Chromosome Studies Of Rabbit Blatocysts Resulting From Spermatozoa Aged In Vivo

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CHROMOSOME STUDIES OF RABBIT BLASTOCYSTS
RESULTING FROM SPERMATOZOA
AGED IN VIVO

by

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Department of Anatomy

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
April 1972

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To my parents and my
husband, Winston.
This work was supported by grants from the Medical Research Council of Canada. The author wishes to express thanks to this granting agency.
ABSTRACT

The effect of ageing rabbit spermatozoa in either the female or the male reproductive tract on the chromosomes of resulting blastocysts was studied. Ageing in the female tract was accomplished by artificially inseminating females at different time in relation to an ovulatory-inducing injection of chorionic gonadotrophin. Ovulation was assumed to occur 10 hours after the injection given at various periods from 0 to 21 hours post-insemination. Thus, spermatozoa were retained in the female tract 10 to 31 hours before ovulation.

The chromosome complement was established in 239 of 251 blastocysts recovered. A significant difference was found between the incidences of chromosomal anomalies in the control group, where ova were fertilized by sperm retained for 10 hours in utero, and the experimental series. The chromosomal anomalies in the experimental series consisted of five 2N/4N mixoploids, five mosaics and three sex chromosome chimaeras. The latter has been hitherto unreported in the rabbit. A 2N/4N mixoploid was the only anomaly in the control group. The sex ratio was not significantly affected by in utero ageing of spermatozoa.
Spermatozoa were aged in the male tract by bilateral ligation of the corpus epididymidis, thus preventing an inflow of fresh spermatozoa from the testis. Females were inseminated with ejaculates collected before ligation and at various post-operative intervals from Day 7 to 35. Ovulation was induced at the time of insemination by exogenous chorionic gonadotrophin.

No blastocysts were recovered from females inseminated with sperm aged 35 days in the males. Eight of 72 blastocysts resulting from sperm aged seven to 27 days were chromosomally abnormal. This incidence of 11.1% was significantly different from the 0.8% incidence of anomalies in blastocysts resulting from sperm collected before ligation. The anomalies were varied, the commonest being autosomal trisomy. Two 2N/4N mixoploids and a triploid arose when ova were fertilized by seven-day-old sperm. One trisomy, one 44/45 mosaic and a deletion were seen in blastocysts resulting from sperm aged 12 to 15 days. Two other trisomic blastocysts were recovered from females inseminated with sperm aged 19 to 21 days. The sex ratio of blastocysts was not affected by ageing of sperm in the male.

The results indicate that ageing of sperm in both male and female tracts leads to a significant increase of chromosome anomalies in resulting blastocysts. The anomalies detected when sperm were aged in the male are mainly those associated with abortions and gross developmental defects. Anomalies observed after sperm ageing in utero were mostly those compatible with extra-uterine life, inflicting less severe phenotypic effects.
ACKNOWLEDGEMENTS

This project was suggested by Dr. E. L. Shaver. The author wishes to thank her sincerely for the opportunity to do the study and for her encouragement and supervision.

Thanks are especially due to Dr. E. B. Gammal who gave unstintingly of his time and of his experience in surgical skills.

Miss Isobel Morrison offered excellent assistance in various technical aspects especially photography and the arrangement of the large number of karyotypes. The author is greatly indebted to her.

The author appreciates the assistance offered by Dr. R. C. Buck in phase contrast microscopy, Mrs. Margaret Corrin in the preparation of illustrations and Dr. R. P. Singh for his critical reading of the manuscript. Credit is due to Mrs. Lyn McLean who typed the manuscript efficiently. Finally, I am indebted to my husband, Winston, who not only endured the preparation of this thesis but offered his encouragement constantly.
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I. INTRODUCTION

In man, microscopically identifiable chromosome change has been related to spontaneous abortions, congenital malformations, abnormal sexual development and function, mental retardation, malignant neo-plasms and behavioural disturbances. The severe effects of chromosome aberrations, as well as the high frequency with which they occur in spontaneous abortions (Carr, 1967, 1971), indicate that the study of their aetiology is important.

Some aetiological aspects of chromosomal abnormalities have been studied in experimental animals. Many genetic and environmental factors have been implicated as causative agents. These aetiological studies can serve two main purposes. They may help to find the means of reducing the incidence of aberrations in the population. On the other hand, they can provide experimentally-produced chromosome aberrations for genetic studies in mammalian species.

