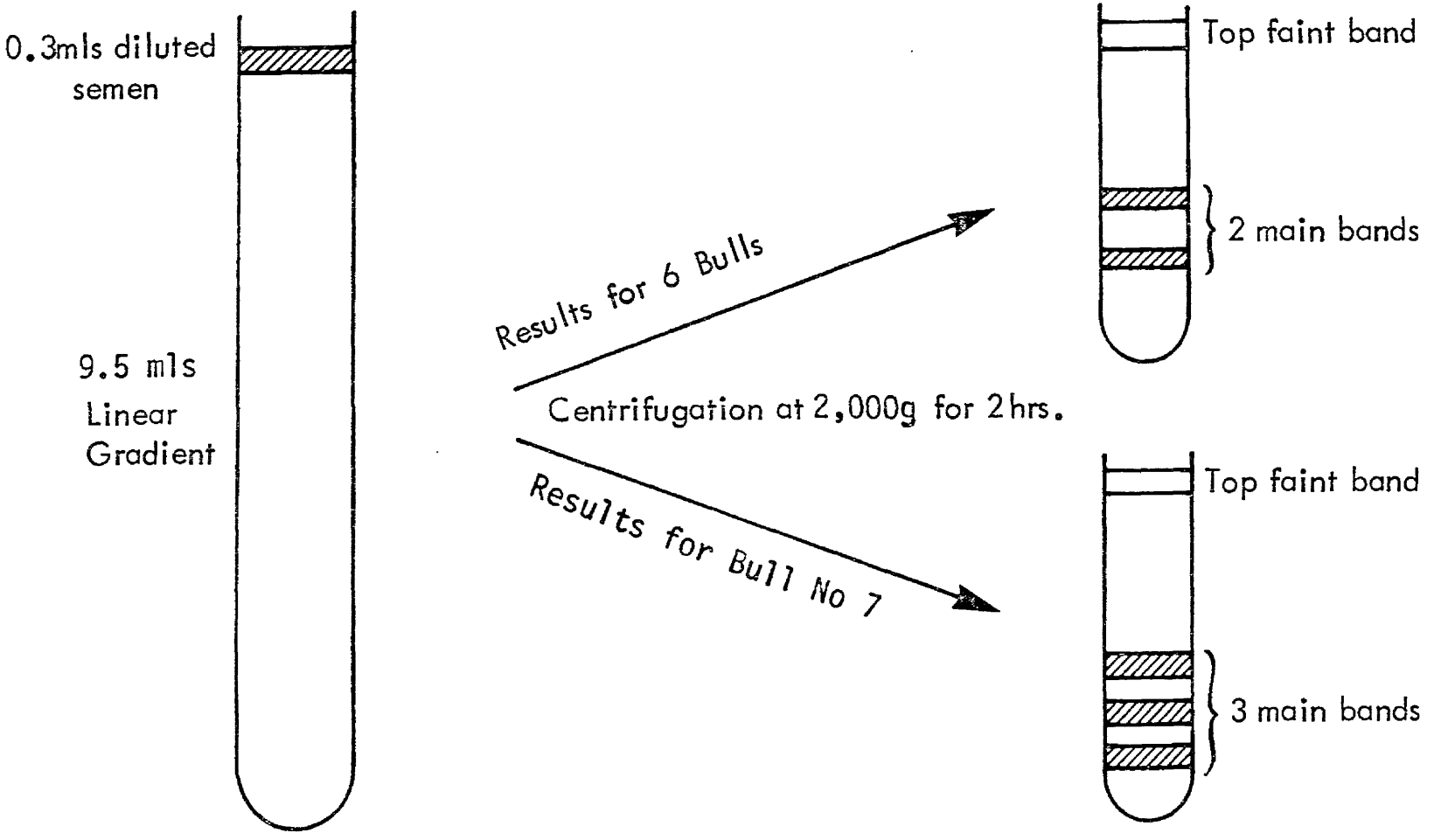
The sperm were recovered from the gradient and washed twice in 0.93 Baker's medium, before staining with quinacrine dihydrochloride. However, no results of fluorescent staining were obtained, because the sperm were obscured by very bright background fluorescence, probably caused by colloidal silica still present in the preparation. Repeated attempts to remove this by washing failed.

5.3.5 Behaviour of Bovine Sperm on the Gradients

The results of experiments using sperm from seven bulls are shown in Fig. 5.4, where it may be seen that, after centrifugation, the sperm of six of these bulls separated into two bands, visible in the gradient. The upper band had an average specific gravity of 1.11 gm/ml and the lower band had an average specific gravity of 1.14 gm/ml. A faint third band was present variably, in the region of the original sperm layer / gradient interface. Microscopic examination of each of the two main bands revealed the presence of sperm, some of which showed weak non-progressive motility, although the majority were immotile. Examination of sperm morphology revealed no obvious differences in structure between these two groups of sperm.

To compare the behaviour of living and dead bovine sperm on colloidal silica gradients, sperm which had been killed by adding a few drops of formalin to the suspension were applied to

Fig. 5.4 Bovine sperm centrifuged on the colloidal silica gradient.


0.3mls diluted semen
9.5 mls Linear Gradient
Results for 6 Bulls
Centrifugation at 2,000g for 2hrs.
Results for Bull No 7
Top faint band
2 main bands
Top faint band
3 main bands

the gradient and centrifuged. The result of this is shown in Fig. 5.5, where it may be seen that a single band of sperm formed in the gradient, at a specific gravity of 1.10 gm/ml.

Because killed sperm formed one band in the gradient while sperm from a normal ejaculate, (presumably a mixture of living and dead sperm), formed two bands, it was considered possible that a separation of living and dead sperm might be taking place on the gradient. To investigate this, nigrosin/eosin staining of the sperm recovered from the two bands was carried out, according to the method of Campbell et al. (1953). However, no clear answer was obtained, as neither band of sperm was found to be either completely eosinophilic or completely unstained. Although it was felt that colloidal silica present in the preparation may have interfered with the staining of sperm, (flakes of silica were seen on the slides examined), washing of the sperm before staining was not carried out, as damage caused to the sperm during washing might have caused more sperm to stain eosinophilically. In order to overcome this problem, a method of distinguishing dead from living sperm by the appearance of the acrosome was adopted. Sperm from the control and sperm recovered from the two bands in the gradient were examined on a thick film of 1% agar in Ringer's solution, under phase contrast, according to the method of Hancock (1952). The sperm were examined under oil, at x 1,000 magnification and the total number observed and the number of these with detachment of the acrosome (and therefore presumed dead), were noted. These results are shown in Table 5.4 and it may be seen that there was no apparent increase in the numbers of dead sperm in either of the two bands recovered from the colloidal silica gradient, compared to the control. Plates 5.1 and 5.2 show the

Fig. 5.5 Killed bovine sperm centrifuged on the colloidal silica gradient.

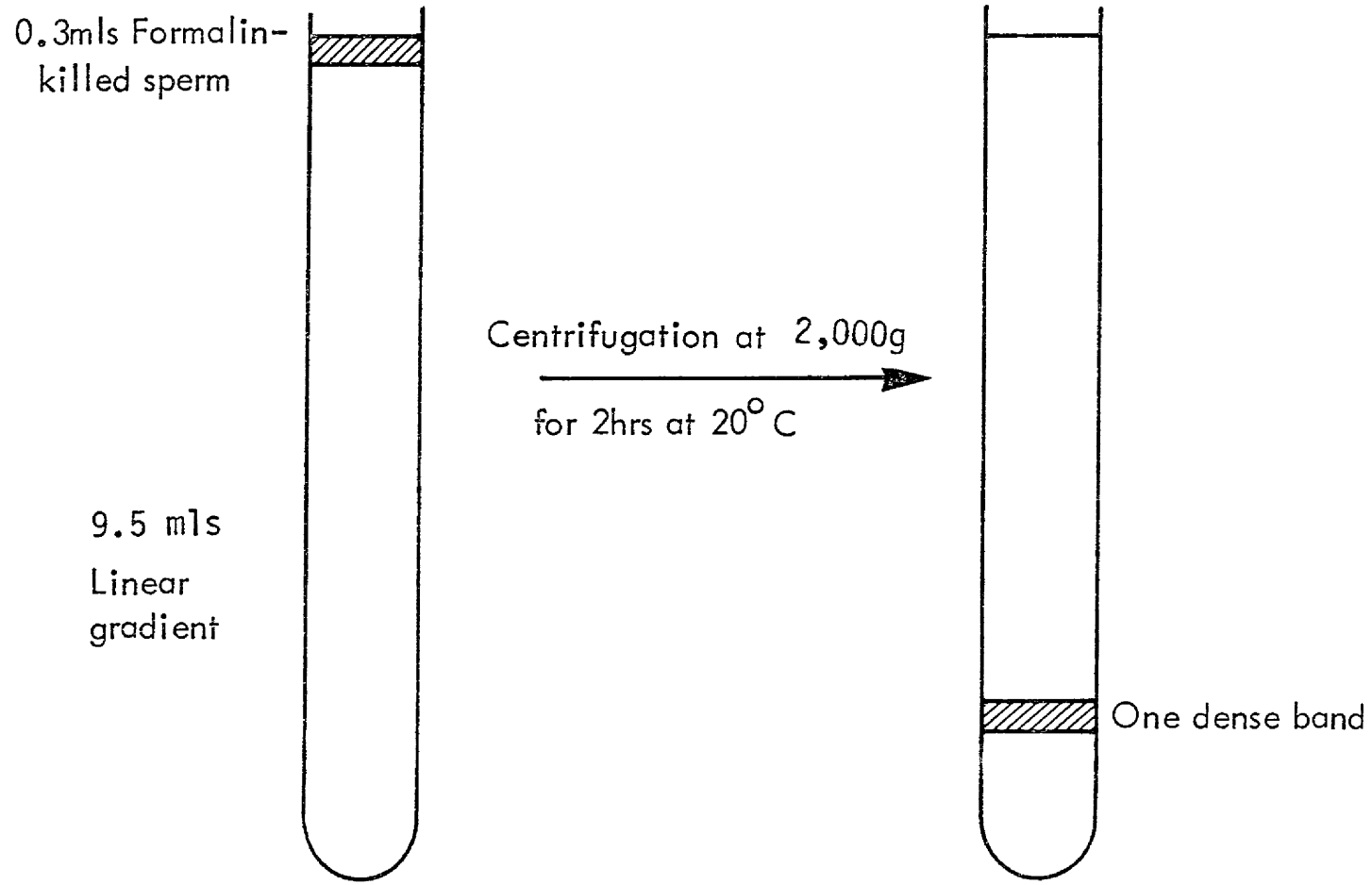

0.3mls Formalin-killed sperm
9.5 mls
Linear gradient
Centrifugation at 2,000g
for 2hrs at 20° C
One dense band

Table 5.4 Estimation of the Numbers of Living and Dead Sperm on a Colloidal Silica Gradient

	Total Number of Sperm Observed	Number of Sperm with Detachment of Acrosome	% Dead Sperm
Control	200	85	42.5
Sperm from Upper Band in Gradient	200	86	43.0
Sperm from Lower Band in Gradient	200	85	42.5

difference in appearance of living and dead bovine sperm, examined under phase contrast.

Sperm from bull number 7 separated into three bands in the lower part of the colloidal silica gradient, as shown in Fig. 5.4, with a fourth faint top band present variably. The lowermost main band had an average specific gravity of 1.16 gm/ml, the middle band, 1.13 gm/ml, and the uppermost band, 1.11 gm/ml. Microscopic examination of the three bands revealed that the lowest band consisted mainly of detached sperm heads, the middle band contained sperm with head and tail attached, and the uppermost band consisted mainly of free tail - midpieces of sperm. When fresh semen from bull number 7 was examined by nigrosin/eosin staining, according to the method of Campbell et al. (1953), it was found that approximately 42% of detached sperm heads were present.

Sperm from an ejaculate of one of the other bulls were subjected to ultrasonic treatment (100 watt ultrasonic disintegrator, Measuring and Scientific Equipment Ltd., Crawley, U.K.) for 20 secs., to produce separation of sperm heads and tails, by fracture of the neck, according to the method of Zaneveld, Wagner, Schlumberger and Schumacher (1974). After microscopic examination, to check that separation had occurred, these sperm were centrifuged on a colloidal silica gradient, under the same conditions as described before. Subsequent examination of the gradient showed that two bands formed in the lower region of the gradient, as shown in Fig. 5.6. The lower band had settled at a specific gravity of 1.16 gm/ml and microscopic examination showed that it consisted of detached sperm heads, as shown in Plate 5.3. The upper band had settled at a specific gravity of 1.10 gm/ml and was found, on examination, to consist of free tail - midpieces of sperm, as shown in Plate 5.4.

Plate 5.1

Living Bovine Sperm examined under phase contrast at x 1,000 magnification

Plate 5.2

Dead Bovine Sperm examined under phase contrast at x 1,000 magnification

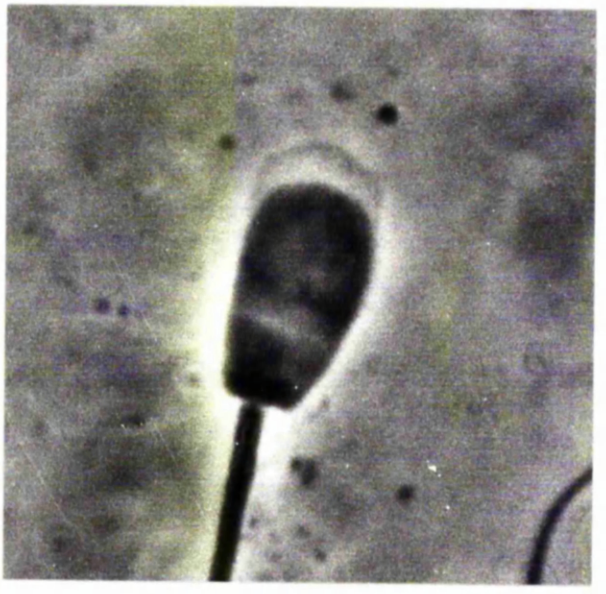


Fig. 5.6 Ultrasonicated bovine sperm centrifuged on the colloidal silica gradient.

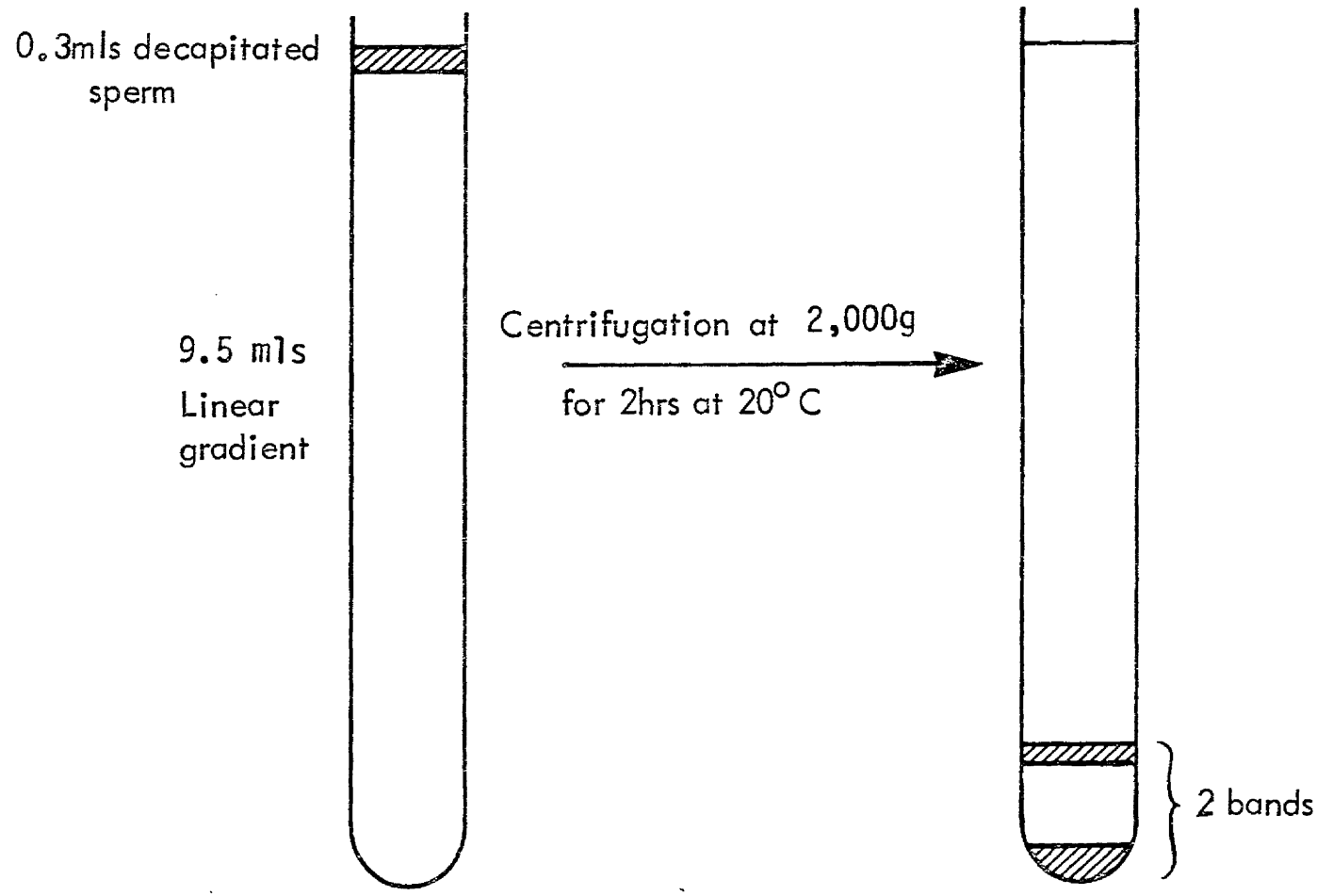
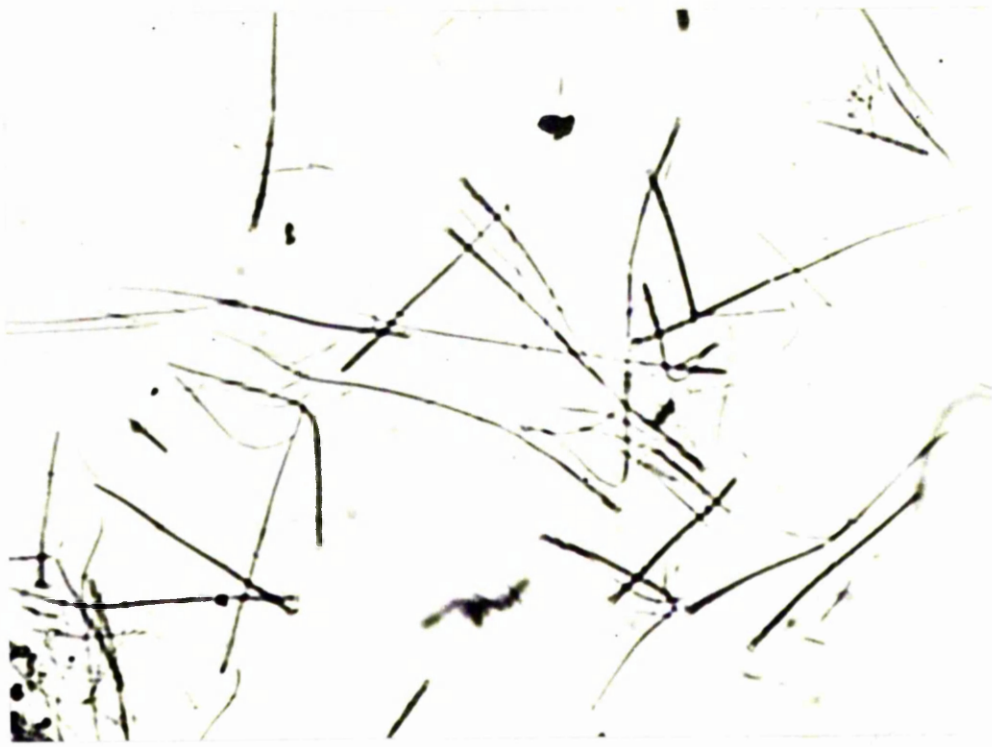

0.3mls decapitated sperm
9.5 mls Linear gradient
Centrifugation at 2,000g
for 2hrs at 20° C
2 bands

Plate 5.3 Detached Bovine Sperm Heads stained with Haematoxylin and Eosin

Plate 5.4 Bovine Sperm Tail - Midpieces stained with Haematoxylin and Eosin



5.4 MATERIALS AND METHODS FOR SUCROSE GRADIENTS

5.4.1 Preparation of Glassware

All glassware was siliconised, by rinsing in a 2% solution of dimethyldichlorosilane in carbon tetrachloride, ("Repelcote", Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.).

5.4.2 Preparation of Sperm Diluent and Sucrose Solutions

As a diluent for sperm, calcium-free Ringer's solution, pH 7.4-7.6, (Krebs-Hensleit-Ringer solution, described by Mann, 1964) was prepared and its osmolarity was measured, using a Knauer semi-micro osmometer, (Shandon Scientific Co. Ltd., Willesden, London, U.K.).

Sucrose solutions of 70%, 60%, 50%, 40% and 30% (W/V) concentrations were prepared in calcium-free Ringer's solution and their osmolarities were measured. The refractive index of each solution was measured, using an Abbé 60 refractometer and its specific gravity was determined directly by weighing.

5.4.3 Preparation of Gradients

Two gradient systems were used in these experiments:

(i) Discontinuous System

Gradients of 4.8 ml volume were prepared by layering 1.2 ml of 60%, 50%, 40% and 30% sucrose solutions, successively, in cellulose nitrate tubes.

(ii) Continuous System

These gradients were prepared according to the method of Stone (1974). 2.4 ml 60% sucrose solution was overlaid with 2.4 ml 30% sucrose solution, in a cellulose nitrate tube held in the vertical position. The tube was closed with plastic sealing tissue ("Parafilm") and tipped to a horizontal position, over 40 seconds. The tube remained in this position at room temperature, for either 6 or 24 hours. At the required time, the tube was returned to the vertical position, over 40 seconds.

Using the same method, gradients were prepared using 2.4 ml 70% sucrose solution, overlaid with 2.4 ml 30% sucrose solution. These gradients were kept in the horizontal position for either 3 or 4 hours, before being returned to the vertical.

5.4.4 Preparation of Semen and Application to the Gradients

Human semen was diluted with an equal volume of calcium-free Ringer's solution and washed six times, at 4°C, for 10 minutes at 110 g. After the final washing, the pellet of sperm was resuspended to 0.8 ml volume in calcium-free Ringer's. 0.5 ml of this suspension was applied to each gradient, using a Pasteur pipette, the remainder being retained as experimental control.

For some experiments, sperm from two ejaculates, from different donors, were combined for application to a gradient. The ejaculates were washed separately, as before, and after the final washing, each was resuspended to 0.5 ml in calcium-free Ringer's solution. Then, 0.25 ml was removed from each suspension and retained separately, as experimental control, the

remaining volumes being combined for application to the gradient.

1 ml bovine semen was diluted with an equal volume of calcium-free Ringer's and washed six times in the manner described for human semen. After the final washing, the sperm were resuspended to 0.5 ml in calcium-free Ringer's solution and applied to the gradient, using a Pasteur pipette.

5.4.5 Conditions of Centrifugation

Gradients of 4.8 ml volume were centrifuged at 4°C for either 30 or 60 minutes at 12,000 g in an SW65K swinging bucket aluminium rotor, in an L2-65B ultracentrifuge (Beckman Ltd.). In addition, continuous gradients were centrifuged at 5,000 g and at 1,300 g, for 30 minutes.

5.4.6 Examination and Harvesting of Gradients

Blank continuous gradients were harvested by downward displacement, after piercing the base of the tube with a 21 gauge needle attached to a peristaltic pump, and 0.5 ml fractions were collected, so that the gradient might be examined for linearity.

At the end of centrifugation, gradients containing sperm were examined for the presence of bands, using a special dark-ground light source. The gradients were photographed under these conditions. In addition, either the gradient itself or the corresponding photographic plate was placed on a densitometer (Model DD2, Kipp and Zonen, Delft, Holland) and examined in visible light.

Harvesting of bands present in the gradient was carried out while the tube was illuminated indirectly. The soft-walled

tube was pierced immediately below the level of each band, with a 21 gauge needle and the contents of the band were withdrawn slowly into an attached 1 ml syringe. The bands were harvested in order of descent. A new needle and syringe were used for each band. After harvesting, needle and syringe were washed out twice with calcium-free Ringer's and the washings were added to the recovered fraction.

5.4.7 Recovery of Sperm from Harvested Fractions

Fractions containing sperm were diluted with an equal volume of calcium-free Ringer's and centrifuged at $4^{\circ}C$ for 20 minutes at 950 g. Each pellet was resuspended in diluent and centrifuged again. Then, the sperm pellet was resuspended, for 20 - 30 minutes, in sufficient fixative (3 : 1, methanol : acetic acid) to produce a milky suspension. Smears were prepared on clean wet slides and dried rapidly.

Sperm, used as control, were also suspended in fixative for 20 - 30 minutes and smears were prepared, as before.

5.4.8 Fluorescent Staining of Human Sperm

The smears were stained with quinacrine dihydrochloride and mounted in water, according to the method described already, for sperm recovered from colloidal silica gradients. The smears were examined in ultra violet light, immediately after mounting, as described before, and the proportion of sperm showing the presence of an F-body was noted.

5.5 RESULTS FOR SUCROSE GRADIENTS

5.5.1 Osmolarity, Refractive Index and Specific Gravity of Solutions

The osmolarities of the sperm diluent and the sucrose solutions were measured by the depression of freezing point produced and the results are shown in Table 5.5. It may be seen that the osmolarities of the more concentrated sucrose solutions were beyond the range of the osmometer, but dilution of these solutions, to reduce the osmolarity, was not possible, because of the change in the activity coefficient of the solution when diluted. The osmolarities of the sucrose solutions were much greater than the value of 302 mOsmols given for human semen, reported by Mann, (1964).

The results of measuring refractive index and specific gravity of sucrose solutions are shown in Table 5.6 and from these values, the relationship between specific gravity and refractive index was examined, as shown in Fig. 5.7. This relationship is direct and linear, so that, as for the colloidal silica dilutions, subsequent measurement of refractive index of sucrose solutions provided a rapid method of assessing their specific gravity.

5.5.2 Examination of Gradients for Linearity

(i) Discontinuous System

A linear gradient was not formed, because the gradient had been prepared by layering four concentrations of sucrose solutions successively within the cellulose nitrate tube. However, the interfaces between the sucrose solutions were visible and these

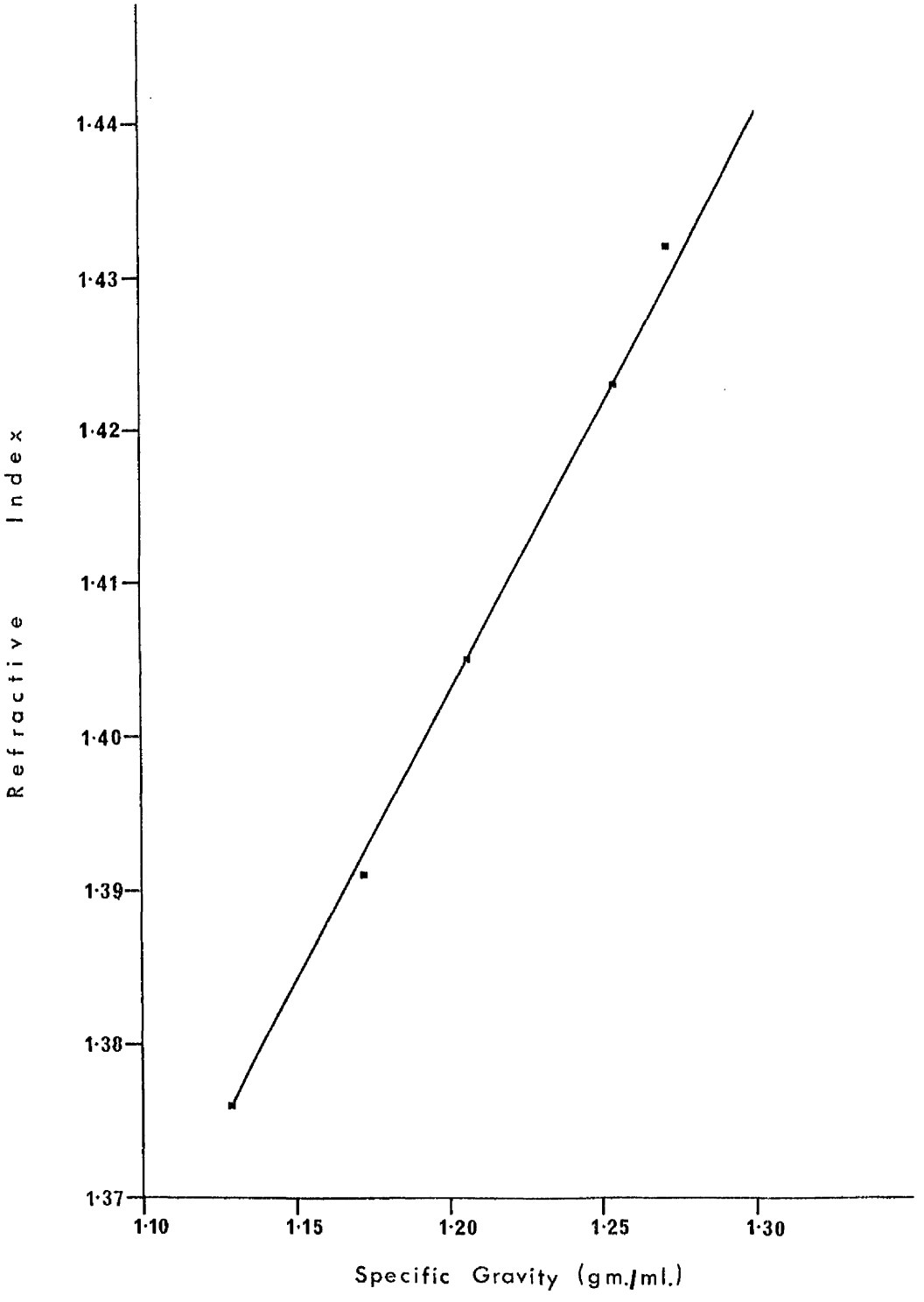
Table 5.5 Osmolarity of Calcium-Free Ringer's Solution and Sucrose Solutions

Solution	Osmolarity (in mOsmols)
Calcium-Free Ringer's	275
30% Sucrose	1,100
40% Sucrose	> 1,600

Table 5.6 Specific Gravity and Refractive Index of Sucrose Solutions

Concentration of Sucrose Solution w/v	Specific Gravity (gm/ml)	Refractive Index
30%	1.1292	1.376
40%	1.1725	1.391
50%	1.2060	1.405
60%	1.2543	1.423
70%	1.2717	1.432

Fig. 5.7 The relationship between specific gravity and refractive index of sucrose solutions.


Refractive Index
1·44
1·43
1·42
1·41
1·40
1·39
1·38
1·37
1·10
1·15
1·20
1·25
1·30
Specific Gravity (gm./ml.)

were harvested by puncturing the soft-walled tube with a needle and withdrawing approximately 0.2 ml of the liquid in the region of the interface into the attached syringe. The refractive index of each interface was measured and the corresponding specific gravity was determined from Fig. 5.7. These results are shown in Table 5.7. The refractive indices shown in Table 5.7 may appear to be lower than would be expected from the values for sucrose solutions shown in Table 5.6, but undoubtedly, some mixing will have taken place, both during gradient preparation and harvesting.

(ii) Continuous System

60% - 30% gradients were harvested in nine 0.5 ml fractions, after 6 or 30 hours' diffusion and the refractive index of each fraction was measured. 70% - 30% gradients were harvested similarly, after 3 or 4 hours' diffusion and the refractive index of each fraction was measured. These results are compared in Fig. 5.8 and the direct linear relationship between refractive index and specific gravity of sucrose solutions allowed the refractive index values of the fractions to be used as an indication of the shape of the density gradient. From Fig. 5.8, it may be seen that the 70% - 30% gradient, prepared by 3 hours' diffusion, was the steepest linear gradient formed and this gradient was chosen for subsequent experiments.

5.5.3 Examination of Gradients containing Sperm, after Centrifugation

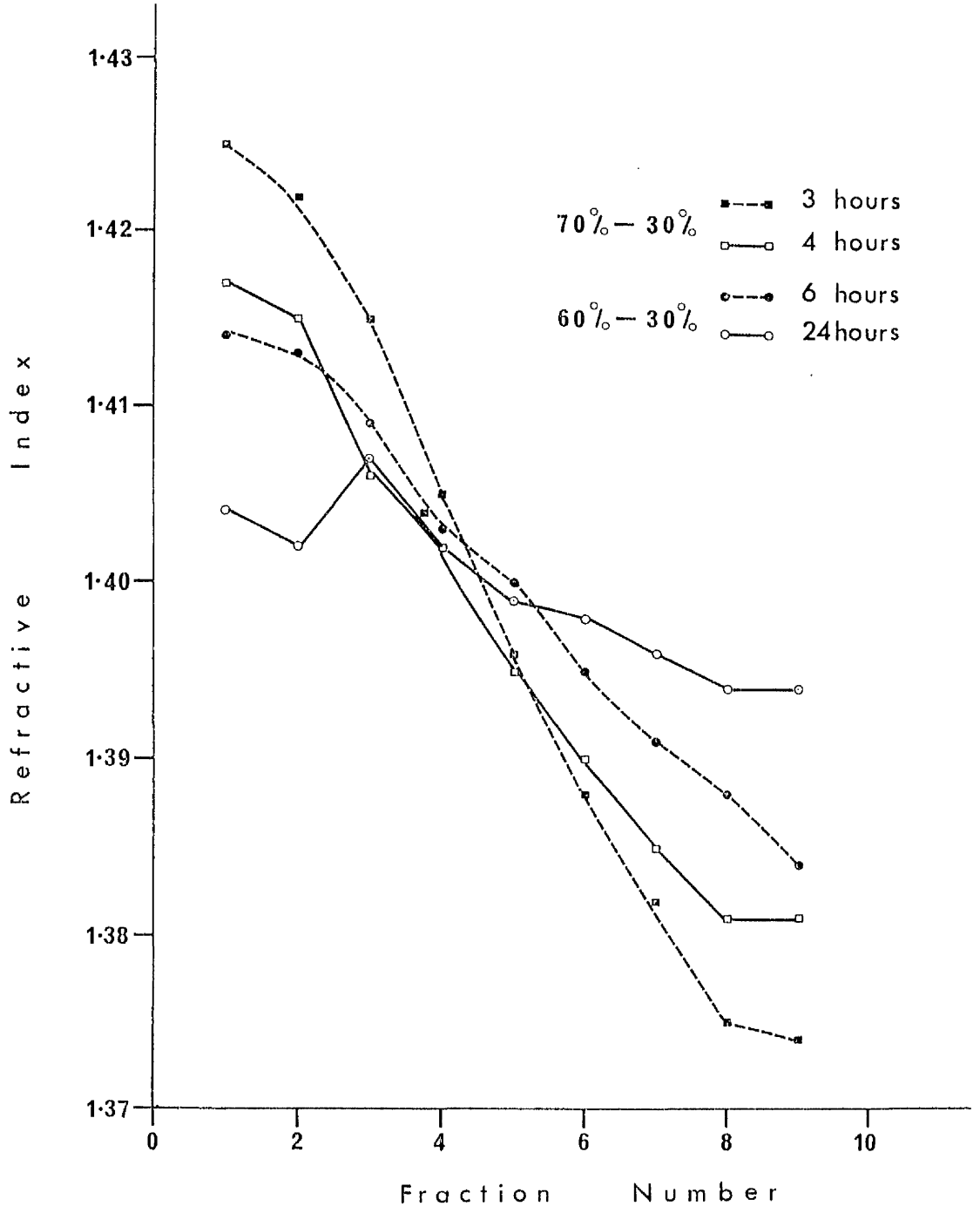
(i) Discontinuous System

Both human sperm and bovine sperm separated into five populations, when centrifuged on the discontinuous sucrose gradient,

Table 5.7 Refractive Indices and Specific Gravities of the Interface Areas of the Discontinuous Sucrose Gradients

Interface between Sucrose Layers	Refractive Index	Specific Gravity (gm/ml)
30 - 40%	1.379	1.137
40 - 50%	1.384	1.151
50 - 60%	1.402	1.198

Fig. 5.8 Four Continuous Sucrose Density Gradients.


1·43
1·42
1·41
1·40
1·39
1·38
1·37
Refractive Index
0
2
4
6
8
10
Fraction Number
70% — 30%
3 hours
4 hours
60% — 30%
6 hours
24 hours

as shown in Fig. 5.9. This separation was readily visible when the gradients were examined in indirect light. When gradients containing sperm were examined by densitometry, a trace was obtained, an example of which is shown in Fig. 5.10. Five peaks are recognisable in Fig. 5.10, corresponding to the populations of either human sperm or bovine sperm in the gradient.

The five populations of sperm were harvested and examined microscopically and each population was found to consist of immotile sperm. The refractive indices of the four bands and precipitate were measured in separation experiments using both human and bovine sperm, and the specific gravity of each was determined from Fig. 5.7. These results are shown in Table 5.8 and it may be noted that similar specific gravity values were obtained for both human and bovine sperm populations, indicating that the sperm of both species had separated at similar positions in the gradient. Comparison of the results in Tables 5.7 and 5.8 shows that the sperm had separated at the interfaces in the gradient.

The distribution of sperm in the gradient was investigated by counting the number of sperm applied to the gradient and the numbers recovered from the four areas of banding and the precipitate, after centrifugation. Human sperm counts were made using a haemocytometer, while bovine sperm counts were carried out using the Coulter counter. These results are shown in Tables 5.9 and 5.10. Using the results for human sperm shown in Table 5.9, it was calculated that 10.45×10^6 sperm (in 0.5 ml suspension) were applied to the gradient and that the total number of sperm recovered after centrifugation was 7.5×10^6, with 2.95×10^6 sperm not recovered after harvesting. Using the

Fig. 5.9 Human sperm centrifuged on a discontinuous sucrose gradient.

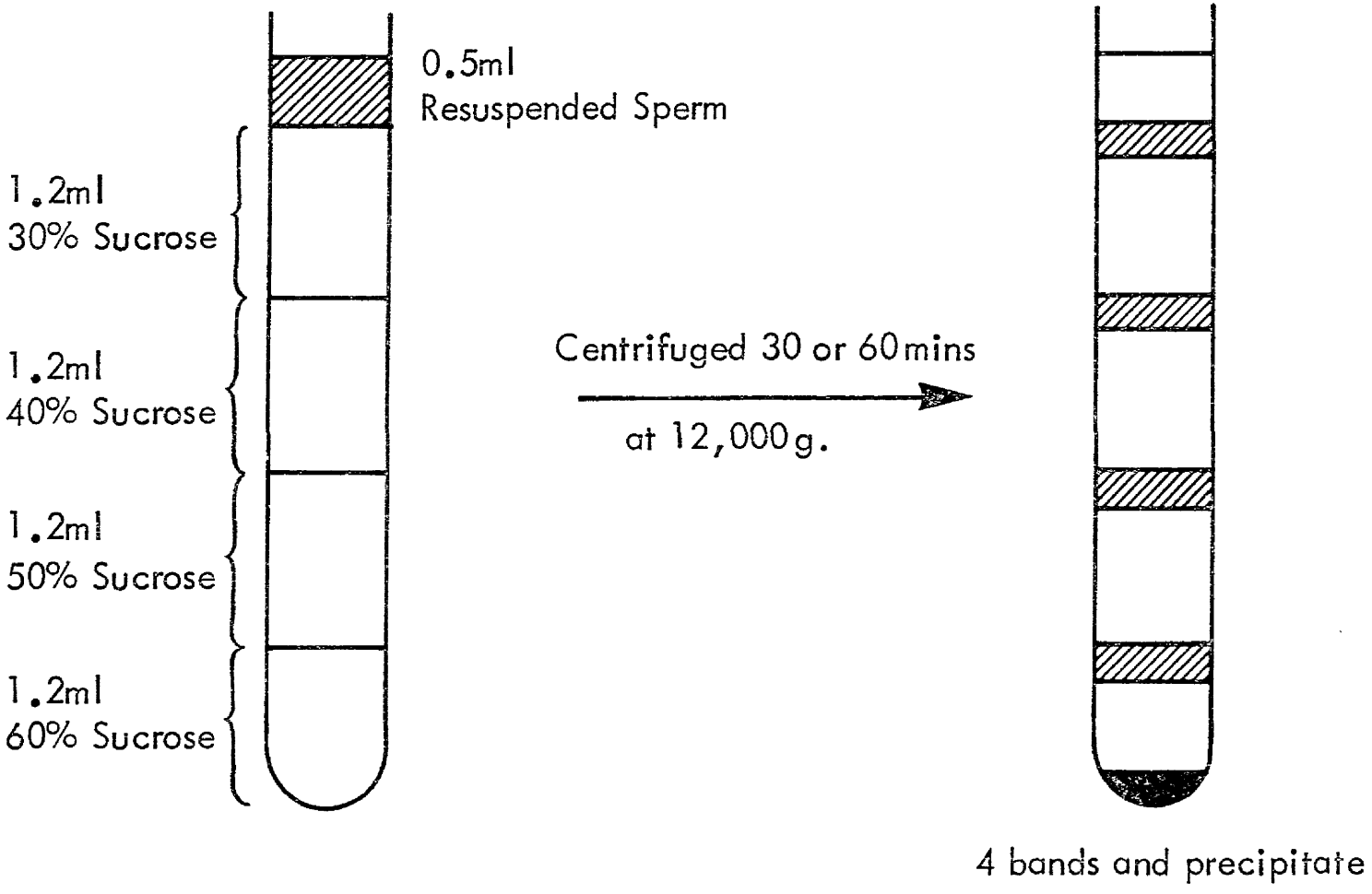

0.5ml
Resuspended Sperm
1.2ml
30% Sucrose
1.2ml
40% Sucrose
1.2ml
50% Sucrose
1.2ml
60% Sucrose
Centrifuged 30 or 60 mins
at 12,000 g.
4 bands and precipitate

Fig. 5.10 Example of the densitometer tracing obtained when a discontinuous sucrose gradient containing sperm was examined.

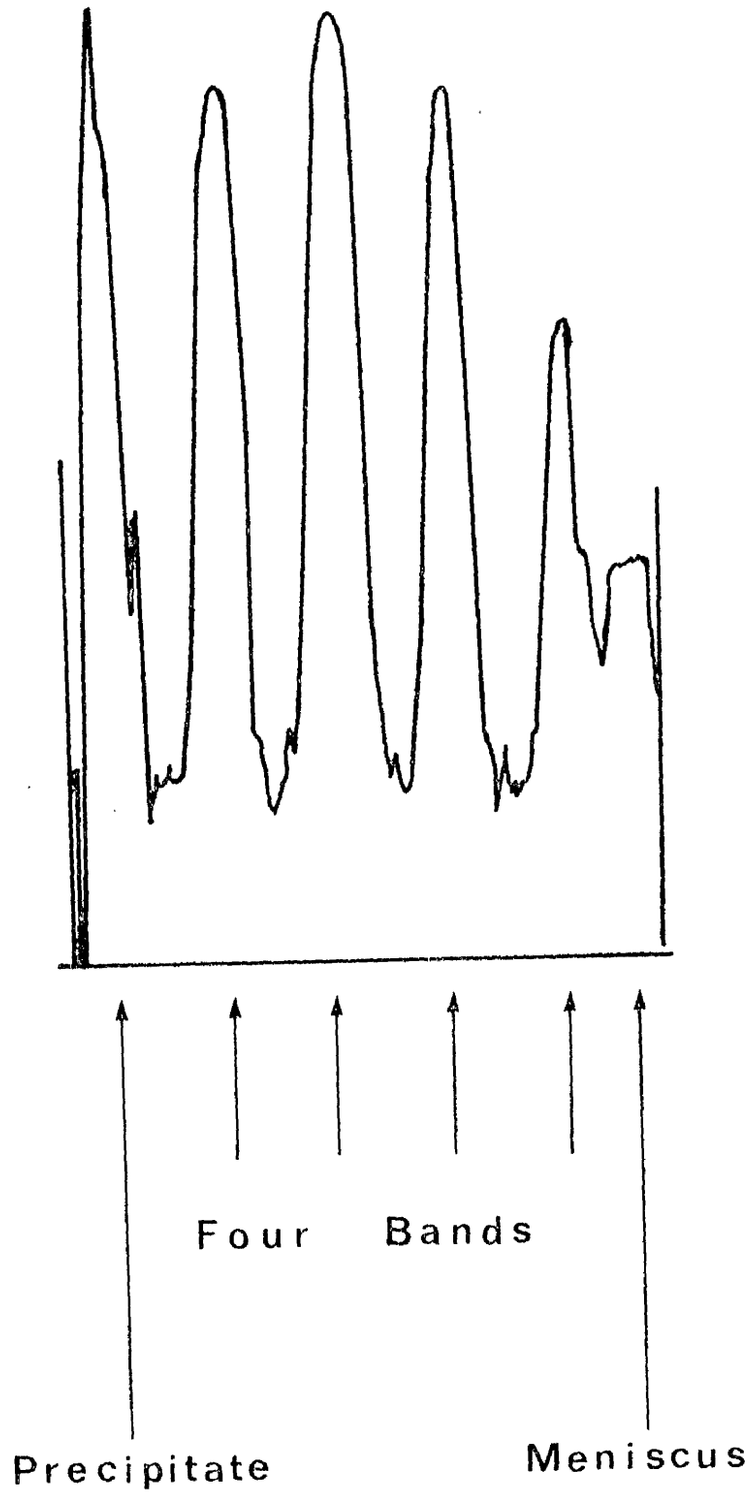

Four Bands
Precipitate
Meniscus

Table 5.8 Refractive Indices and Specific Gravities of Human and Bovine Sperm Populations, separated on the Discontinuous Sucrose Gradient

Sperm Population Band Number	Human Sperm		Bovine Sperm	
	Refractive Index	Specific Gravity (gm/ml)	Refractive Index	Specific Gravity (gm/ml)
1	1.355	1.072	1.359	1.082
2	1.378	1.135	1.376	1.128
3	1.386	1.156	1.384	1.150
4	1.399	1.190	1.392	1.172
Precipitate	1.413	1.228	1.409	1.217

Table 5.9 Distribution of Human Sperm on a Discontinuous Sucrose Gradient, after 60 minutes at 12,000 g

Sperm Band Number	Volume Recovered (ml)	Count per ml (Average of 2)	No. of Sperm Recovered
1	0.8	5,625	4,500
2	0.7	5,000	3,500
3	0.8	15,625	12,500
4	1.0	282,500	282,500
Precipitate	0.15	47.75×10^6	7.2×10^6
Control (Resuspension after washing)	-	20.9×10^6	-

results for bovine sperm shown in Table 5.10, 282.95 x 10^6 sperm were applied to the gradient, while 202.8 x 10^6 sperm were recovered, after centrifugation, and 80.2 x 10^6 sperm were not recovered.

The distributions of human and bovine sperm on discontinuous gradients are compared in Fig. 5.11, the numbers of sperm counted in each band being expressed as a percentage of the number originally applied to the gradient. From Fig. 5.11, it would appear that the greatest number of sperm, of both species, precipitated through the gradient during centrifugation. In the case of human sperm, the lowest band contained a much greater number of sperm than either of the three upper bands, while for bovine sperm, the two middle bands contained fewer sperm than either the upper or the lower band.

Human sperm recovered from the discontinuous sucrose gradient were stained with quinacrine dihydrochloride and examined in ultra violet light for the presence of an F-body. These results are shown in Table 5.11 and it may be seen that, in six experiments, there was no increase in the numbers of Y sperm in the two uppermost bands (Bands 1 and 2), in the gradient, compared to the controls. Also from Table 5.11, it may be seen that the numbers of sperm counted in these two upper bands were sometimes considerably lower than the numbers of sperm examined in the controls, making comparison rather difficult. To try to overcome this, three experiments were carried out using a combination of two ejaculates, but although some improvement was obtained, the result was not completely successful, probably because most of the sperm continued to precipitate through the gradient, during centrifugation.

Table 5.10 Distribution of Bovine Sperm on a Discontinuous Sucrose Gradient, after 60 minutes at 12,000 g

Sperm Band Number	Volume Recovered (ml)	Count per ml (Average of 4)	No. of Sperm Recovered
1	0.6	48.17×10^6	28.9×10^6
2	0.6	28.55×10^6	17.1×10^6
3	0.7	26.15×10^6	18.3×10^6
4	0.9	28.37×10^6	25.5×10^6
Precipitate	0.3	376.67×10^6	113.0×10^6
Control (Resuspension after washing)	-	565.90×10^6	-

Fig. 5.11 The distribution of human and bovine sperm on discontinuous sucrose gradients.

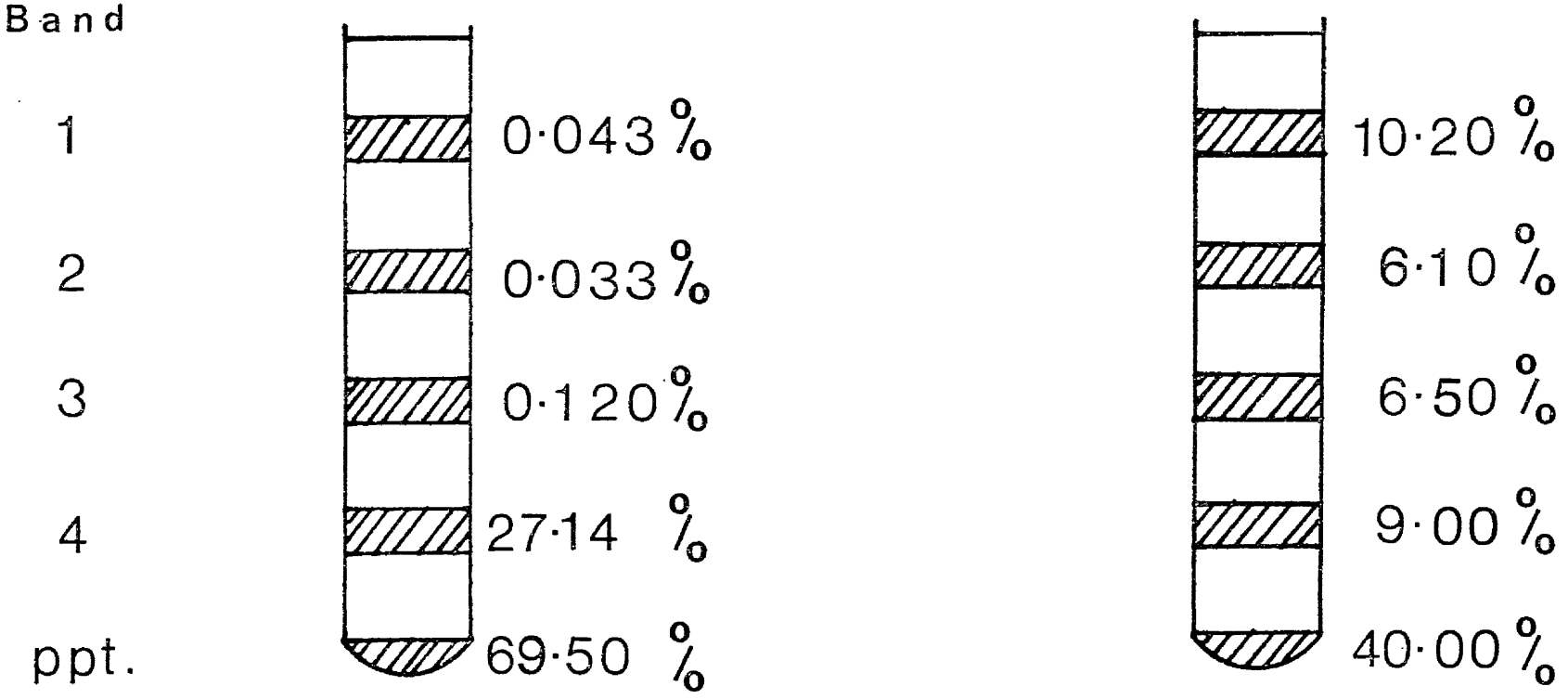

Band
1
2
3
4
ppt.
0·043 %
0·033 %
0·120 %
27·14 %
69·50 %
10·20 %
6·10 %
6·50 %
9·00 %
40·00 %
Human Sperm
Bovine Sperm

Table 5.11 Fluorescent Staining of Human Sperm Recovered from Discontinuous Sucrose Gradients

Centrifugal Force (g)	Time (Mins)	Control		Band 1		Band 2		Band 3		Band 4		Precipitate	
		Total Sperm Counted	Y Sperm Counted	Total Sperm Counted	Y Sperm Counted	Total Sperm Counted	Y Sperm Counted	Total Sperm Counted	Y Sperm Counted	Total Sperm Counted	Y Sperm Counted	Total Sperm Counted	Y Sperm Counted
12,000	60	311	125	273	84	88	27	300	94	307	96	465	132
12,000	60	520	178	565	135	126	33	403	102	491	145	921	281
12,000	60	517	183	101	22	118	31	349	82	250	64	303	112
Experiments with 2 ejaculates													
12,000	30	(i) 300 (ii) 305	111 121	320	89	234	71	250	77	320	82	304	131
12,000	30	(i) 314 (ii) 408	127 150	300	85	302	78	269	70	400	118	319	126
12,000	30	(i) 279 (ii) 415	122 138	112	38	144	32	350	101	405	140	400	149

Because both human and bovine sperm had behaved similarly on the gradient and separated into five populations, it was considered possible that the sperm might have been held back at the three interfaces between the sucrose solutions and at the sperm layer/gradient interface. If this were true, most sperm would precipitate eventually, if centrifugation were continued for long enough. Evidence for this was obtained in an experiment where centrifugation was allowed to continue for $3\frac{1}{2}$ hours, with photographs of the gradient being taken after 30 minutes and at subsequent hourly intervals. The photographs showed that, as centrifugation continued, the upper bands of sperm in the gradient became fainter, while the lowest band and precipitate increased in size. As a result of this, the use of a continuous sucrose gradient was investigated, to see if a population of sperm could be produced without the mechanical aid of interfaces.

(ii) Continuous System

The result of centrifuging human sperm on a continuous sucrose gradient, at 12,000 g, is shown on Fig. 5.12. The washed sperm formed a single band, in the region of the sperm layer/gradient interface, and a precipitate.

The centrifugal force was reduced to 5,000 g, in an attempt to produce a further separation of sperm and the result is shown in Fig. 5.13. The washed sperm separated into two populations in the gradient, and a precipitate. The upper band lay in the region of the sperm layer/gradient interface, while the lower band was wide and ill-defined.

Sperm were recovered from both gradients, stained with quinacrine dihydrochloride and examined for the presence of an

Fig. 5.12 Human sperm centrifuged on a continuous sucrose gradient at 12,000 g.

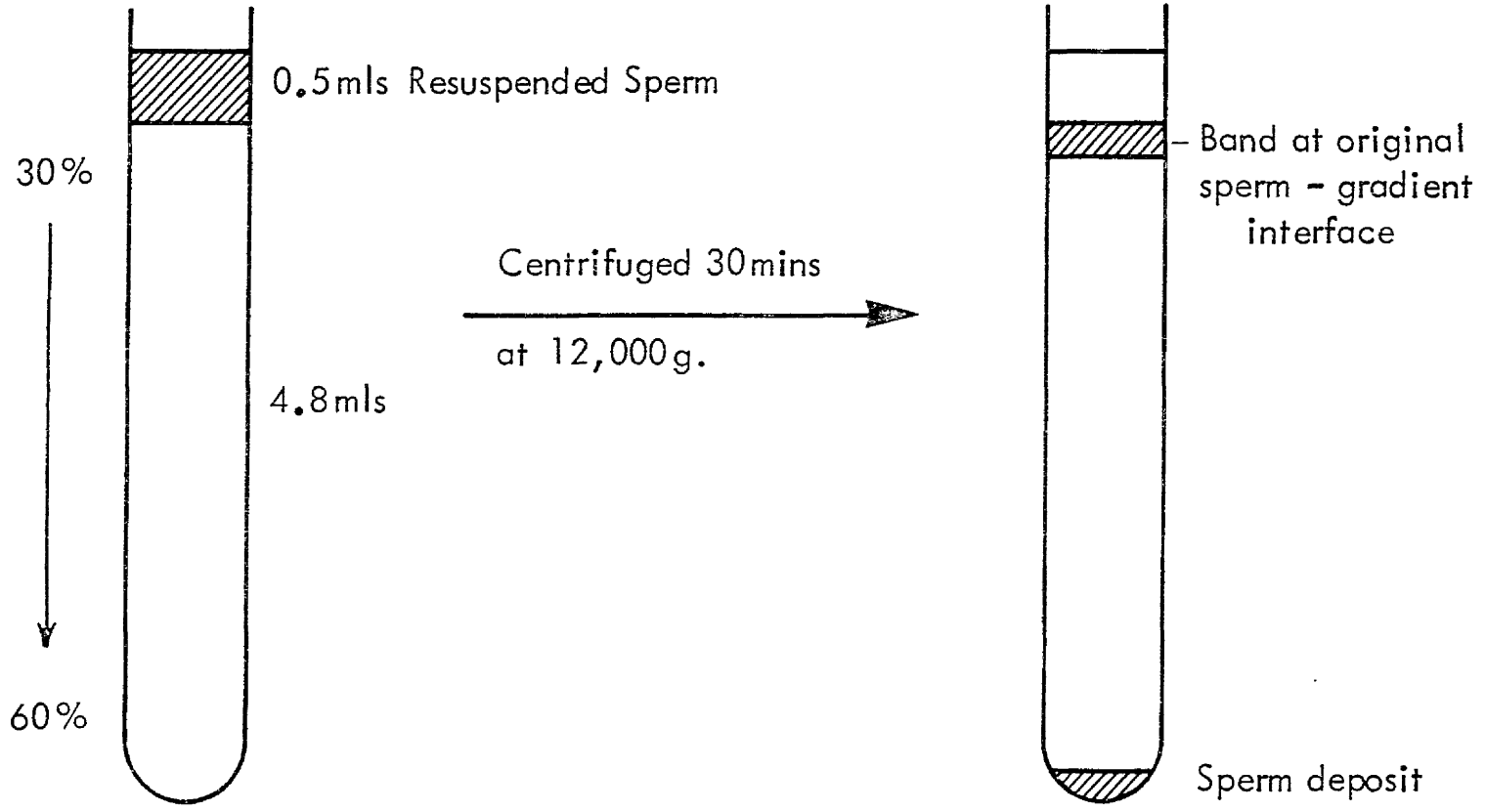
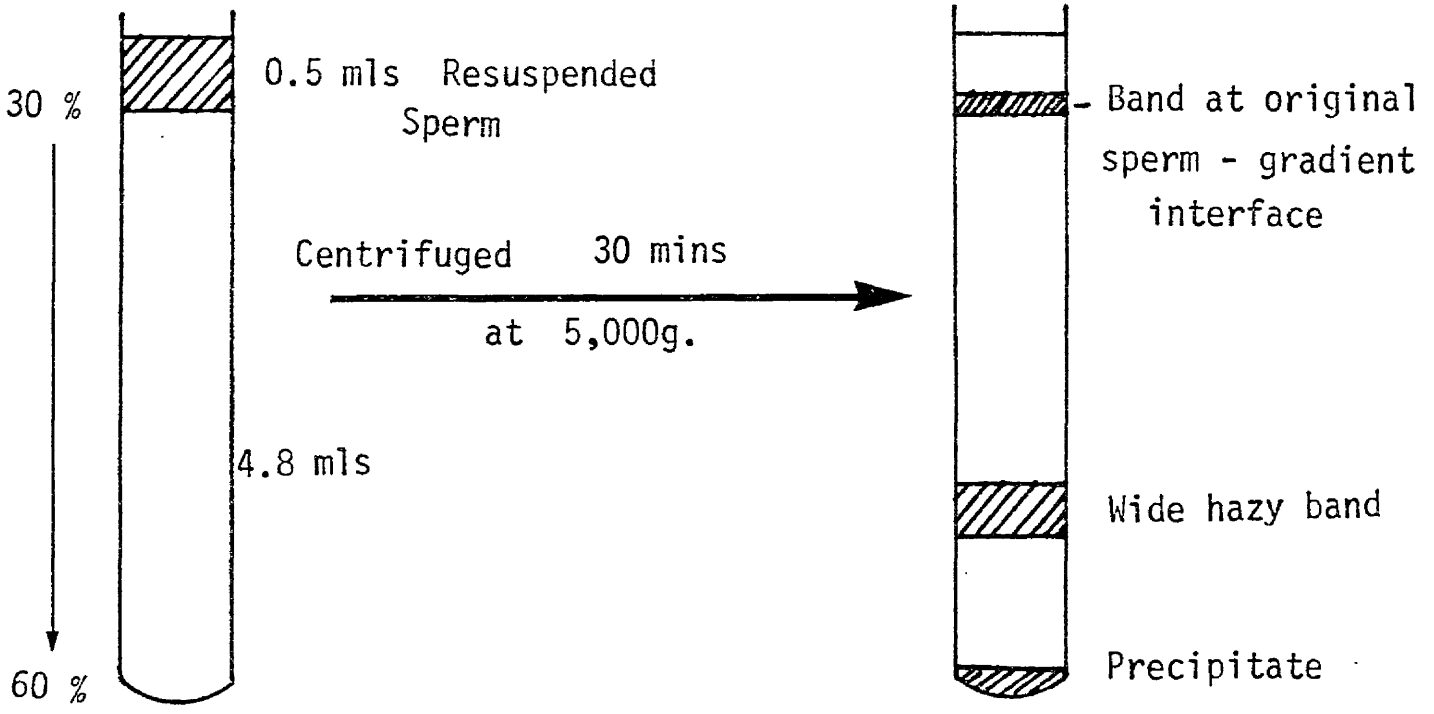

0.5mls Resuspended Sperm
30%
4.8mls
60%
Centrifuged 30mins
at 12,000g.
Band at original sperm - gradient interface
Sperm deposit

Fig. 5.13 Human sperm centrifuged on a continuous sucrose gradient at 5,000 g.


30 %
0.5 mls Resuspended Sperm
Centrifuged 30 mins
at 5,000g.
4.8 mls
60 %
Band at original sperm - gradient interface
Wide hazy band
Precipitate

F-body. The results are shown on Table 5.12, where it may be seen that there was no significant difference ($p = \leq 0.05$) between the numbers of Y sperm recovered from the bands or the precipitate formed in the gradients and the controls.

Table 5.12 Fluorescent Staining of Human Sperm Recovered from Continuous Sucrose Gradients

Centrifugal Force (g)	Time (Mins)	Control		Upper Band		Lower Band		Precipitate	
		Total Sperm Counted	Y Sperm Counted	Total Sperm Counted	Y Sperm Counted	Total Sperm Counted	Y Sperm Counted	Total Sperm Counted	Y Sperm Counted
12,000	30	(i) 403 (ii) 406	140 174	401	126	-	-	401	138
12,000	30	(i) 400 (ii) 404	151 162	400	145	-	-	400	152
12,000	30	(i) 400 (ii) 406	163 168	402	158	-	-	401	161
5,000	30	(i) 400 (ii) 417	139 161	239	80	500	153	369	135
5,000	30	(i) 403 (ii) 409	148 139	306	123	400	129	400	140
5,000	30	(i) 400 (ii) 402	135 170	256	98	400	127	403	134

5.6 MATERIALS AND METHODS FOR METRIZAMIDE GRADIENTS

Metrizamide, (2-(3-acetamido - 5-N- methylacetamido - 2, 4, 6 - tri-iodo - benzamido -) - 2 deoxy - D - glucose) was supplied as a white powder, (Nyegaard and Co. Ltd., Oslo, Norway).

5.6.1 Preparation of Sperm Diluent and Metrizamide Solutions

As a diluent for sperm, calcium-free Ringer's solution was prepared, as described before. Metrizamide solutions of 5%, 10%, 20%, 30%, 40%, 50%, 55%, 60% and 70% (w/v) concentrations were prepared in calcium-free Ringer's solution. The specific gravity of each solution was measured directly, by weighing, and its refractive index was measured on an Abbé 60 refractometer.

5.6.2 Preparation of Gradients

Two methods were used:

(i) A self-generating gradient was prepared, according to the method of Rickwood, Hell and Birnie (1973). 5.5 ml of a 40% Metrizamide solution were centrifuged at 4°C for 48 hours at 161,000 g, in a swinging bucket aluminium SW65K rotor, using an L2-65B ultracentrifuge, (Beckman Ltd.).

(ii) Density gradients of 5.5 ml volume were prepared in a Perspex double-chambered gradient maker, starting with equal volumes of 5% and 55% Metrizamide solutions. The gradients were pumped, by a peristaltic pump, into cellulose nitrate tubes and then centrifuged at 4°C for 24 hours at 161,000 g.

5.6.3 Preparation of Sperm and Application to the Gradients

At the beginning of each experiment, 0.5 ml human semen was retained from each ejaculate, as experimental control. Control and experimental volumes were diluted with equal volumes of calcium-free Ringer's solution and washed three times, at $4^{o}C$, for 10 minutes at 110 g. After the final washing, the experimental sperm were resuspended to 0.5 ml volume in calcium-free Ringer's solution and this suspension was applied to the gradient carefully, using a Pasteur pipette.

One ml bovine semen was washed three times in calcium-free Ringer's, under the same conditions as above. After the final washing, the sperm pellet was resuspended in calcium-free Ringer's solution to 0.5 ml, and applied to the gradient, using a Pasteur pipette.

5.6.4 Conditions of Centrifugation

The gradients were centrifuged, at $4^{o}C$ for 30 minutes at 2,500 g in an SW65K swinging bucket rotor, using the L2-65B ultracentrifuge.

5.6.5 Examination and Harvesting of Gradients

At the end of centrifugation, the gradients were examined against darkground illumination, for the distribution of sperm.

Blank gradients were harvested by downward displacement, after puncturing the base of the tube with a 21 gauge needle, attached to a peristaltic pump. Fractions of 0.20 ml volume were collected.

Harvesting of populations of sperm, visible within the gradient, was carried out as described in the section on sucrose gradients.

5.6.6 Recovery of Sperm from Harvested Fractions

Fractions containing sperm were diluted with an equal volume of calcium-free Ringer's and centrifuged, at $4^{\circ}C$, for 20 minutes at 950 g. Each pellet of sperm was washed once more, then resuspended in fixative (3 : 1, methanol : acetic acid) for 20 - 30 minutes, before smears were prepared on clean wet slides, and dried rapidly.

5.6.7 Experimental Controls

Sperm used as experimental controls were fixed for 20 - 30 minutes in 3 : 1, methanol : acetic acid, and smears were prepared, as described before.

5.6.8 Fluorescent Staining of Human Sperm

The smears were stained for 5 - 6 minutes in 0.5% aqueous quinacrine dihydrochloride and mounted in water, as described before. The slides were examined, immediately, in ultra violet light, as before.

5.7 RESULTS FOR METRIZAMIDE GRADIENTS

5.7.1 Osmolarity, Specific Gravity and Refractive Index of Solutions

The values obtained for specific gravity, refractive index and osmolarity of Metrizamide solutions are shown in Table 5.13, where it may be seen that, with increasing concentration, the osmolarity rose from 345 mOsmols for the 5% solution to 1,020 mOsmols for the 70% solution. The osmolarities of all the Metrizamide solutions were greater than the values of 302 mOsmols, for human semen, and 339 mOsmols, for bovine semen reported by Mann (1964).

From the values shown in Table 5.13, the relationship between specific gravity and refractive index for Metrizamide solutions was determined and this is shown in Fig. 5.14. This relationship was direct and linear, so that measurement of the refractive index of any Metrizamide solution provided a rapid method of assessing its specific gravity.

5.7.2 Survival of Sperm in Metrizamide Solutions

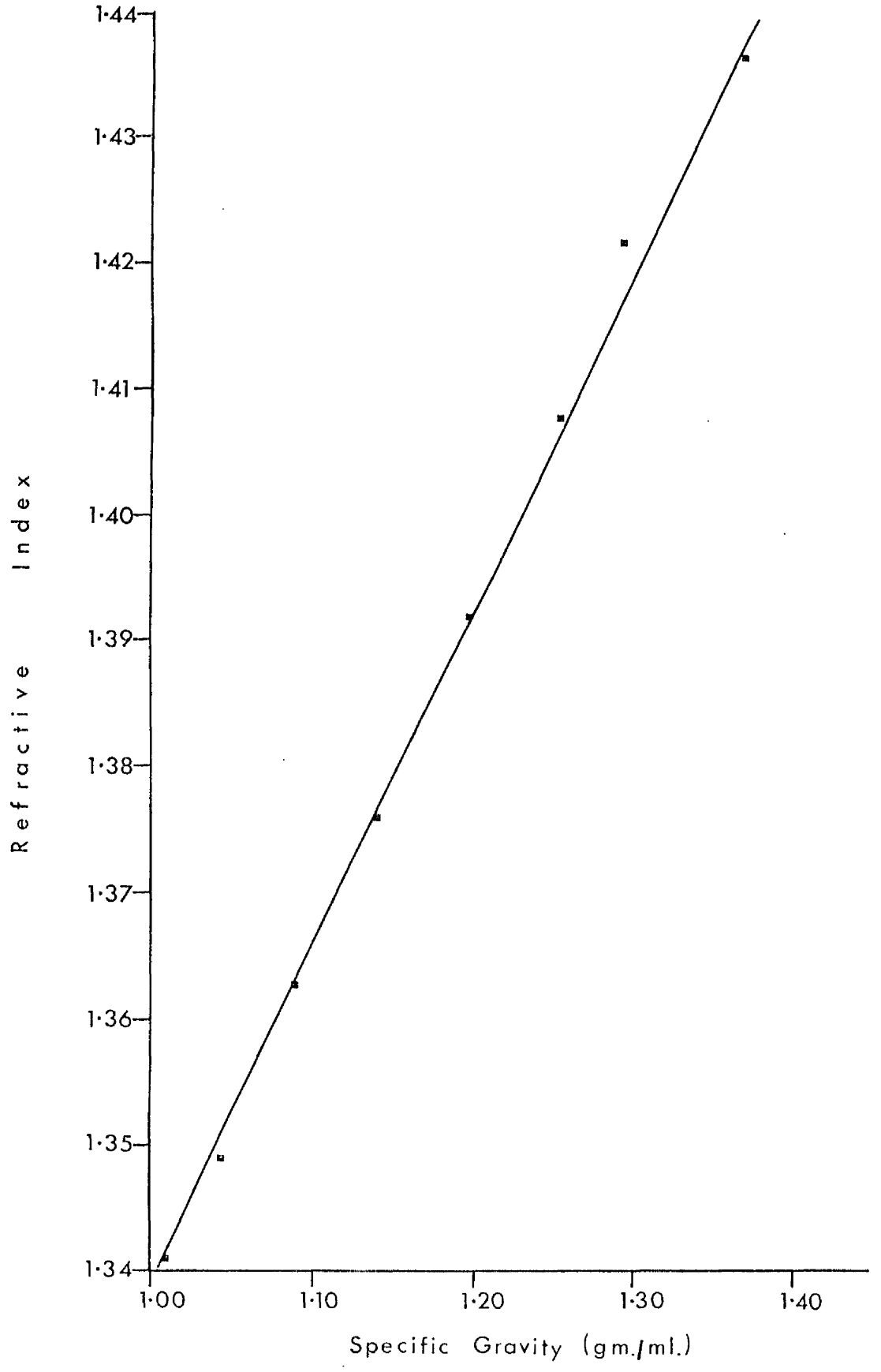
(a) Human Sperm

Eight test tubes were prepared, each containing 1 ml of Metrizamide solution of a particular concentration. 0.2 ml fresh human semen was added to each solution, mixed gently and left to stand for two hours. After this time, one drop from each tube was placed on a clean slide and examined microscopically. Survival of sperm was assessed by the presence or absence of motility.

Table 5.13 Specific Gravity, Refractive Index and Osmolarity of Metrizamide Solutions

Concentration of Metrizamide Solution (w/v)	Specific Gravity (gm/ml)	Refractive Index	Osmolarity (mOsmols)
5%	1.0094	1.341	345
10%	1.0433	1.348	400
20%	1.0888	1.363	425
30%	1.1400	1.376	480
40%	1.1966	1.392	575
50%	1.2533	1.408	690
60%	1.2933	1.422	810
70%	1.3700	1.437	1,020

Fig. 5.14 The relationship between specific gravity and refractive index of Metrizamide solutions.


1·44
1·43
1·42
1·41
1·40
1·39
1·38
1·37
1·36
1·35
1·34
Refractive Index
1·00
1·10
1·20
1·30
1·40
Specific Gravity (gm./ml.)

(b) Bovine Sperm

The experiment described above was repeated, using bovine semen.

The results of both experiments are shown in Table 5.14 and it may be seen that motile sperm of both species were present in solutions of up to 50% concentration, with the most vigorous progressive motility shown in the 5% solution. In the 60% and 70% solutions, most of the sperm were immotile, although occasionally, an individual sperm showed weak, non-progressive motility.

5.7.3 Examination of Gradients for Linearity

(i) Self-Generating Gradients

After centrifugation for 48 hours at 161,000 g, the gradient was harvested in 0.2 ml fractions and the refractive index of each one was measured. These results are shown in Fig. 5.15 and it may be seen that, as the Metrizamide redistributed in the tube, during centrifugation, a sigmoid gradient was formed. Using Figs. 5.14 and 5.15, the specific gravity range of this gradient was found to be 1.089 - 1.432 gm /ml.

(ii) Pre-Formed Gradients

The pre-formed gradient was centrifuged for 24 hours at 161,000 g, then harvested in 0.2 ml fractions and the refractive index of each fraction was measured. These results are shown in Fig. 5.16, and it may be seen that a relatively smooth linear gradient was formed. From Figs. 5.14 and 5.16, the specific gravity range of this gradient was found to be 1.016 - 1.404 gm /ml.

Table 5.14 Survival of Sperm after Two Hours in Metrizamide Solutions

Concentration of Metrizamide Solution (w/v)	Human Sperm Survival	Bovine Sperm Survival
5%	++	++
10%	+	+
20%	+	+
30%	+	+
40%	+	+
50%	+	+
60%	-	-
70%	-	-

++ = vigorously motile

\+ = motile

\- = immotile

Fig. 5.15 A self-generated Metrizamide gradient.

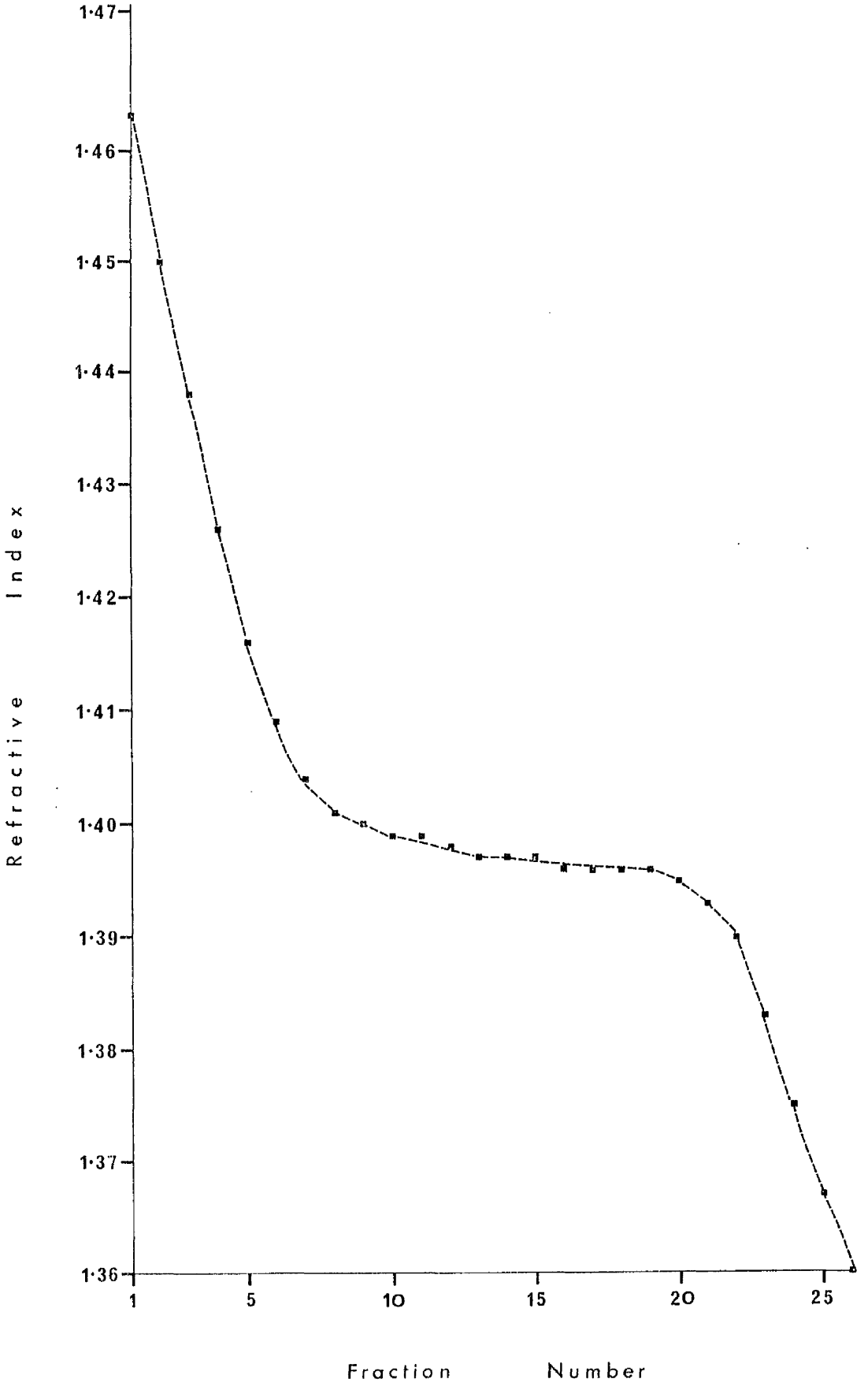
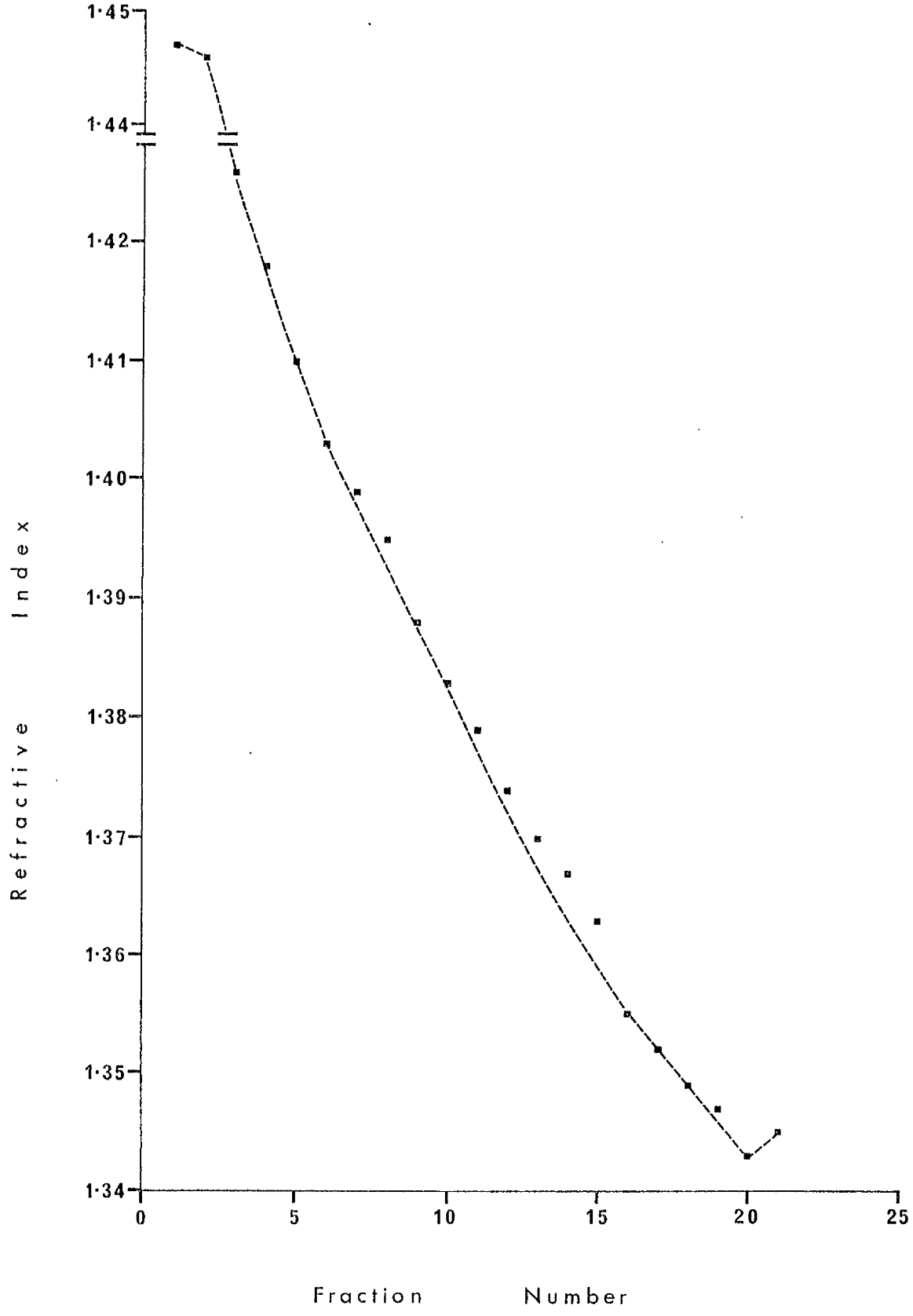

1·47
1·46
1·45
1·44
1·43
1·42
1·41
1·40
1·39
1·38
1·37
1·36
Refractive Index
1
5
10
15
20
25
Fraction Number

Fig. 5.16 A pre-formed Metrizamide gradient.


1·45
1·44
1·42
1·41
1·40
1·39
1·38
1·37
1·36
1·35
1·34
Refractive Index
0
5
10
15
20
25
Fraction Number

5.7.4 Examination of Gradients containing Sperm, after Centrifugation

(i) Self-Generating Gradients

Human sperm were applied to the gradient and centrifuged for 30 minutes at 2,500 g. After this time, the gradient was examined in indirect light and, as shown in Fig. 5.17, two populations were visible in the gradient. These two populations were harvested and microscopic examination showed that each contained sperm showing weak, non-progressive motility. The refractive index of each band of sperm was measured and, from Fig. 5.14, the specific gravity at which the sperm had settled in the gradient was estimated.

The refractive index values for the two sperm bands were super-imposed on the sigmoid gradient shown in Fig. 5.15, as shown in Fig. 5.18, and it may be seen that the two sperm populations had separated in the steep parts of the sigmoid gradient, at specific gravities of 1.116 gm/ml and 1.228 gm/ml. The two sperm populations were recovered by centrifugation, washed and stained with quinacrine dihydrochloride. The smears were examined in ultra violet light and the number of sperm showing the presence of an F-body was noted. The results of six experiments are shown in Table 5.15, and it may be seen that there was no significant difference ($p \leq 0.05$) between the numbers of Y sperm recovered from the bands of sperm in the gradient and the controls, except for a significant but small decrease in Y sperm in the upper band of experiment 5, compared to the control.

Human sperm had separated into two populations on the sigmoid gradient, but no increase in the numbers of Y sperm had been observed in either population. Consequently, the behaviour

Fig. 5.17 Human sperm centrifuged on a self-generated Metrizamide gradient.

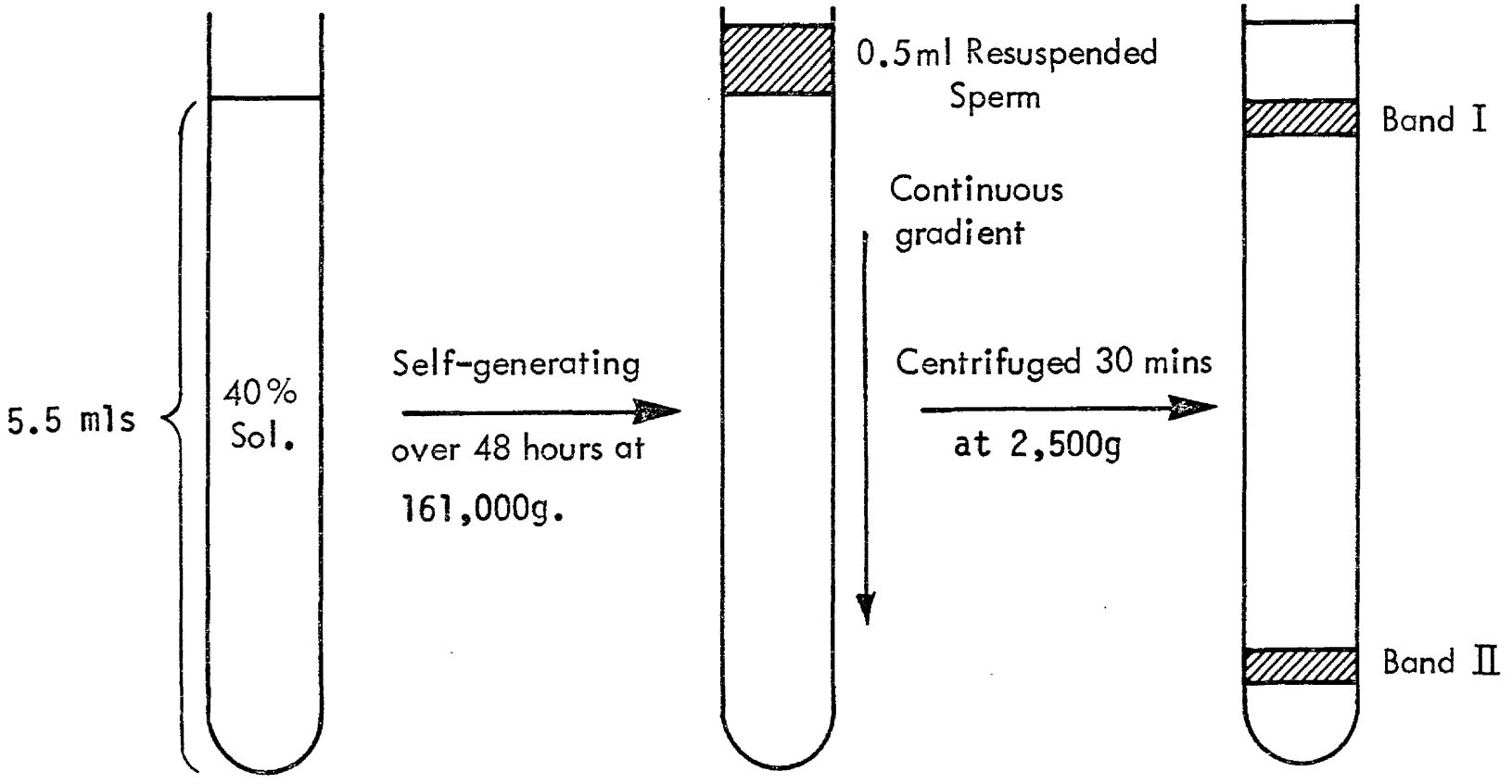

5.5 mls
40%
Sol.
Self-generating
over 48 hours at
161,000g.
0.5ml Resuspended
Sperm
Continuous
gradient
Centrifuged 30 mins
at 2,500g
Band I
Band II

Fig. 5.18 The sites of banding of human sperm on a self-generating Metrizamide gradient.

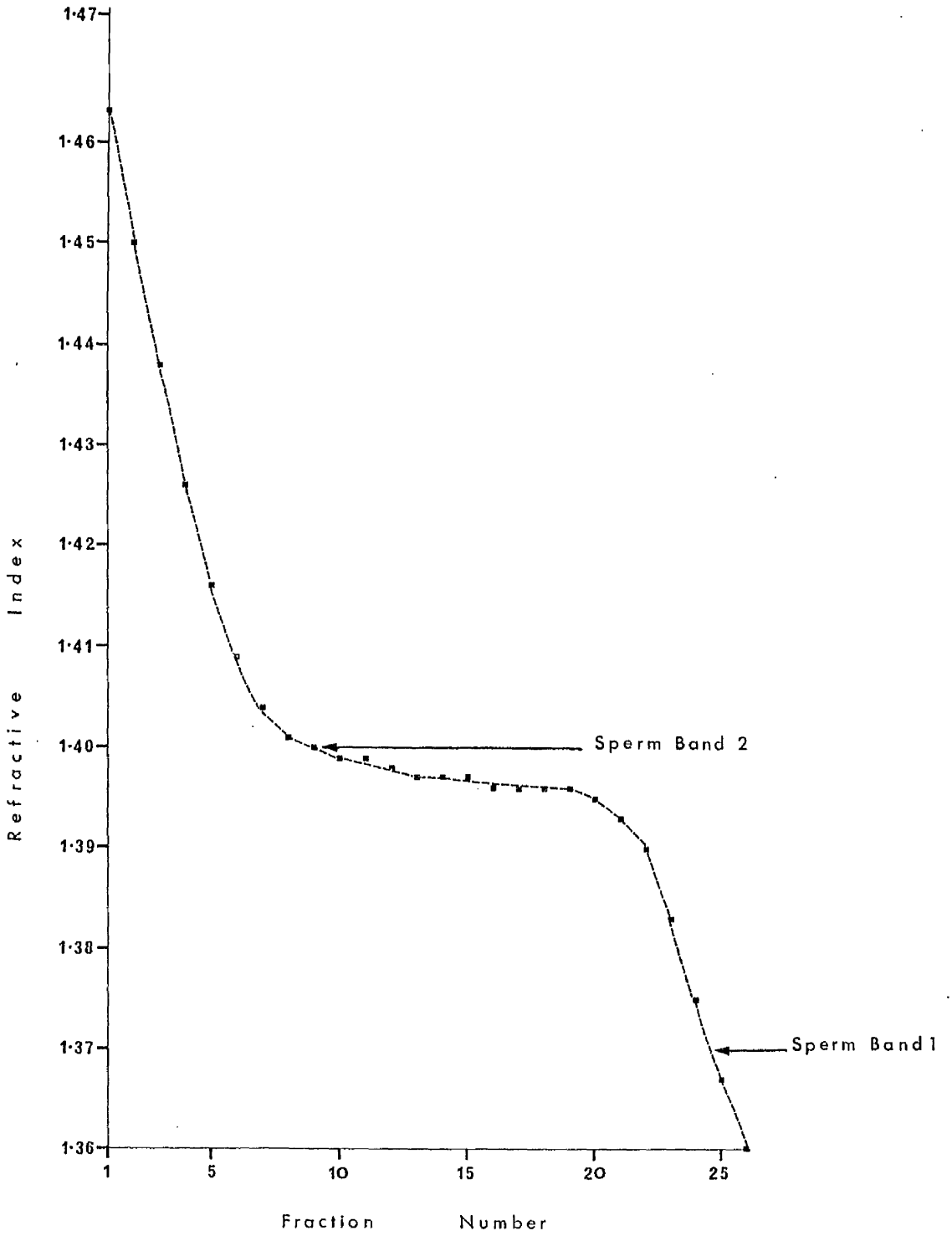

1·47
1·46
1·45
1·44
1·43
1·42
1·41
1·40
1·39
1·38
1·37
1·36
Refractive Index
Sperm Band 2
Sperm Band 1
1
5
10
15
20
25
Fraction Number

Table 5.15 Fluorescent Staining of Human Sperm Recovered from Self-Generating Metrizamide Gradients

Expt. Number	Control			Band 1			Band 2		
	Total Sperm Counted	Y Sperm Counted	% Y Sperm	Total Sperm Counted	Y Sperm Counted	% Y Sperm	Total Sperm Counted	Y Sperm Counted	% Y Sperm
1	406	159	39.2	409	135	33.0	693	226	32.6
2	400	159	39.8	414	158	38.2	642	210	32.5
3	404	138	34.2	419	153	36.5	330	118	35.8
4	400	129	32.3	364	126	34.6	415	147	35.4
5	423	182	43.0	402	130	32.3	223	77	34.5
6	476	162	34.0	401	105	26.2	406	126	31.0

of sperm on pre-formed linear gradients was examined.

(ii) Pre-Formed Gradients

Following centrifugation on this gradient for 30 minutes at 2,500 g, human sperm separated into two populations in the gradient, as shown in Fig. 5.19. These sperm populations were harvested and microscopic examination showed that most of the sperm present were immotile, although an occasional sperm showed weak, non-progressive motility. There were no obvious differences between the sperm in the two bands. Refractive index measurements showed that the sperm bands lay at specific gravities of 1.164 gm/ml and 1.264 gm/ml.

The two sperm populations were recovered by centrifugation, washed and stained with quinacrine dihydrochloride. The sperm were examined in ultra violet light and the number of these showing the presence of an F-body was noted. The results of four experiments are shown in Table 5.16, and it may be seen that, for three of these experiments, there was no significant difference ($p \geq 0.05$) between the numbers of Y sperm in the sperm bands and in the control. However, in experiment 3, there was a significantly lower number of Y sperm in the bands, compared to the control.

Human sperm, killed by immersion in liquid nitrogen at -196°C for one minute, were centrifuged on a similar pre-formed gradient. The results are shown in Fig. 5.20 and it may be seen that a single floccular band formed in the gradient, in contrast with the two bands formed when living sperm were applied to the gradient, (see Fig. 5.19). Refractive index measurement showed that the floccular band had formed at a specific gravity of 1.234 gm/ml.

Fig. 5.19 Human sperm centrifuged on a pre-formed Metrizamide gradient.

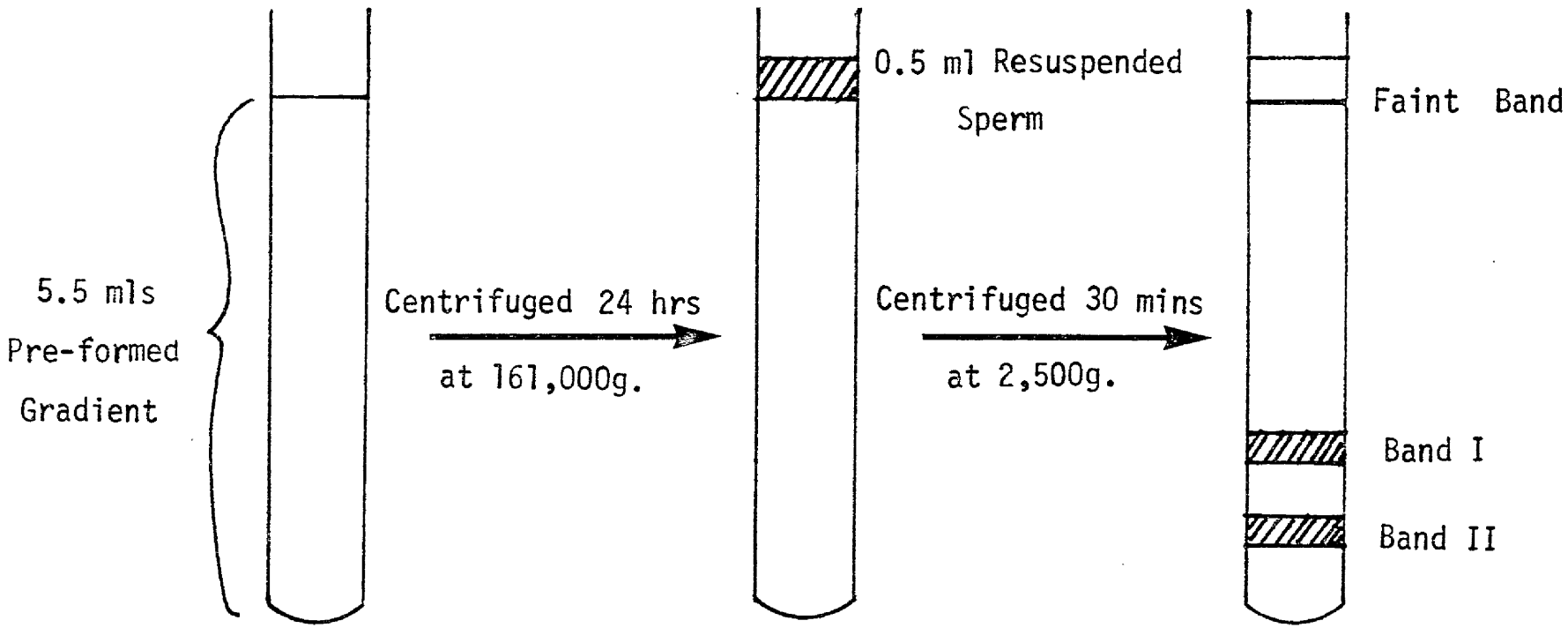
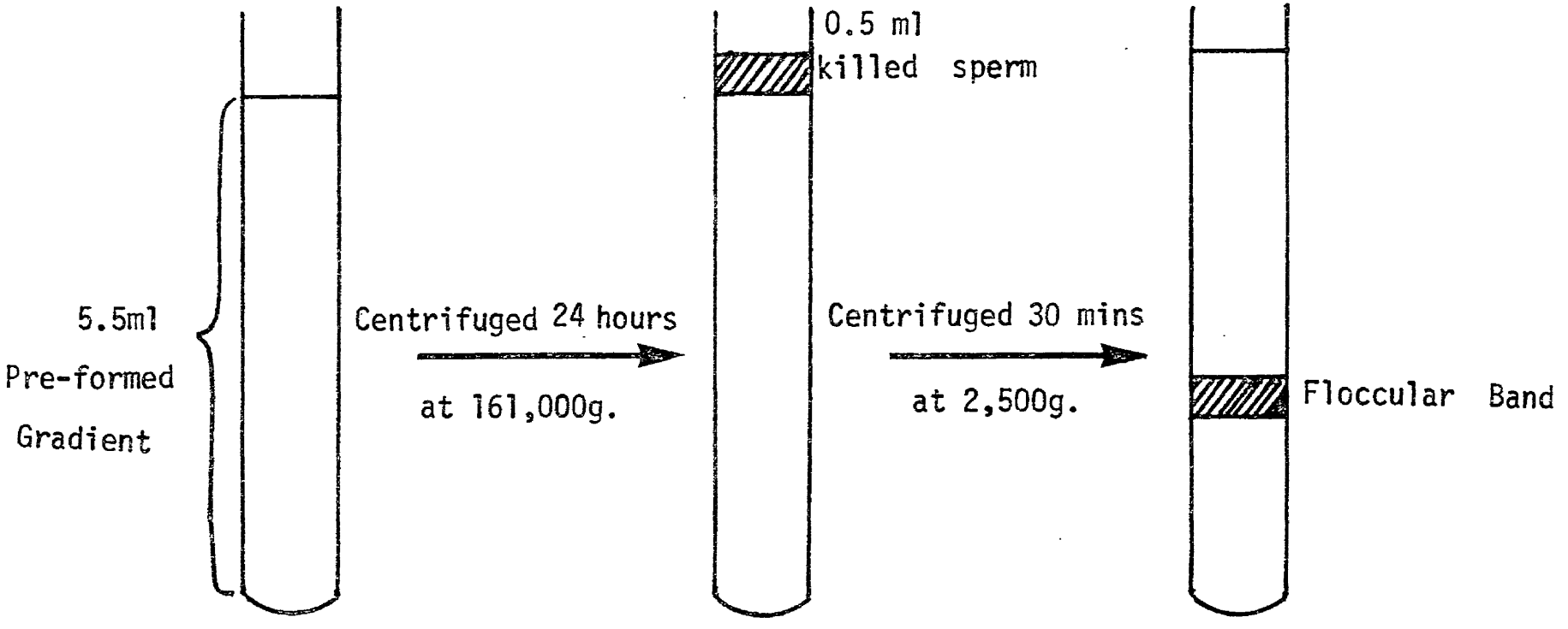

5.5 mls
Pre-formed
Gradient
Centrifuged 24 hrs
at 161,000g.
0.5 ml Resuspended
Sperm
Centrifuged 30 mins
at 2,500g.
Faint Band
Band I
Band II

Table 5.16 Fluorescent Staining of Human Sperm Recovered from Pre-Formed Metrizamide Gradients

Expt. Number	Control			Band 1			Band 2		
	Total Sperm Counted	Y Sperm Counted	% Y Sperm	Total Sperm Counted	Y Sperm Counted	% Y Sperm	Total Sperm Counted	Y Sperm Counted	% Y Sperm
1	411	147	36.0	402	136	34.0	403	146	36.2
2	401	139	34.7	801	241	30.1	403	111	27.5
3	415	165	39.8	404	118	29.2	416	115	27.6
4	400	145	36.3	395	140	35.4	406	144	35.5

Fig. 5.20 Killed human sperm centrifuged on a pre-formed Metrizamide gradient.


5.5ml
Pre-formed
Gradient
Centrifuged 24 hours
at 161,000g.
0.5 ml
killed sperm
Centrifuged 30 mins
at 2,500g.
Floccular Band

As a comparison with human sperm, washed bovine sperm were applied to similar pre-formed Metrizamide gradients and centrifuged. Two populations of sperm separated in the gradient, similar to those obtained with human sperm, (see Fig. 5.19). When bovine sperm, killed by immersion in liquid nitrogen for one minute, were centrifuged on the gradient, a single floccular band was formed in the gradient, similar to that obtained with killed human sperm, (see Fig. 5.20).

It has been shown that centrifugation of both washed human sperm and washed bovine sperm on pre-formed Metrizamide gradients produced a separation of two populations of sperm. Examination of the two separated populations of human sperm by fluorescent staining showed little apparent difference in the numbers of Y sperm in the bands, compared to the controls and, when killed sperm of either species were centrifuged on the gradient, a single sperm population formed in the gradient. This situation was similar to the findings obtained when bovine sperm were centrifuged on colloidal silica gradients and, once again, the possibility was considered that a separation of living and dead or damaged sperm might be taking place, this time on Metrizamide gradients.

To investigate this possibility, the method described by Hancock (1952) for examination of the appearance of the acrosome in unstained bovine sperm was used, as described previously. The results of phase contrast examination of bovine sperm recovered from the Metrizamide gradient are shown in Table 5.17 and it may be seen that the highest percentage of dead sperm (assessed by detachment of the acrosome) occurred in the lower sperm band formed in the Metrizamide gradient.

These observations on the acrosomes of unstained sperm,

Table 5.17 Phase Contrast Examination of Unstained Bovine Sperm, Recovered from Pre-Formed Metrizamide Gradients

Sperm Population	Total No. of Sperm Counted	No. of Sperm Showing Detachment of Acrosome	% Dead Sperm
Washed Sperm (Control)	200	124	62
Sperm from Upper Band	200	144	72
Sperm from Lower Band	200	176	88
Washed Sperm, killed in Liquid Nitrogen (Control)	208	192	92.3
Sperm Recovered from Floccular Band	202	182	90.1

under phase contrast, were carried out also on the human sperm populations recovered from Metrizamide gradients and the appearances of living and dead human sperm under phase contrast are shown in Plates 5.5 and 5.6. The distinction between living and dead human sperm by detachment of the acrosome, proved to be much more difficult to determine than for bovine sperm (see Plates 5.1 and 5.2). The results of phase contrast examination of human sperm populations recovered from Metrizamide gradients are shown in Table 5.18 and it may be seen that the highest percentage of dead sperm occurred in the lower sperm band formed in the gradient. The results obtained with human sperm would appear to agree with those obtained with bovine sperm and lend support to the hypothesis that a partial separation of living and dead sperm has occurred on the Metrizamide gradient.

Plate 5.5

Living Human Sperm examined under phase contrast at x 1,000 magnification

Plate 5.6

Dead Human Sperm examined under phase contrast at x 1,000 magnification

Table 5.18 Phase Contrast Examination of Unstained Human Sperm, Recovered from Pre-Formed Metrizamide Gradients

Sperm Population	Total No. of Sperm Counted	No. of Sperm Showing Detachment of Acrosome	% Dead Sperm
Washed Sperm (Control)	200	30	15
Sperm from Upper Band	210	82	39.0
Sperm from Lower Band	208	120	57.6
Washed Sperm, killed in Liquid Nitrogen (Control)	202	166	82.2
Sperm Recovered from Floccular Band	200	160	80.0

5.8 DISCUSSION

When using the technique of density gradient separation, it is desirable to know the expected density range of the cells or particles to be separated, so that a suitable gradient may be constructed. Harvey (1946) reported that the average specific gravity of human sperm was 1.0715 gm/ml, but gave no values for the specific gravity range of human sperm, whereas Oresnik (1969) found the specific gravity range of bovine sperm to be 1.083 - 1.102 gm/ml. This difference in the specific gravities of bovine and human sperm may perhaps be attributed to the greater number of chromosomes present, there being 60 in cattle compared to 46 in man, and to the greater size of the bovine sperm head, which is 9.2 μm long (Ortavant, Courot and Hochereau, 1969), compared with the average 4.6 μm head length of human sperm (Monesi, 1972). From the preliminary results reported in this thesis, it would seem that the specific gravity range of the colloidal silica gradients used (1.01 - 1.17 gm/ml) was suitable for the separation of sperm of both species.

When human sperm were centrifuged on the colloidal silica gradient, a single wide floccular band of sperm formed in the middle of the gradient, at a specific gravity of 1.08 gm/ml, (see Fig. 5.3), whereas bovine sperm separated into two, or in one case, three bands in the gradient, (see Fig. 5.4). Even when the force and time of centrifugation were reduced, no further separation of human sperm occurred. There could be several explanations for this difference in the behaviour of human and bovine sperm on the gradient, one possibility being the viscous nature of human seminal plasma. In the bull, the ejaculate remains liquid, but human semen appears to be

"clotted" immediately after ejaculation. This clotting of human semen is thought to be produced by the action of a clotting enzyme, secreted by the prostate, on a fibrinogen-like protein produced by the seminal vesicles, with the formation of a fibrin-like clot. Spontaneous liquefaction takes place when a plasmin-like enzyme has formed from an inactive precursor, also of prostatic origin, and lyses the clot. A third state in this process involves further proteolysis of the lysed fibrin, with the formation of free amino acids and ammonia, (Mann, 1964). The liquefaction of semen occurs approximately 20 minutes after ejaculation, although the exact time may vary with the ejaculate. In these experiments, the examination of fresh human semen was delayed until after liquefaction had occurred, so it would seem unlikely that the phenomenon of gelation was involved in the failure of human sperm to separate on colloidal silica gradients. A more likely reason for this failure may have been the high mucoprotein content of human seminal plasma, compared to that of the bovine. Attempts were made to remove this mucinous seminal plasma from human sperm by washing, before the sperm were applied to the gradient. After three washes, the sperm resuspended readily in 0.93 Baker's medium and it appeared that most of the mucinous material had been removed. Nevertheless, after centrifugation on colloidal silica, exactly the same wide floccular band of sperm was formed in the gradient. This would tend to suggest that the presence of seminal plasma was not the sole reason for human sperm forming a single band in the gradient.

The lack of separation of human sperm on colloidal silica gradients may be related to specific gravity. The specific gravity range of human sperm may turn out to be narrower than that reported for the bovine by Oresnik (1969), although no information on this

point is available to date. If indeed this were so, a better separation of human sperm might perhaps have been achieved using a gradient of narrower range.

Because human and bovine sperm have been shown to behave differently on colloidal silica gradients, the possibility that the sperm of these two species might differ in surface properties should be considered. However, in subsequent density gradient separations using sucrose and Metrizamide reported in this thesis, human and bovine sperm appeared to behave similarly, so that this possibility would seem to be remote, unless the surface properties of sperm were influenced somehow by the nature of the gradient medium. This should perhaps be borne in mind when human sperm are used in pilot separation experiments, in the expectation that bovine sperm will behave in the same way.

As mentioned earlier, the majority of the samples of bovine sperm examined separated into two bands on the colloidal silica gradient, although sperm from one bull, whose ejaculate contained a high percentage of decapitated sperm, separated into three bands on the gradient. In this case, the lower band contained mainly detached sperm heads, the middle band consisted of sperm with heads and tails attached and the upper band consisted mainly of free sperm tail - midpieces. The separation of bovine sperm on colloidal silica gradients appeared to be isopycnic, since with increased time of centrifugation, no change in the position of the sperm bands in the gradient was produced. Therefore, it would appear that the bovine ejaculates contained two populations of sperm, differing in specific gravity. This was surprising, because previous microscopic examination of bovine semen had shown that the sperm

were remarkably uniform in appearance and in size, compared to the diversity in appearance of human sperm.

A possible explanation for the division of bovine sperm into two populations on the colloidal silica gradient could be that a separation of living and dead cells had taken place. Benedict et al. (1967) reported that dead bovine sperm showed an increased permeability and absorbed surrounding gradient media, with a consequent increase in their specific gravity. The lower band of bovine sperm in the colloidal silica gradients might have contained a greater number of dead or dying sperm and to investigate this, nigrosin/eosin staining was carried out on sperm recovered from the gradient. Nigrosin/eosin staining has been described by Campbell et al. (1953), as a method of differentiating between living and dead bovine sperm. The increased permeability of dead sperm allows the entry of eosin, so that dead sperm are stained pink against the dark blue background of nigrosin, while living sperm remain unstained. However, one drawback to this method is that sperm in the process of dying may either fail to stain or stain only partially, so that they may not be recognised as dying cells. For this reason, the more sophisticated technique of identifying dead sperm by detachment of the acrosome (Hancock, 1952) was also used to examine sperm recovered from colloidal silica gradients. Nevertheless, it may be seen from the results that the question of whether living and dead bovine sperm had separated on colloidal silica gradients was not answered clearly by either of these techniques. Further investigation of this problem might have included an examination of the uptake of radio-actively labelled glucose by sperm recovered from the gradients, according to the method of Van der Horst, (cited by Perera, 1975). Living sperm would metabolise

this glucose to lactic acid, which could be recovered and examined for radioactivity, while in the presence of dead sperm, the glucose would remain unchanged.

Another possibility is that a separation of sperm according to age had occurred on the colloidal silica gradient. Lindahl and Kihlström (1952) and Lavon, Volcani, Amir and Danon (1966) reported that the specific gravity of bovine sperm increased during the maturation process. Therefore, it seemed possible that a separation of either immature and mature sperm or mature and aged sperm might have taken place on the gradient. Immature sperm, characterised by the presence of a cytoplasmic droplet attached at some point along the length of the sperm tail, are retained in the epididymis and are not normally present in the ejaculate. However, they may appear in the ejaculate when the bull is used repeatedly, to the point of exhaustion although for this reason, it would seem unlikely that any immature sperm were present in the ejaculates used in these experiments. On the other hand, a separation of mature and aged sperm might have occurred, but unfortunately, it would seem to be impossible, as yet, to distinguish aged from mature sperm, by examination under the light microscope. If, at some future date, a method of differentiating mature and aged sperm does become available, further investigation of the separation of bovine sperm on colloidal silica gradients might be warranted.

The most serious disadvantage of using colloidal silica was that it could not be removed from sperm harvested from the gradients, even by repeated washing. When nigrosin/eosin stained smears of bovine sperm were examined, large flakes of colloidal silica were observed on the slides. Colloidal silica was present also in the preparations of human sperm, even after repeated washing and,

when these were stained with quinacrine dihydrochloride, the bright fluorescence of the colloidal silica in ultra violet light prevented the examination of the sperm head for the presence of an F-body. This meant that no technique was available to monitor any change in the ratio of X- and Y-bearing sperm. For this reason and because of the lack of separation of human sperm, no further investigations were conducted using colloidal silica gradients.

At this time, Rohde et al. (1975) reported the successful fluorescent staining of human sperm recovered from discontinuous sucrose gradients, and it was felt that an investigation of their separation technique would prove worthwhile, even though Benedict et al. (1967) had shown that the high osmotic pressure of sucrose gradients was lethal to rabbit and bovine sperm. It was appreciated at the outset that the osmotic pressure of the sucrose gradient was likely to be lethal to human sperm, but it was hoped that if similar results to those of Rohde et al. (1975) could be achieved, a search for a more suitable gradient medium might then be made, in an attempt to recover living sperm.

The experiments described in this thesis showed that human and bovine sperm behaved similarly on the discontinuous sucrose gradient and appeared to separate at the interfaces in the gradient. Recovery of sperm from the upper bands was poor, mainly because of the low numbers of sperm retained here, as most of them had precipitated to the bottom of the tube, (see Fig. 5.11). It is probable that the sperm, killed by the high osmotic pressure of the sucrose solutions, absorbed some of the gradient medium, with consequent increase in specific gravity. When centrifuged, only small numbers of sperm were held back at the interfaces, with most of the

sperm precipitating through the gradient. However, contrary to the results of Rohde et al. (1975), there was no significant increase in the numbers of Y sperm in the two uppermost bands in the gradient. Indeed, in all the cases shown in Table 5.11, fewer Y sperm were recovered from the four bands in the gradient, compared to the controls. This decrease in the numbers of Y sperm was small, even though significant in some cases. Logically, the next step would have been to apply the separation technique to the sperm of a laboratory animal, with insemination of the separated sperm and sexing of the offspring, to see if a real change in the ratio of X and Y sperm had been produced, but because the sperm recovered from the sucrose gradients were dead, no opportunity to do this was available.

To try to improve sperm recovery from the upper bands in the gradient, reductions in the time and force of centrifugation might have been examined, but this was not carried out in the present series of experiments, the reason being that the sucrose gradients were lethal to sperm. Preliminary experiments using continuous sucrose gradients were also carried out, to see if separation of sperm could be produced without the mechanical aid of interfaces, but although separation did take place, there was no indication of a significant change in the ratio of X and Y sperm in the separated populations.

The study of density gradient separations of sperm continued with an investigation of the use of Metrizamide as a density gradient medium. Solutions of Metrizamide have a much lower osmotic pressure than sucrose solutions of corresponding density and preliminary experiments showed that both human and bovine sperm could

survive in all but the most concentrated Metrizamide solutions. Further studies using the sigmoid Metrizamide gradient showed that human sperm tended to collect in the two steep areas of the gradient, where there was a large density span over a few fractions of the gradient. Shortman (1972) has pointed out that cells or particles may separate artifactually in such regions of a gradient. Human sperm were recovered from these gradients and stained with quinacrine dihydrochloride, but no consistent significant change in the ratio of X and Y sperm was shown in the separated populations, in the small number of experiments carried out.

The use of a preformed Metrizamide gradient was examined also and this gradient was found to separate both human and bovine sperm into two main populations. However, quinacrine staining of human sperm harvested from this gradient showed that no consistent significant change in the ratio of X and Y sperm was produced. As before with colloidal silica gradients, the possibility arose that this separation of sperm on Metrizamide gradients occurred between living and dead or damaged cells. This possibility was strengthened when it was found that sperm killed by immersion in liquid nitrogen formed only one band in the gradient. In order to examine this further, nigrosin/eosin staining of sperm recovered from Metrizamide gradients might have been carried out, but because of the difficulties with this method outlined before (e.g. partial staining of sperm), it was decided to use the technique of examining sperm for detachment of the acrosome, as described by Hancock (1952). The appearance of the acrosome in living and dead bovine sperm has been well documented by Hancock (1952) and the technique for examining the acrosome proved to be relatively straightforward

when used in the present series of experiments. However, the appearance of the acrosome in living and dead human sperm does not seem to have been reported previously and proved to be more difficult to examine, (see Plates 5.5 and 5.6), as separation of the acrosome from the sperm was much less obvious than in bovine sperm. This difference between human and bovine sperm might be explained by differences in their structure. In the living bovine sperm, the acrosome fits very closely to the sperm head (see Plate 5.7) and, when death of the sperm occurs, shrinkage of the acrosome away from the sperm causes a gap to be left between it and the sperm head. On the other hand, in the living human sperm, the acrosome appears to be less closely applied to the sperm head (Aughey, 1976), as shown in Plate 5.8, so that when death of the sperm occurs, any shrinkage of the acrosome away from the sperm head may perhaps be less noticeable.

The separation of sperm into bands on density gradients is intriguing and no real explanation of what these bands represent has been forthcoming from the present experiments, although some separation of living and dead sperm in an ejaculate may be taking place. Further investigation of this separation may be warranted in the future, as new techniques of examining sperm become available. No significant change in the ratio of X and Y sperm has been produced in these density gradient separations and it is considered to be extremely unlikely that a complete separation of X and Y sperm will be achieved using this technique, because the calculated difference in the specific gravity of X and Y sperm is extremely small. However, the possibility that some partial degree of separation of X and Y sperm might be achieved using a density gradient technique should not be ruled out.

Plate 5.7 Electron Micrograph of Bovine Sperm stained with Uranyl Acetate and Lead Citrate at x 25,000 magnification

Plate 5.8 Electron Micrograph of Human Sperm stained with Uranyl Acetate and Lead Citrate at x 25,000 magnification

CHAPTER SIX

THE SEPARATION OF HUMAN SPERM USING SEPHADEX G-50

CHAPTER SIX

THE SEPARATION OF HUMAN SPERM USING SEPHADEX G-50

6.1 INTRODUCTION

Gel chromatography is a well-recognised means of separating particles which differ in size. The tool for obtaining the separation is a chromatographic bed, consisting of minute gel particles, swollen in an aqueous solution and packed into a column. When a mixture of particles differing in size is placed on the chromatographic column, the particles separate by their different rates of flow through the column: large particles pass between the beads of chromatographic material and emerge first, while smaller particles penetrate the gel beads and are retained. The technique of gel chromatography has been used extensively (Fischer, 1969) and some applications include enzyme and antibody purifications, the separation and purification of peptide and protein hormones, to determination of molecular weights, (especially of proteins), and the removal of allergens from pharmaceutical preparations, but its most recent use has been for the separation of cells.

Ly and Mishell (1974) reported that the passage of mouse spleen cells through a column of Sephadex G-10 resulted in the selective retention of antibody-forming cells, while Schwarz, Bianco, Handwerger and Kahn (1975) used a similar column of

Sephadex G-10 to separate lymphocytes and monocytes. The separation of cells using Sephadex would not appear to be based on differences in cell size, since the Sephadex particles used were too small to permit the entry of cells and therefore, another explanation has been sought. Rabinowitz (1964) used glass beads to separate lymphocytes and monocytes and found that the monocytes adhered preferentially to the glass beads. Ly and Mishell (1974) have suggested that a similar mechanism may account for the separation of cells on Sephadex, with some cells adhering more readily to the Sephadex particles and being retained in the column.

The use of Sephadex for the separation of sperm was reported recently by Steeno and Adimoelja (1975a) and by Steeno, Adimoelja and Steeno (1975b). These authors described the separation of human X- and Y-bearing sperm on columns of Sephadex G-50 fine grade and claimed that Y sperm (identified by quinacrine staining) were retained in the Sephadex column, while most of the X sperm passed through the gel. Using this technique, they found that an almost pure fraction of X sperm could be isolated, with only 4 - 5% Y sperm passing through the gel. In view of this reported success, it was decided that further investigation of this separation method was warranted.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of the Sephadex Gel

Sephadex G-50 is a polysaccharide gel, supplied in bead form (Pharmacia Ltd., Uppsala, Sweden). 1 gm of Sephadex G-50, fine grade, was mixed with 50 ml Locke's fluid, pH 8.4, and left to swell, at room temperature, for a minimum time of 4 hours. Then, when the gel had settled, the supernatant was removed and the gel was resuspended in excess fresh Locke's fluid. This decanting procedure was repeated twice more, to remove fine particles, which did not sediment, then the supernatant was removed until the volume of liquid remaining was approximately half that of the sedimented gel. Gel and liquid were mixed gently to form a slurry.

6.2.2 Preparation of the Chromatographic Column

A transparent acrylic column, 0.9 cm x 15 cm (Type K9/15, Pharmacia Ltd.), was clamped in a vertical position. At its lower end, the column was closed with an end-piece, holding a nylon bed support, and attached to a peristaltic pump (LKB-Produkter, Bromma 1, Sweden). At the top end of the column was a reservoir (Type R9, Pharmacia Ltd.).

Initially, the tubing below the nylon bed support (the dead space) and the column, to a depth of 1 cm, were filled with Locke's fluid, to remove air bubbles. The gel slurry was poured into the column down a glass rod. The upper part of the slurry was stirred, to remove air bubbles, and the gel was allowed to settle for 5 minutes before the flow of fluid through the column was started at a

rate of approximately 14 ml/hour. The reservoir was filled with Locke's fluid and connected to a Mariotte flask, also containing Locke's fluid. The Sephadex was allowed to pack until the gel surface became stationary and a column 12 cm long had formed.

6.2.3 Application of Human Semen to the Column

Human semen was washed three times in excess Locke's fluid and centrifuged at 4°C for 10 minutes at 110 g. After the third wash, the supernatant was removed and the sperm were re-suspended in Locke's fluid to 1 ml volume.

0.5 ml of either this sperm suspension or unwashed semen was applied to the Sephadex column, using a Pasteur pipette, with care being taken not to disturb the surface of the gel. The sperm were eluted into the column with small volumes of Locke's fluid, then the flow of eluant was started and the sperm allowed to pass through the column. Fluid emerging from the column was collected automatically in 1 ml fractions. After fifteen 1 ml fractions had been harvested, the flow of eluant was stopped.

6.2.4 Recovery of Sperm from Harvested Fractions

Fractions containing sperm were centrifuged at 4°C for 20 minutes at 950 g. The supernatant were discarded and each cell deposit was resuspended in a few drops of fixative (3 : 1, methanol : acetic acid) to produce a milky suspension. The suspensions were allowed to stand for 20 - 30 minutes, then smears were prepared on clean, wet slides and dried rapidly.

6.2.5 Recovery of Sperm from the Sephadex Column

The Sephadex column was inverted and the gel allowed to slide into a large test tube. The Sephadex was mixed with Locke's fluid and allowed to settle for 10 minutes, before the supernatant was removed with a Pasteur pipette. Gel particles were allowed to settle from this supernatant in a second test tube and the clear supernatant was recovered.

The two supernatants (which formed a fifth large sperm fraction) were combined and centrifuged at 4°C for 20 minutes at 950 g, and the cell deposit was resuspended in fixative, as described before. After 20 - 30 minutes, smears were prepared on clean, wet slides and dried rapidly.

6.2.6 Experimental Controls

The remaining volume of untreated ejaculate or of washed sperm suspension was centrifuged, to recover sperm, which were fixed for 20 - 30 minutes. Smears of fixed sperm were prepared as described before.

6.2.7 Fluorescent Staining of Sperm

All smears were stained for 5 - 6 minutes, in 0.5% aqueous quinacrine dihydrochloride solution, followed by brief rinses in running tap water and in distilled water. The smears were mounted, as described before, coded and examined "blind", under the microscopic conditions described before, and the proportion of sperm carrying a fluorescent body was noted.

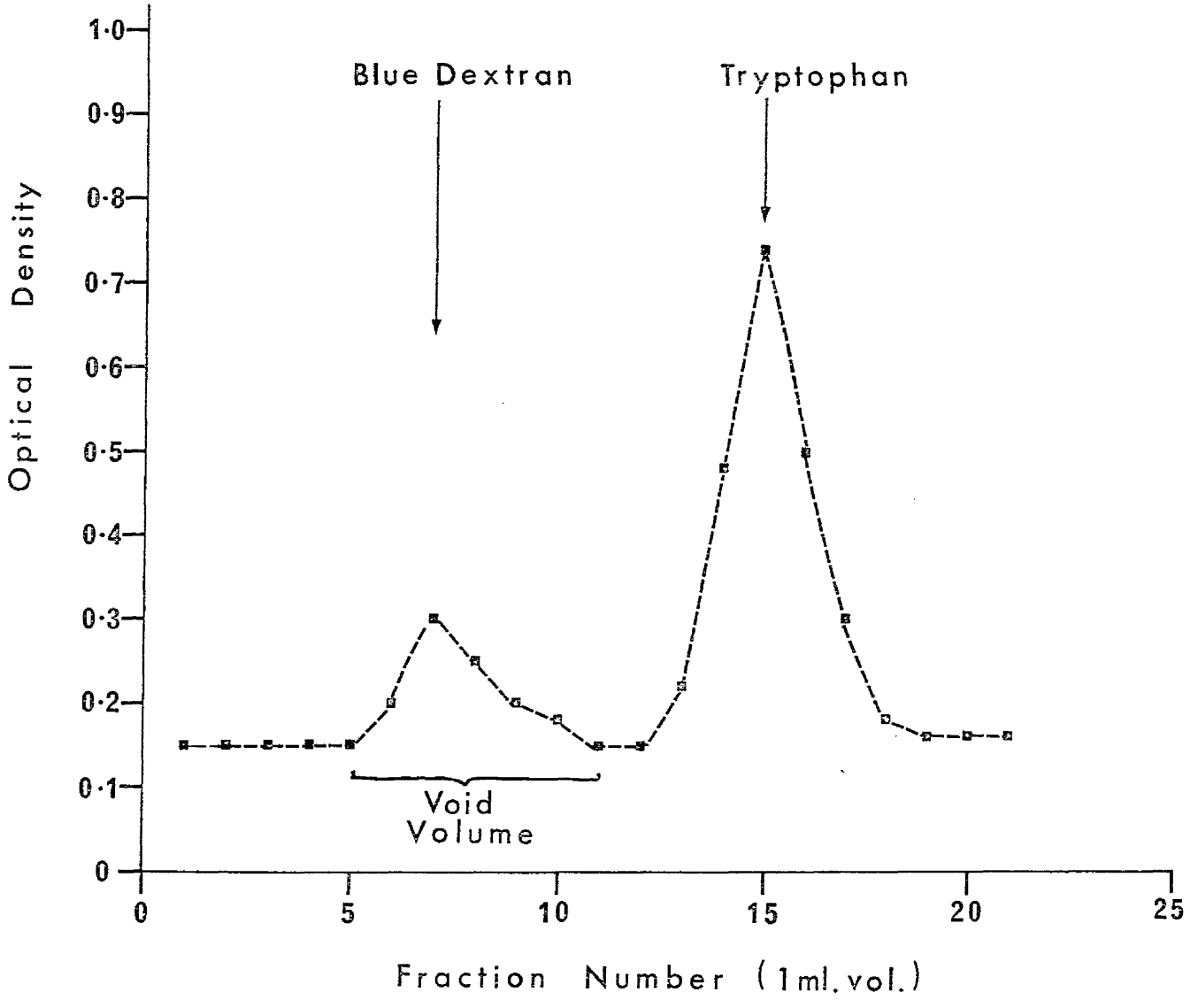
6.3 RESULTS

6.3.1 Determination of the Characteristics of the Column

Before the column was used for the separation of sperm, its characteristics were examined by passing a solution of 0.3% (w/v) Blue Dextran 2,000, (Pharmacia Ltd.), containing 0.1% (w/v) tryptophan through the gel. Approximately 0.3 ml of this solution was applied to the surface of the gel and eluted into the column with Locke's fluid. The liquid emerging from the column was pumped through an ultra violet absorptiometer (Uvicord II, LKB-Produkter) and collected in 1 ml fractions.

The Blue Dextran was seen to move gradually down the Sephadex column as a narrow coloured zone, which showed that the column was packed evenly. The optical density of each fraction harvested was measured and these results are shown in Fig. 6.1, where it may be seen from the two peaks of optical density that the gel had separated the two components of the test sample. Blue Dextran, with a molecular weight of 2 million, was excluded from entering the gel beads and emerged from the column first, between fractions 5 and 10. From the elution volume of Blue Dextran, the void volume of the chromatographic bed (i.e. the volume of liquid in the interstitial space between the beads of Sephadex) was found to be 6 mls. The second component of the test sample, tryptophan, with a molecular weight of 204, penetrated into the gel beads and passed more slowly through the column, to emerge between fractions 13 and 18.

Fig. 6.1 The separation of Blue Dextran 2,000 and Tryptophan on a column of Sephadex G-50.


Blue Dextran
Tryptophan
Optical Density
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Void Volume
0
5
10
15
20
25
Fraction Number (1ml. vol.)

6.3.2 Separation of Sperm on the Sephadex Column

Human sperm were applied to the chromatographic column either as untreated semen or as washed sperm suspended in Locke's fluid.

(i) Untreated Semen

0.5 ml untreated human semen was applied to the Sephadex column, the rest of the ejaculate being retained as experimental control. Motile sperm appeared first in the eluant in fraction 5 and initially, 140 fractions, each of 1 ml volume, were harvested. The fractions containing sperm were passed through the ultra violet absorptiometer, but no peaks were observed on the tracing, although if present in sufficient numbers, sperm might have been expected to scatter the light. There was no evidence that sperm were separating on the basis of size, unlike the separation of the molecules of Blue Dextran and tryptophan. The fractions harvested were examined microscopically for the presence of sperm and practically all of them were found to contain sperm, although sometimes in very low numbers. In subsequent experiments, fifteen 1 ml fractions were harvested. Sperm counts were made using a haemocytometer and these showed that the majority of sperm recovered were present in fractions 5, 6 and 7, which were cloudy in appearance. The later fractions seemed to be comparatively clear, although microscopic examination showed that sperm were present, but in greatly reduced numbers.

When the customary fifteen fractions had been harvested, the eleven fractions containing sperm were combined as follows: the first two fractions, to form a single large fraction and the remaining nine fractions in threes, giving a total of four fractions

containing sperm. Sperm retained in the Sephadex column were recovered by washing and formed a fifth fraction. These five fractions and the sperm from the experimental control were stained with quinacrine dihydrochloride and examined in ultra violet light. The results of staining are shown in Table 6.1. From this table, it may be seen that the numbers of Y sperm in the first fraction of all three experiments were significantly reduced, compared to the controls, ($p =< 0.05$). However, the numbers of Y sperm in fraction V of the same experiments were also significantly lower than those in the controls, ($p =< 0.05$).

In all the experiments carried out with untreated human semen, a darkened area was observed immediately below the surface of the gel after the sperm had been applied to the column and this area remained visible throughout the experiment. On several occasions, this part of the gel was sampled and microscopic examination showed that, at this point, the gel contained a large number of closely packed sperm. For this reason, consideration was given to the possibility that overloading of the column had occurred, with resulting poor separation of the sperm. Consequently, the numbers of sperm applied to the gel and the numbers recovered from the column between fractions 5 and 10 were counted. The haemocytometer counts showed that when 55×10^6 sperm were applied to the gel, approximately 22×10^6 sperm were recovered in the six fractions examined (i.e. 40% of the total number of sperm applied were recovered). Subsequently, 20×10^6 sperm were applied to the column and in this case, 3×10^6 sperm were recovered in the fractions examined (i.e. 15% of the total number of sperm applied). Even when this smaller number of sperm were applied to the gel, the darkened area at the top of the column remained visible. When

Table 6.1 Untreated Human Sperm on Sephadex Columns

Experiment Number	Fraction Number	Total No. of Sperm Counted	No. of Y Sperm Counted	% Y Sperm
1	Control	400	152	38.0
	I	400	104	26.0
	II	349	85	24.4
	III	188	51	27.3
	IV	400	112	28.0
	V (Sperm retained on Sephadex)	400	108	27.0
2	Control	400	131	32.75
	I	403	80	19.85
	II	400	91	22.75
	III	404	84	20.79
	IV	400	97	24.25
	V (Sperm retained on Sephadex)	400	61	15.25
3	Control	400	137	34.25
	I	400	99	24.75
	II	400	125	31.25
	III	400	107	26.75
	IV	410	100	24.39
	V (Sperm retained on Sephadex)	600	142	23.67

untreated semen was applied to the column, the sperm were suspended in seminal plasma. The presence of this mucinous material might have prevented the entry of sperm into the gel, with a reservoir of closely packed sperm being retained at the top of the column. In view of this, human sperm were washed in Locke's fluid before being applied to the column in a second series of experiments.

(ii) Washed Sperm

When washed sperm were applied to the Sephadex column, the darkened area below the surface of the gel was observed, as before, and this remained visible throughout the experiment. Motile sperm appeared in the eluant between fractions 5 and 15, as before and visual observation showed that most of these sperm were present in fractions 5, 6 and 7. Although the sperm recovered were motile, this motility was reduced, compared to that of the sperm harvested when untreated semen was applied to the column.

The eleven fractions containing sperm were combined, as described before, to form four large fractions and sperm recovered from the Sephadex column by washing formed the fifth fraction. Sperm from these five fractions and sperm from the experimental controls were stained with quinacrine dihydrochloride and examined in ultraviolet light, as before. The results are shown in Table 6.2, where it may be seen that the numbers of Y sperm identified in fraction I of the four experiments were significantly lower than those in the controls ($p =< 0.05$). In experiments 1 to 3, fraction V also contained a significantly lower number of Y sperm, compared to controls, although in experiment 4, the number of Y sperm did not differ significantly between fraction V and the control ($p =< 0.05$). It may be noted also that in experiments 3 and 4, there was a

Table 6.2 Washed Sperm on Sephadex Columns

Experiment Number	Fraction Number	Total No. of Sperm Counted	No. of Y Sperm Counted	% Y Sperm
1	Control	900	349	38.8
	I	405	63	15.6
	II	801	135	16.85
	III	404	147	36.4
	IV	508	128	25.2
	V (Sperm retained on Sephadex	400	120	30.0
2	Control	300	119	39.7
	I	556	128	23.9
	II	476	88	17.9
	III	300	73	24.3
	IV	400	100	25.0
	V (Sperm retained on Sephadex)	301	74	24.6
3	Control	400	152	38.0
	I	404	47	11.6
	II	400	58	14.5
	III	300	48	17.0
	IV	119	25	21.0
	V (Sperm retained on Sephadex)	400	80	20.0
4	Control	803	203	25.3
	I	809	134	16.6
	II	502	122	24.3
	III	500	125	25.0
	IV	550	143	26.0
	V (Sperm retained on Sephadex)	600	145	24.2

pattern of low numbers of Y sperm in fraction I, with increased numbers of Y sperm identified in fractions II to V.

6.3.3 Investigation of the Interference of Sephadex G-50 with the Quinacrine Staining of Human Sperm

Sperm retained in the Sephadex column were recovered by washing the gel twice in excess volumes of Locke's fluid and harvesting the supernatants after the gel had precipitated. However, a small number of gel particles remained in suspension and were harvested with the supernatant. When smears of the sperm released from the gel were made (fraction V in the staining experiments), these Sephadex particles were present also and stained in outline with the quinacrine dye. When low numbers of Y sperm were identified in fraction V as well as in fraction I of the experiments, it was considered that the presence of the Sephadex particles might have interfered with the quinacrine staining of the sperm and therefore, with the identification of the F-body of the Y sperm. Two smears were prepared from the same ejaculate, as described previously. A few drops of Sephadex G-50 suspension were applied to cover one of the smears and both smears were left to dry. When dry, the Sephadex-covered smear and the control smear were coded, stained with quinacrine dihydrochloride and examined in ultraviolet light, as before. 300 sperm were counted on each slide and the numbers of Y sperm identified were noted. The results are shown in Table 6.3, where it may be seen that the presence of Sephadex appeared to cause no interference with quinacrine staining and identification of the human Y sperm.

Table 6.3 An Assessment of the Interference of Sephadex G-50 with Quinacrine Staining of Human Spermatozoa

Smear	Total No. of Sperm Counted	No. of Y Sperm Counted	% Y Sperm
Sperm stained in the presence of Sephadex G-50	300	96	32.00
Untreated Sperm - Control	300	98	32.67

6.3.4 Investigation of the Adsorption of Sperm on to the Sephadex Particles

The recovery of low numbers of Y sperm from the Sephadex column might be explained by the preferential adsorption of Y sperm on to the Sephadex particles. If this were so, increased time of contact between the sperm and Sephadex might create a better opportunity for the adsorption to take place. Washed human sperm were resuspended in Locke's fluid to 2 ml volume. Eight test tubes, each containing 1 ml Sephadex G-50 suspension, were prepared. 0.2 ml of the washed sperm suspension was added to each tube and mixed gently. The tubes were sealed with plastic film and placed horizontally on a rotary mixer, (Matburn Surgical Equipment Ltd., Portsmouth, U.K.) and the sperm were mixed gently with the Sephadex suspensions. At intervals of 15 minutes, one tube was removed from the mixer and the Sephadex allowed to precipitate for 5 minutes. Then, the supernatant was removed and the sperm recovered by centrifugation, for fluorescent staining. Washed sperm were used as control. Duplicate smears were prepared from each supernatant and the slides were coded and examined "blind". These results are shown in Table 6.4 and it may be seen that, with increased length of time of contact between sperm and Sephadex suspension, fewer Y sperm were identified, compared to the control. After 80 minutes and 120 - 160 minutes of contact, significantly lower numbers of Y sperm were identified, compared to controls, ($p = < 0.05$).

Table 6.4 The Effect of Contact with Sephadex G-50 on the Identification of Human Y-bearing Sperm

Fraction Number	Slide Number	Length of Time of Contact with Sephadex G-50 (mins)	Total No. of Sperm Counted	Y Sperm Counted	% Y Sperm	Average % Y Sperm
Control	1 2	0	300 300	109 106	36.3 35.3	35.80
1	1 2	20	301 300	89 87	29.6 29.0	29.0
2	1 2	40	300 305	96 95	32.0 31.2	31.6
3	1 2	60	310 301	91 95	29.4 31.7	30.55
4	1 2	80	302 300	85 81	28.2 27.0	27.6
5	1 2	100	300 301	92 102	30.7 33.9	32.3
6	1 2	120	300 300	86 77	28.7 25.7	27.2
7	1 2	140	300 300	87 83	29.0 27.7	28.35
8	1 2	160	300 300	72 93	24.0 31.0	27.5

6.4 DISCUSSION

Sephadex is a bead-formed dextran gel. It is strongly hydrophilic and the beads swell in water or in electrolyte solutions to form a three-dimensional network, capable of separating substances according to their molecular size. Large molecules move easily through the network and emerge first from the chromatographic bed, but small molecules are trapped in the network and retained. No known chromatographic material has an absolutely homogeneous particle size and the fine grade of Sephadex G-50 has a dry particle diameter range of 20 - 80 μm. This gel has been used successfully to fractionate peptides, globular proteins and dextrans, but these molecules are very much smaller than human sperm, where the average head length is 4 - 5 μm and the average head width is 2.5 - 3.5 μm, (Monesi, 1972). Using the minimum and maximum sizes of the dry Sephadex G-50 beads, it was calculated that the interstitial spaces between the beads would be large enough to allow the passage of spherical particles of 8 - 24 μm diameter, and would probably be sufficiently large to permit the passage of human sperm. However, the gel is used in a wet state and the beads swell, although retaining their circular shape. This suggests that the interstitial spaces would be larger than calculated for the dry state and would probably allow the passage of human sperm with ease. Steeno and Adimoelja (1975a) and Steeno et al. (1975b) have not reported why Sephadex G-50 fine grade was chosen particularly for the separation of human sperm, nor whether other types of gel were examined during initial experiments on sperm separation. Since, in addition to Sephadex G-50 fine grade, other Sephadex G-types have a similar dry particle diameter range (e.g. Sephadex G-25, fine),

it may be possible that a range of gels will prove suitable for human sperm separation.

Using the quinacrine dihydrochloride stain to identify human Y sperm, Steeno et al. (1975a and b) have claimed that only 4 - 5% Y sperm were present in the fraction recovered from the column which contained the greatest number of sperm, with 65% Y sperm being adsorbed on to the gel particles and remaining in the column. They compared these results with a control value of 38% Y sperm in a normal human ejaculate. In the work described in this thesis, a similar, but not so dramatic, trend was observed. From Tables 6.1 and 6.2, it may be seen that the numbers of Y sperm recovered in the first fractions after gel chromatography were significantly lower than the control values. In the seven experiments recorded, the average control value for Y sperm was 35.25% and in the first fractions recovered from the Sephadex column, the percentage of Y sperm was reduced to an average of 19.32%, with the lowest number of Y sperm recovered being 11.6%. From these results, there would appear to be some evidence that the column of Sephadex G-50 is able to produce a degree of separation of human X- and Y-bearing sperm, but how this separation occurs is not clear. It would seem unlikely that this separation of X and Y sperm can be explained by the theory of gel chromatography and therefore some other explanation must be considered. Ly and Mishell (1974) used Sephadex G-10 gel for the separation of mouse spleen cells of different types (in this case, antibody forming from non-antibody forming) and they suggested that the separation of these cells might occur because of differences in their adhesion to the gel beads. Steeno et al. (1975b) also advanced this theory to explain the separation of X and Y sperm on Sephadex G-50 and suggested that,

in this instance, Y sperm were adsorbed selectively on to the gel particles and were retained, while actively motile X sperm passed through the column. The adhesive properties of cells have long been recognised and used in separation procedures involving columns of glass beads. Rabinowitz (1964) showed that lymphocytes, polymorphonuclear leucocytes and monocytes could be fractionated from a mixed leucocyte population, using a column of glass beads, and recently, Schwarz et al. (1975) described the separation of lymphocyte and monocyte populations on a Sephadex column. Similarly, Bangham and Hancock (1955) and Whitfield (1976) used glass bead columns to separate living and dead bovine sperm, with dead sperm being retained on the glass beads, while Graham, Vazquez, Schmehl and Evensen (1976) have shown that a similar separation can be achieved on columns of Sephadex. This separation of cells on columns of glass beads is thought to take place because some of the cells adhere preferentially to the beads. In the instances just given, the same types of cells have separated on both glass bead and Sephadex columns and this would tend to suggest that the glass beads and Sephadex are acting in a similar fashion. However, one drawback to the theory advanced by Steeno et al. (1975b) for the separation of sperm is that there is no conclusive evidence to show that X and Y sperm differ in their surface properties or powers of adhesion, but if a pure population of either X or Y sperm could be isolated, further studies might provide an answer to this question.

During the experiments reported in this thesis, a darkened area was observed immediately below the surface of the gel and this was shown to consist of very large numbers of closely packed sperm. Washing of the sperm in Locke's fluid, to remove seminal plasma, prior to application to the column produced no change in the situation.

This collection of sperm at the top of the column may have been caused by overloading the gel with sperm, as in most cases, approximately 50 x 10^6 sperm were applied to the columns in each experiment. The first sperm applied would presumably enter the column and Y sperm would adsorb on to the gel beads, with X sperm moving down the column, partly by swimming and partly aided by the flow of eluant, to emerge in fractions 5, 6 and 7. Then, as more sperm entered the column, competition between the Y sperm for the gel beads near the top of the column might be intensified, with the result that the gel beads would become surrounded by clusters of Y sperm, which would tend to block the interstitial spaces. The passage of X sperm through the column might then be slowed to a trickle, with only small numbers of sperm being harvested in later fractions. One answer to this problem might be to use a very dilute suspension of sperm for application to the gel, but it should also be taken into account that considerable numbers of harvested sperm might be needed for further studies or for artificial insemination.

The main difference between the results reported in these experiments and those of Steeno et al. (1975a and b) is that examination of sperm retained on the Sephadex column did not show that large numbers of Y sperm were present, Steeno et al. (1975a and b) claimed that when sperm retained in the gel were recovered and examined, they were found to lie with their heads orientated into the Sephadex beads and that when stained with quinacrine, the majority of these sperm were identified as Y-bearing. During the experiments described in this thesis, some difficulty was experienced in harvesting sperm retained in the gel. If some of the Sephadex suspension was included in fraction V, it was found that the circular

beads of Sephadex stained with quinacrine dye and sperm retained with their heads orientated into the beads were obscured. Thus, it proved impossible to assess whether or not an F-body was present in the sperm head. Consequently, in the method adopted for harvesting these sperm, the Sephadex gel was washed twice in Locke's fluid and allowed to precipitate, the two supernatants being combined and centrifuged to recover sperm released from the gel. Undoubtedly, some sperm remained trapped within the rapidly sedimenting gel and this might possibly account for the low numbers of Y sperm identified in fraction V of these experiments, since if Y sperm were indeed preferentially adsorbed on to the Sephadex beads, it might be expected that more Y sperm would be trapped as the gel precipitated.

The possibility that Y sperm are adsorbed preferentially on to the Sephadex beads might be investigated further in one or two ways. Firstly, better adsorption of Y sperm on to the gel particles might take place if more time were allowed for the separation. In this case, sperm could be applied and eluted into the column with small volumes of fluid, then the flow of eluant stopped and the column left for, say, 30 or 60 minutes, before restarting the flow of eluant. This might allow time for sperm to swim downwards into the column and reduce the blocking effect at the top of the column. The results of a control experiment reported in Table 6.4 lend some support to this hypothesis, as increased time of contact between Sephadex and sperm apparently caused significantly lower numbers of Y sperm to be recovered from Sephadex suspension compared to the controls. Secondly, the use of Sephadex columns in series might lead to a more complete separation of X and Y sperm.

Sperm could be applied to one column, harvested in the usual way and numbers of Y sperm recovered could be noted. Then, sperm recovered from the first column could be placed on a second column of the gel and the process repeated. If selective adsorption of Y sperm were taking place, it would be expected that an even lower number of Y sperm would be recovered from the second column. For example, if a normal ejaculate containing 40% Y sperm was placed on the first Sephadex column and only 10% Y sperm were present in the recovered fractions, it would be expected that when these sperm were placed on the second column, only 2.5% Y sperm would be recovered. This process might even be continued until a virtually pure fraction of X sperm was isolated. However, although the results obtained so far, with quinacrine staining as a monitor, are intriguing, final proof of the separation must still be shown: as living sperm have been recovered using this method, ultimate proof of separation may be given by clinical trials with insemination of the separated sperm fractions.

The Sephadex separation process is simple and easy to carry out, needing no expensive or elaborate equipment. If conclusive evidence of the separation of human X and Y sperm can be obtained, the next step might be to determine whether the process could separate X and Y sperm of other species, where field trials would probably be necessary to monitor the technique. The method could be adapted easily for use in the field; fresh semen could be mixed with the Sephadex suspension, the gel allowed to precipitate, then the supernatant, now enriched with X sperm, harvested. The process might eventually reach such a degree of refinement that a sterilised Sephadex suspension could be added to, say, bovine semen prior to storage and inseminated together with the sperm, eliminating

the time-consuming process of setting up Sephadex columns in a laboratory and reducing the need to handle sperm between collection and insemination.

CHAPTER SEVEN

GENERAL DISCUSSION

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GENERAL DISCUSSION

At the end of the nineteenth century, the view prevailed that the embryo was initially sexually undifferentiated and that its sex was determined subsequently by such agents as temperature and nutrition. McClung (1902) was first to suggest that chromosomes might be concerned with the determination of sex and it was established later that, in higher organisms, sex is determined by a difference in chromosome constitution. In general, sex is determined at fertilisation: one of the sexes produces gametes of two kinds, which in turn give rise to offspring of either sex. In the majority of cases, the male is the heterogametic sex and the eggs are sexually undifferentiated until fertilised by either a male- or a female-producing sperm. This situation prevails in most mammals, including man, although in birds the opposite is true, with the female being the heterogametic sex (Ohno, 1967).

In man, there are 46 chromosomes, consisting of 22 pairs of autosomes and one pair of sex chromosomes. In comparison, the bovine has 60 chromosomes, composed of 29 pairs of autosomes and one pair of sex chromosomes. The sex chromosomes of the males in both species are X and Y and these two specialised chromosomes differ from each other in both size and genetic content,

although it has been shown that they were originally a homologous pair of autosomes (Ohno, 1967). In both species, the Y chromosome is much smaller than the X chromosome and in humans, its length is known to vary considerably between individuals (Pearson, 1971). The mammalian Y chromosome is known to exert a very strong male-determining influence and induces the undifferentiated embryonic gonad to develop as a testis,(Ohno, 1967), while in the mature male, the presence of the Y chromosome seems to be essential for mammalian spermatogenesis (Short, 1972).

During the meiotic divisions of spermatogenesis, X and Y chromosomes are separated, with half the sperm receiving the X chromosome and half receiving the Y. By this segregation of the sex chromosomes, two populations of sperm are produced. These two sperm populations are known to differ in sex chromosome status, but as yet, there is no definite proof that they differ in size, shape, motility, surface properties, electrical charge or antigenicity. If it could be shown that X and Y sperm differed phenotypically, the difference might be attributed to the sex chromosome carried by the sperm. Sex chromosomes (and therefore the genes they carry) are present in the sperm in the haploid state and it is doubtful whether they are able to exert an influence on the phenotype of the sperm. It has been generally concluded from biochemical studies that there is a virtually complete stoppage of demonstrable gene activity at meiosis, (Monesi, 1965), although Beatty (1975a) has concluded from circumstantial evidence that haploid gene action in animals probably does occur in extremely rare cases.

Because the X chromosome is larger than the Y chromosome, it was logical to consider that X and Y sperm might differ in size,

but after extensive research, Beatty (1972) found that there was no evidence that a bimodal size distribution of sperm existed. Relatively recent evidence on this point has come from Pearson, Geraedts and Pawlowitzki (1973) who claimed that human X sperm have a 7% larger surface area than Y sperm (identified by quinacrine staining). The difference in size between X and Y chromosomes was the basis of the belief that X and Y sperm might differ in specific gravity and numerous attempts have been made to separate them by sedimentation (e.g. Schilling, 1971) and by using density gradients (e.g. Benedict et al., 1967). Using a sedimentation technique, Schilling (1971) reported that a shift in the bovine sex ratio was maintained over seven years, but other workers have not had the same success (Lavon et al., 1971). Benedict et al. (1967) used the methylglucamine salt of umbradilic acid as a gradient medium for the separation of rabbit sperm, but observed no effect on the sex ratio of the offspring produced after insemination of the separated sperm populations. In the experiments reported in this thesis, colloidal silica, sucrose and Metrizamide were used as density gradient media to separate both human and bovine sperm into populations. Colloidal silica proved to be unsuitable, at least for the separation of human sperm and had the added drawback that it prevented staining of the sperm with quinacrine. The high osmotic pressure of sucrose solutions resulted in the death of the sperm, but the use of the recently developed Metrizamide, with a lower osmotic pressure than sucrose solutions, seemed hopeful. However, although the Metrizamide gradient separated the sperm into two populations, no consistent significant change in the ratio of X- and Y-bearing sperm was observed. From these

results, it would appear that density gradient techniques are unsuitable for the separation of X and Y sperm, which differ by only one chromosome. Harvey (1946) calculated density values of 1.07132 for human Y sperm and 1.0715 for human X sperm and emphasised that their separation would require a very slight density gradient, while Bahr (1971a) suggested that, because of inter-chromosomal variability, there may be no difference in mass or only a very small difference between the average haploid X-containing and Y-containing sets of chromosomes. Beatty (1974b) has proposed that although X sperm may contain more DNA, Y sperm may compensate by having extra cytoplasm, so that the actual difference in specific gravity between the sperm may be even less than the theoretical value and this would certainly make it unlikely that a density gradient could be developed for the separation of X and Y sperm.

The discovery by Barlow and Vosa (1970) that human Y sperm could be identified by quinacrine staining stimulated new interest in the possibility of separating X and Y sperm, for sex control of the offspring. Unfortunately, the quinacrine staining technique has proved to be suitable only for the identification of human Y sperm. In an attempt to apply the technique to other species, Pearson et al. (1971) examined the chromosomes of a number of mammalian species and showed that areas of intensely fluorescent chromatin (similar to that found on the human Y chromosome) were confined to the chromosomes of the gorilla (Gorilla gorilla) and the chimpanzee (Pan troglodytes), but only in the gorilla did the long arms of the Y chromosome fluoresce brightly, as in man. Up to the present time, there appears to have been no extension of this work to include an examination of gorilla sperm,

although the evidence obtained from staining the chromosomes would suggest that the Y-bearing gorilla sperm might be expected to contain an F-body. The staining of bovine chromosomes with quinacrine and with other acridine derivatives has been reported in this thesis, but no specific fluorescence of the bovine Y chromosome was observed. However, Bhattacharya (1976) has claimed that quinacrine can be used to identify the bovine Y sperm after treatment of the sperm with a proteinase enzyme, which apparently allows penetration of the dye into the sperm. He identified the Y chromosome within the sperm head as a fluorescing "B-body", but produced no evidence to show that the bovine Y chromosome fluoresced intensely in mitotic chromosome preparations.

The intensely fluorescent regions of the human Y chromosome may vary in size between individuals and can even be completely absent in normal fertile males. As there is no obvious external phenotypic manifestation of the absence of this fluorescent region, it is presumed to be of little or no genetic consequence (Evans, 1972). However, the reason why the distal ends of the long arms of the human Y chromosome stain intensely with quinacrine remains obscure. Caspersson et al. (1968) suggested that the bright fluorescence might be a characteristic of DNA with a high guanine - cytidine content, but Evans (1972) argued that the fluorescence might be a property of the physical state of the DNA rather than its chemical constitution. Although the exact mechanism of staining is unknown, it appears that the quinacrine staining technique for the human Y sperm has become accepted by many research workers. Initially, the belief that the F-body represented the human Y chromosome within the sperm head was based on

circumstantial evidence - the distal ends of the long arms of the chromosome were known to fluoresce intensely (Zech, 1969) and the proportions of sperm containing an F-body corresponded approximately to the theoretical 50 : 50 distribution of X and Y chromosomes at meiosis. Support for this belief was gained from the work of Sumner et al. (1971) who showed that sperm lacking an F-body had a higher Feulgen - DNA content than sperm with an F-body, while recent evidence from Sumner and Robinson (1976) on dry mass measurements of human sperm would seem to make the identification certain. On the other hand, the average percentage of Y sperm scored in normal ejaculates does appear to vary widely between observers. For example, Barlow and Vosa (1970) scored 40 - 45% Y sperm in a normal human ejaculate, while Rohde et al. (1975) scored an average of 38% Y sperm, but Beatty (1975b) produced values of 35.5% and 50% Y sperm for the same ejaculate, depending on whether strict or relaxed criteria were followed. In the present work, the % Y sperm scored in a normal human ejaculate also varied widely, although a comparison of staining and counting techniques between this laboratory and that of another observer showed only an average difference of 4%. With increasing experience, the % Y sperm scored in a human ejaculate appeared to become more consistent, although, using strict criteria, values of more than 40% were rarely noted. Pearson et al. (1973) stated that the % of Y sperm scored is always less than 50% and that this discrepancy between the theoretical and the observed can be attributed to staining difficulties. Differences in optical conditions and the "eye" of the observer may play a part, while anyone working in this field will be familiar with understained or faded slides, which may account for some of the variation reported. Barlow and

Vosa (1970) remarked that the position of the F-body within the sperm head was variable, although it was found usually at the boundary between the densely and the less densely stained regions of the sperm head. If the Y chromosome lies within the more dense region of the sperm head, it is certainly more difficult to score. The sperm head may perhaps vary in thickness and Goodall and Roberts (1976) suggested that when the Y chromosome lies deep within the sperm head, it may be more difficult to recognise with certainty. It should be remembered too that some of the separation procedures used may interfere with the subsequent identification of recovered Y sperm (notably colloidal silica in these experiments, although bovine serum albumin when present in high concentrations was also found to interfere with quinacrine staining) and this must be taken into account when trying to assess the results of a separation process.

The discovery that the human Y sperm could be identified by staining was undoubtedly a major advance, but the human is not always a suitable experimental animal. A means of identifying the Y sperm of a laboratory or domestic animal would be most useful, as the semen might be more readily available and because any apparently successful results of a separation process could be confirmed readily, by carrying out artificial insemination in field trials. Therefore, the search continues for another experimental animal, whose Y sperm can be identified. Tates, Pearson and Geraedt (1975) used Leishman stain to identify the Y chromosome of the northern vole (Microtus oeconomus). The Y chromosome stains darkly and can be identified in interphase nuclei and in sperm, so it would appear that the animal could be used for research into the separation of X and Y sperm, although at present, the human still

remains more convenient. However, the use of this stain is a reminder that non-fluorescent compounds may be of value in this field and that attention should not be focused exclusively on fluorescent stains.

Most of the methods used in attempts to control the sex of the offspring have involved some process of separating X and Y sperm, usually on the basis of their supposed physical differences. For example, Ericsson et al. (1973) claimed that the separation of human sperm on albumin columns depended on the superior motility of Y-bearing sperm, a theory advanced initially by Roberts (1972). Support for this theory comes from the circumstantial evidence of the natural sex ratio in most species being weighted in favour of male offspring, which might suggest that the Y-bearing sperm is able to reach the ovum faster than the X sperm. However, both the results of experiments described in this thesis and those of Ross et al. (1975) differed from the findings of Ericsson et al. (1973), although recent evidence from Goodall and Roberts (1976), also using the quinacrine staining technique, suggests that human Y sperm tend to swim upwards in a column of fluid in greater numbers than X sperm. Since the concept of superior motility of the Y sperm remains controversial, further investigation of this point would seem to be necessary.

The separation of human sperm on columns of Sephadex G-50 may be another instance where some physical difference between X and Y sperm is being exploited. The results of Steeno et al. (1975a and b) showed that Y sperm adhered more strongly to the gel beads than X sperm and the results obtained in experiments described in this thesis appear to support their findings, although to less marked

degree. Neither the experiments of Steeno et al. (1975a and b) nor the experiments described in this thesis have explained why this separation takes place, but it would seem that some surface property of the Y sperm is involved. Because some sperm, (actually observed by Steeno et al. (1975b) as Y-bearing), adhere to the gel beads, one might speculate that human Y sperm could be "roughened" and therefore, more likely to adhere to the beads, but it can only be concluded that further investigation of this phenomenon is warranted.

Although there is no clear evidence that X and Y sperm do differ antigenetically, immunological methods of sex control have been investigated extensively (Beatty, 1960; McLaren, 1964, 1965; Bennett and Boyse, 1973), although the results are controversial. Nevertheless, this situation might be changed if some separation procedure (such as the Sephadex separation) resulted in the isolation of a pure fraction of either X or Y sperm. This would be a major advance and would enable researchers to work on the production of an antiserum against either X- or Y-bearing sperm. Such a development would enable offspring of the desired sex to be produced in most species simply by mixing the antiserum with semen prior to insemination. In the future, it might even be possible to extend this technique to the living animal, with the production of a vaccine against X or Y sperm. Although not practical in the human species, this technique would be most useful in certain husbandry situations where the sire could be vaccinated against either X or Y sperm and would produce only offspring of one sex for the rest of its reproductive life.

REFERENCES

ANDERSEN, H. and ROTTENSTEN, K. (1962)
Aarsberetn. Inst. Sterilitetsforskn.
K. vet-og Landbohojsk. (KBH.) 1962, 125.
(Anim. Breed. Abstr. (1965) 31, 245)

ANON. (1974)
Lancet i, 203

APPELLA, E. and ZITO, R.
cited by: Stambaugh, R. and Buckley, J. (1971b)
in: Sex Ratio at Birth - Prospects for Control,
Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.),
59, Amer. Soc. Anim. Sci., Albany, U.S.A.

ASDELL, S. A. (1927)
J. Am. med. Ass. 89, 509

AUGHEY, E. (1976)
Personal communication

BAHR, G. F. (1971a)
in: Sex Ratio at Birth - Prospects for Control,
Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.),
28, Amer. Soc. Anim. Sci., Albany, U.S.A.

BAHR, G. F. (1971b)
in: Sex Ratio at Birth - Prospects for Control,
Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.),
57, (statement in course of discussion), Amer. Soc.
Anim. Sci., Albany, U.S.A.

BAIER, W. (1957)
Tierzüchter 9, 13

BAIER, W. and HAEGER, O. (1958)
Dt. tierärztl. Wschr. 71, 426

BAKER, F. N., SALISBURY, G. W. and FECHHEIMER, N.S.
(1965)
Nature, Lond. 208, 97

BANE, A. (1952)
Cornell Vet. 42, 518

BANGHAM, A. D. (1962)
J. Reprod. Fert. 4, 231

BANGHAM, A. D. and HANCOCK, J. L. (1955)
Nature, Lond. 176, 656

BARLOW, P. W. and VOSA, C. G. (1970)
Nature, Lond. 226, 961

BEATTY, R. A. (1960)
Mem. Soc. Endocr. No.7, 93

BEATTY, R. A. (1961)
Proc. R. Soc. Edinb. B 68, 72

BEATTY, R. A. (1964)
in: Proc. 5th Int. Congr. Anim. Reprod. & A.I., Trento, 1964, Vol. 3, 276

BEATTY, R. A. (1969)
J. Reprod. Fert. 19, 379

BEATTY, R. A. (1970)
Biol. Rev. 45, 73

BEATTY, R. A. (1972)
Atti del VII Symposio Internazionale di Zootecnia, Milan, April 1972, 239

BEATTY, R. A. (1974a)
Biblphy. Reprod. 23, 1

BEATTY, R. A. (1974b)
Personal communication

BEATTY, R. A. (1975a)
in: Suppl. 1, Biol. J. Linnean Soc. Lond. 7, 291

BEATTY, R. A. (1975b)
in: The Functional Anatomy of the Spermatozoon, Afzelius, B. A. (ed.), 1975, Pergamon Press, Oxford and New York

BEATTY, R. A. and FECHHEIMER, N. S. (1972)
Biol. Reprod. 7, 267

BECK, K. J., HERSCHEL, S., HUNGERSHÖFER, R. and SCHWINGER, E. (1976)
Fert. Steril. 27, 407

BEDFORD, J. M. and BIBEAU, A. M. (1967)
J. Reprod. Fert. 13, 167

BENEDICT, R. C., SCHUMAKER, V. N. and DAVIES, R.E. (1967)
J. Reprod. Fert. 13, 237

BENNETT, D. and BOYSE, E. A. (1973)
Nature, Lond. 246, 308

BHATTACHARYA, B. C. (1958)
Z. Tierzücht. Zücht Biol. 72, 250
(Anim. Breed. Abstr. (1962) 30, 570)

BHATTACHARYA, B. C. (1962)
Z. wiss Zool. A166 (3/4) : 204

BHATTACHARYA, B. C. (1964)
New Scient. 24, 151

BHATTACHARYA, B. C. (1976)
in: Proc. 8th Int. Congr. on Anim. Reprod. & A.I.,
Krakow, 1976, (in press)

BHATTACHARYA, B.C., BANGHAM, A. D., CRO, R. J.,
KEYNES, R. D. and ROWSON, L. E. A., (1966)
Nature, Lond. 211, 863

BISHOP, D. W. (1960)
Nature, Lond. 187, 256

BLANDAU, R. J. and YOUNG, W. C., (1939)
Am. J. Anat. 64, 303

BROTHERTON, J. (1969a)
Cytobios 1b, 95

BROTHERTON, J. (1969b)
Cytobios 3, 307

BROTHERTON, J. and BARNARD, G. (1974)
J. Reprod. Fert. 40, 341

BURKOV, I. (1968)
Mater 6 vses. Konf. Fiziol. Biokhim. Osnov.
Povysh. Prod. Sel'-Khoz. Zhivot. 1968, Borovsk. 390-1
(Anim. Breed. Abstr. (1971) 39, 351)

CAMPBELL, R. C. HANCOCK, J. L. and ROTHSCHILD, LORD
(1953)
J. exp. Biol. 30, 44

CASPERSSON, T., FARBER, S., FOLEY, G. E., KUDYNOWSKI, J., MODEST, E. J., SIMONSSON, E., WAGH, U. and ZECH, L. (1968) Expl. Cell Res. 49, 219

CASPERSSON, T. and ZECH, L. (1972) in: Karolinska Symposia on Research Methods in Reproductive Endocrinology, 5th symposium. Gene Transcription in Reproductive Tissue, May 1972, 67

COHEN, M. R. (1967) Int. J. Fert. 12, 32

CREW, F. A. E. (1927) Br. med. J. ii, 917

DIASIO, R. B. and GLASS, R. H. (1971) Fert. Steril. 22, 303

EDWARDS, A. W. F. (1963) Nature, Lond. 198, 1106

EDWARDS, R. G. (1963) cited by: Tyler, A. and Bishop, D. W. in Mechanisms concerned with Conception, Hartmann, C. G. (ed.), chapt. 8, 397, Pergamon, Oxford, 1963

EICHWALD, E. and SILMSER, C. R., (1955) Transplantn. Bull. 2, 148

ERICSSON, R. J., LANGEVIN, C. N. and NISHINO, M. (1973) Nature, Lond. 246, 421

ETZIONI, A., (1968) Science 161, 1107

EVANS, H. J. (1972) in: Proc. Int. Symp., The Genetics of the Spermatozoon, Edinburgh, 1971, Beatty, R. A. and Gluecksohn-Waelsch, S. (eds.), 144

FELDMAN, M. (1958) Transplantn. Bull. 5, 15

FELLOUS, M. and DAUSSET, J. (1970) Nature, Lond. 225, 191

FISCHER, L. (1969) in: Laboratory Techniques in Biochemistry and Molecular Biology, Vol. I, Work, T. S. and Work, E. (eds.), North-Holland Publishing Company, Amsterdam and London

FOOTE, R. H. and HALL, A. (1954)
J. Dairy Sci. 37, 673

FOOTE, R. H. and MILLER, P. (1971)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 1, Amer. Soc. Anim. Sci., Albany, U.S.A.

FOOTE, W. D. and QUEVEDO, M. M. (1971)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 55, Amer. Soc. Anim. Sci., Albany, U.S.A.

FOWLER, A. K. and HELLMAN, A. (1965)
Fert. Steril. 16, 778

GARDNER, K. E. (1950)
J. Dairy Sci. 33, 391

GLOVER, F. A. and PHIPPS, L. W. (1962)
J. Reprod. Fert. 4, 189

GOLDBERG, E. and HAWTREY, C. (1967)
cited by: Stambaugh, R. and Buckley, J. (1971b) in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 59, Amer. Soc. Anim. Sci., Albany, U.S.A.

GOODALL, H. and ROBERTS, A. M. (1976)
Personal communication

GORDON, D. L., MOORE, D. J., THORSLUND, T., and PAULSEN, C. A. (1965)
J. Lab. clin. Med. 65, 506

GORDON, M. J. (1957)
Genetics, N. Y. 43, 913

GORDON, M. J. (1958)
Scient. Am. 199, No.5, 87

GRAHAM, C. F. (1970)
Nature, Lond. 226, 165

GRAHAM, E. F., VAZQUEZ, I. A., SCHMEHL, M. K. L. and EVENSEN, B. K. (1976)
in: Proc. 8th Int. Congr. on Anim. Reprod. & A. I., Krakow, 1976, (in press)

HAFS, H. D. and BOYD, L. J. (1971)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 85, Amer. Soc. Anim. Sci., Albany, U.S.A.

HAMMOND, J. (1934)
Br. J. exp. Biol.

HANCOCK, J. L. (1952)
J. exp. Biol. 29, 445

HANSEN, K. M. (1972)
Hereditas 70, 225

HART, D. and MOODY, J. D. (1949)
Ann. Surg. 129, 550

HARVEY, E. N. (1946)
J. Hered. 37, 71

HEATH, C. W. (1954)
Hum. Biol. 26, 337

IVERSEN, S. (1964)
J. agric. Sci., Camb. 62, 219

IVERSEN, S. (1965)
J. Reprod. Fert. 9, 197

JAMES, W. H. (1971)
Lancet i, 112

JOHANSSON, I. (1932)
cited by: Van Demark, N. L. and Malven, P. V. (1960), A. I. Dig. 8 (12), 11.

KAISER, R., BROER, K. H., CITOLER, P. and LEISTER, B. (1974)
Geburtsh. Frauenheilk. 34, 426

KIDDY, C. A. and BAILEY, L. F. (1972)
J. Dairy Sci. 55, 681 (abstr. only)

KILHSTRÖM, J. E. and FJELLSTRÖM, D. (1967)
J. Reprod. Fert. 14, 155

KLEEGMAN, S. (1954)
Fert. Steril. 5, 7

KNAACK, J. (1968)
cited by: Schilling, E. (1971) in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 76, Amer. Soc. Anim. Sci., Albany, U.S.A.

KOLTZOFF, N. K. and SCHRÖDER, V. N. (1933)
Nature, Lond. 131, 329

KORDTS, E. (1952)
Z. Tierzücht. Zücht Biol. 60, 221

KRZANOWSKI, M. (1970)
J. Reprod. Fert. 23, 11

LAMAR, J. K., SHETTLES, L. B. and DELFS, E. (1940)
Am. J. Physiol. 129, 234

LAURENCE, K. A. and CARPUK, O., (1963)
Fert. Steril. 14, 451

LAVON, U., VOLCANI, R. and DANON, D. (1971)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 19, Amer. Soc. Anim. Sci., Albany, U.S.A.

LAVON, U., VOLCANI, R., AMIR, D. and DANON, D. (1966)
J. Reprod. Fert. 11, 447

LEWIN, S. (1956)
Br. vet. J. 112, 549

LINDAHL, P. E. (1948)
Nature, Lond. 161, 648

LINDAHL, P. E. (1956)
Nature, Lond. 178, 491

LINDAHL, P. E. (1958a)
Nature, Lond. 181, 784

LINDAHL, P. E. (1958b)
Acta Agric. scand. 8, 226

LINDAHL, P. E. (1971)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 69, Amer. Soc. Anim. Sci., Albany, U.S.A.

LINDAHL, P. E. and KIHLSTRÖM, J. E. (1952)
J. Dairy Sci. 35, 393

LOVELOCK, J. E. (1960)
in: Lewin, S. (1960) Mem. Soc. Endocr., No.7, 113, (statement in course of discussion)

LUSH, J. L. (1925)
J. agric. Res. 30, 893

LY, I. A. and MISHELL, R. I. (1974)
J. Immunol. Methods 5, 239

McCLUNG, C. E. (1902)
cited by: Ohno, S. (1967) in: Monographs on Endocrinology, I, Sex Chromosomes and Sex-Linked Genes, (1967), Springer-Verlag, Berlin, Heidelberg and New York

McLAREN, A. (1964)
Nature, Lond. 201, 582

McLAREN, A. (1965)
Transplantation, 3, 259

McLAREN, A. (1972)
in: Proc. Int. Symp., The Genetics of the Spermatozoon, Edinburgh, 1971, Beatty, R. A. and Glucksohn-Waelsch, S. (eds.), 313

MacPHERSON, J. W. and VESSELINOVITCH, S. D. (1959)
Can. J. comp. Med. 23, 375

McWHIRTER, K. G. (1960)
Mem. Soc. Endocr. No. 7, 98

MANN, T. (1964)
The Biochemistry of Semen and of the Male Reproductive Tract, Methuen, London

MATHAI, E. and NAMBOODIRIPAD, T. R. B. (1971)
Anim. Breed. Abstr. 39, 85

METCHNIKOFF, E. (1899)
Annls. Inst. Pasteur, Paris, 13, 737

METCHNIKOFF, E. (1900)
Annls. Inst. Pasteur, Paris, 14, 1

MOGHISSI, K. S. (1966)
Fert. Steril. 17, 663

MONESI, V. (1965)
cited by: Beatty, R. A. in: (1975a), Suppl. 1, Biol. J. Linnean Soc. Lond. 7, 291

MONESI, V. (1972)
in: Reproduction in Mammals, vol. I, Austin, C. R. and Short, R. V. (eds.), Cambridge University Press

MOORE, C. R. and PRICE, D. (1948)
J. exp. Zool. 108, 171

MORE O'FERRALL, G. J., MEACHAM, T. N. and FOREMAN, W. E. (1968)
J. Reprod. Fert. 16, 243

MUDD, S., and MUDD, E. B. H. (1929)
J. Immun. 17, 39

MUNTHE-KAAS, A. C. and SEGLEN, P. O. (1974)
FEBS Lett. 43, 252

NEVO, A. C., MICHAELI, I. and SCHINDLER, H. (1961)
Expl. Cell Res. 23, 69

O'DONNELL, J. M. (1969)
J. Reprod. Fert. 19, 263

OHNO, S. (1967)
Monographs on Endocrinology, I, Sex Chromosomes and Sex-Linked Genes, (1967), Springer-Verlag, Berlin, Heidelberg and New York

ORESNIK, A. (1969)
cited by: Schilling, E. (1971) in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 76, Amer. Soc. Anim. Sci., Albany, U.S.A.

ORTAVANT, R., COUROT, M. and HOCHEREAU, M. T. (1969)
in: Reproduction in Domestic Animals, 2nd edition, Cole, H. H. and Cupps, P. T. (eds.), Chapt. 10, Academic Press, New York and London

PAWLOWITZKI, I. H. and BOSSE, H. G. (1971)
Humangenetik 13, 338

PEARL, R. and PARSHLEY, H. M. (1913)
cited by: Van Demark, N. L. and Malven, P. V. (1960), A. I. Dig. 8 (12), 11

PEARSON, P. L. (1971)
New Scient. 49, 606

PEARSON, P. L. (1973)
in: New Techniques in Biophysics and Cell Biology, Vol. I, chapter 7, Pain, R. H. and Smith, B. J., (eds.), Wiley and Sons, London and New York.

PEARSON, P. L. and BOBROW, M. (1970)
J. Reprod. Fert. 22, 177

PEARSON, P. L., BOBROW, M., and VOSA, C. G. (1970)
Nature, Lond. 226, 78

PEARSON, P. L., BOBROW, M., VOSA, C. G. and BARLOW, P. W. (1971)
Nature, Lond. 231, 326

PEARSON, P. L., GERAEDTS, J. P. M. and PAWLOWITZKI, I. H. (1973)
in: Proc. I.N.S.E.R.M. Symp., Paris, 1973, Chromosome Errors in Relation to Reproductive Failure, 219

PERTOFT, H. (1970)
Analyt. Biochem. 38, 506

PERTOFT, H. and LAURENT, T. C. (1969)
in: Progress in Separation and Purification, Gerritsen, T. (ed.), Vol. 2, 71, Wiley and Sons, London and New York

PILZ, Z. (1952)
Z. Tierzücht. Zücht Biol. 60, 315

PINCUS, G. (1930)
Proc. R. Soc. B 107, 132

QUINLIVAN, W. L. G., and SULLIVAN, H. (1974)
Fert. Steril. 25, 315

RABINOWITZ, Y. (1964)
Blood 23, 811

RAJAMMANAN, A. H. J. (1971)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 58, (statement in course of discussion), Amer. Soc. Anim. Sci., Albany, U.S.A.

RENKONEN, K. O. (1956)
Annls. Med. exp. Biol. Fenn. 34, 447

RENKONEN, K. O., MÄKELÄ, O. and LEHTOVAAHA, R. (1962)
Nature, Lond. 194, 308

RICHARDSON, D. W. (1975)
Personal communication

RICKWOOD, D., HELL, A. and BIRNIE, G. D. (1973)
FEBS Lett. 33, 221

ROBERTS, A. M. (1972)
Nature, Lond. 238, 223

ROHDE, W., PORSTMANN, T. and DORNER, G. (1973)
J. Reprod. Fert. 33, 167

ROHDE, W., PORSTMANN, T., PREHN, S. and DORNER, G. (1975)
J. Reprod. Fert. 42, 587

ROSS, A., ROBINSON, J. A. and EVANS, H. J. (1975)
Nature, Lond. 253, 354

ROTHSCHILD, LORD (1960)
Nature, Lond. 187, 253

SACHS, L. and HELLER, E. (1958)
J. natn. Cancer Inst. 20, 555

SALISBURY, G. W., BRATTON, R. W. and FOOTE, R. H. (1952)
J. Dairy Sci. 35, 256

SCANLON, P. E. (1971)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 58, (statement in course of discussion), Amer. Soc. Anim. Sci., Albany, U.S.A.

SCHILLING, E. (1965)
Naturwissenschaften 12, 353
(Anim. Breed. Abstr. (1966) 34, 50)

SCHILLING, E. (1966)
J. Reprod. Fert. 11, 469

SCHILLING, E. (1971)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 76, Amer. Soc. Anim. Sci., Albany, U.S.A.

SCHILLING, E., JAZBEC, I. and SCHMID, P. (1967)
Z. Tierzücht. Züchtbiol. 83, 331
(Anim. Breed. Abstr. (1968) 36, 234)

SCHRÖDER, V. N. (1934)
Biol. Zh. 3, 465

SCHRÖDER, V. N. (1941)
Z. Tierzücht. Züchtbiol. 50, 1

SCHWARTZ, R. H., BIANCO, A. R., HANDWERGER, B. S. and KAHN, C. R. (1975)
Proc. natn. Acad. Sci. U.S.A., 72, 474

SEGAL, S. J. and LAURENCE, K. A. (1962)
Ann. N. Y. Acad. Sci. 99, 271

SEVINÇ, A. (1968)
J. Reprod. Fert. 16, 7

SHETTLES, L. B. (1960a)
Nature, Lond. 186, 648

SHETTLES, L. B. (1960b)
Nature, Lond. 187, 254

SHETTLES, L. B. (1961)
Fert. Steril. 12, 20 and 516

SHETTLES, L. B. (1970)
Int. J. Gynec. Obstet. 8, 643

SHISHITO, S., SHIRAI, M. and MATSUDA, S. (1974)
Andrologia 6 (i), 17

SHISHITO, S., SHIRAI, M. and SASAKI, K. (1975)
Int. J. Fert. 20, 13

SHORT, R. V. (1972)
in: Proc. Int. Symp., The Genetics of the Spermatozoon, Edinburgh, 1971, Beatty, R. A. and Gluecksohn - Waelsch, S. (eds.), 325

SHORTMAN, K. (1972)
Annu. Rev. Biophys. & Bioeng. 1, 93

SIEGEL, P. W. (1916)
cited by: James, W. H. (1971), Lancet i, 112

STAMBAUGH, R. and BUCKLEY, J. (1967)
J. biol. Chem. 242, 4053

STAMBAUGH, R. and BUCKLEY, J. (1971a)
J. Reprod. Fert. 25, 275

STAMBAUGH, R. and BUCKLEY, J. (1971b)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 59, Amer. Soc. Anim. Sci., Albany, U.S.A.

STEENO, O. and ADIMOELJA, A. (1975a)
Annalen von der Vereniging voor Fertiliteitsstudie, Tweede Jaargang Deel I Voorjaarsvergadering gehouden te Utrecht, op. 10 mei, 1975

STEENO, O., ADIMOELJA, A. and STEENO, J. (1975b)
Andrologia 7, 95

STONE, A. B. (1974),
Biochem. J. 137, 117

SUMNER, A. T. and ROBINSON, J. A. (1976)
J. Reprod. Fert. 48, 9

SUMNER, A. T., ROBINSON, J. A. and EVANS, H. J. (1971)
Nature New Biology 229, 231

TARKOWSKI, A. K., WITKOWSKA, A. and NOWICKA, J. (1970)
Nature, Lond. 226, 162

TATES, A. D., PEARSON, P. L. and GERAEDTS, J. P. M. (1975)
J. Reprod. Fert. 42, 195

TYLER, A. (1961)
4th Oliver Bird Lecture, in: J. Reprod. Fert. 2, 473

ULSTEIN, M. (1972)
Acta obstet. gynec. scand. 51, 335

VAN DER HORST (personal communication to PERERA, B.M.A.O., 1975)
cited in: "Some effects of vasectomy on the Reproductive System of the Ram."
Ph.D. thesis, University of Glasgow, Faculty of Veterinary Medicine, 1975

VAN DEMARK, N. L. and MALVEN, P. V. (1960)
A. I. Dig. 8 (12), 11

VAN DUIJN, C. (jnr.), (1961)
Fert. Steril. 12, 509

VLADIMIRSKAYA, E. M. (1966)
Tsitol. Genet. respub. mezhved. Sb. (2): 153 Rus.
(Anim. Breed. Abstr. (1967) 35, 568)

VOSA, C. G. (1970)
Chromosoma (Berl.) 30, 366

WEIR, J. A. (1955)
J. Hered. 46, 277

WEIR, J. A. (1971)
in: Sex Ratio at Birth - Prospects for Control, Symp. 1970, Kiddy, C. A. and Hafs, H. D. (eds.), 43, Amer. Soc. Anim. Sci., Albany, U.S.A.

WHITFIELD, C. H. (1976)
Personal communication

ZANEVELD, L. J. D., WAGNER, L., SCHLUMBERGER, H. D. and SCHUMACHER, G. F. B. (1974)
J. Reprod. Fert. 38, 411

ZECH, L. (1969)
Expl. Cell Res. 58, 463