Many chromosomal abnormalities are due to anomalies of fertilization. It is logical, therefore, to examine the factors affecting the gametes and the timing of the events which lead to fertilization. Fertilization of ageing eggs, delayed fertilization, has been shown to lead to an increased incidence of chromosomal anomalies in amphibians (Witschi and Laguens, 1963), rabbits (Shaver and Carr, 1967; Austin, 1967), and mice (Vickers, 1969). On the other
hand the chromosomal effects of ageing spermatozoa have never been reported in the literature. Nevertheless, indirect evidence suggested that in cattle structural anomalies in embryos resulting from spermatozoa aged *in vitro* may be due to chromosome disorders (Salisbury, 1965; Salisbury and Hart, 1970).

In the rabbit, ageing of spermatozoa in the female reproductive tract prior to ovulation (Tesh, 1969) and in the male tract prior to ejaculation (Tesh and Glover, 1969) has resulted in a decline in fertility. Increased pre- and post-implantation loss were observed in these two studies. There is a high correlation between reproductive failure and chromosomal abnormalities. Therefore, the present investigation was conducted to study the consequences of ageing rabbit spermatozoa in the male and female reproductive tracts, on the chromosome constitution of resulting blastocysts. The rabbit, *Oryctolagus cuniculus* (L.), is particularly suited for this study since the time of ovulation can be determined with accuracy, its reproductive biology is well studied, artificial insemination can be carried out with some ease, and the blastocysts grow to an unusually large size prior to implantation.
II. HISTORICAL REVIEW

A. Consequences of Sperm Ageing with Particular Reference to the Rabbit

Excellent reviews have been published on the effects of ageing spermatozoa in invertebrates and infra-mammalian vertebrates by Lanman (1968) and Salisbury and Hart (1970). The present section will be confined to ageing of spermatozoa in mammals. The effects of ageing vary greatly according to the environment.

(i) Ageing of spermatozoa in utero

Spermatozoa may be aged in the female tract by prolonging the interval between insemination and ovulation. The first comprehensive investigation of this was conducted in the rabbit (Hammond and Asdell, 1926). A decrease occurred in the litter size when spermatozoa had remained in the female tract for 22 hours before ovulation. After 30 to 32 hours, the fertilizing ability of the spermatozoa was completely lost. No increase in abnormal off-spring was detected. Noyes and Thibault (1962), who corroborated the above finding for the fertile life-span of rabbit spermatozoa, showed that sperm motility is maintained for 50 hours after insemination.

Observations on the functional life of sperm in utero were extended later to other species. When spermatozoa were aged for 22 hours in the guinea-pig (Soderwall and Young, 1940) and 14 hours in
rat (Soderwall and Blandau, 1941), no litters were produced. A decline in litter size occurred after 17 hours in the former and after 10 hours in the latter. No increase in developmental anomalies was observed in either species. In the bat, horse, ferret and mouse, complete infertility was shown to occur when sperm resided in the female tract for 135 days (Wimsatt, 1944), six days (Day, 1942), 126 hours (Chang, 1965) and 12 hours (McGaughey, Marston and Chang, 1968), respectively.

The above observations suggested that ageing of spermatozoa in utero affects fertility only, with development proceeding normally if fertilization occurs. However, the occurrence of live-births was the only criterion of fertility used in most of the studies. Direct observation of the contents of the reproductive tract early after fertilization is a more reliable indicator of fertilization rate. This procedure was followed in later studies.

Maurer, Whitener and Foote (1969) used initial cleavage of oocytes in vivo and their subsequent development during culture as test criteria of ageing rabbit spermatozoa. After 20 hours storage of spermatozoa in utero, fertilization and cleavage rates were reduced and fewer zygotes developed into blastocysts. Studying fertilization rate, pre- and post-implantation loss, Tesh (1969) confirmed the original finding of Hammond and Asdell (1926) of a 30 to 32 hour fertile life-span of rabbit spermatozoa. In addition, he demonstrated that pre- and post-implantation losses contribute to the decline in fertility observed with ageing. The losses appeared to occur at progressively earlier stages with increasing spermatozoal
(ii) **Ageing of spermatozoa in the male genital tract**

In the male genital tract, spermatozoa may be aged after prolonged periods of sexual rest or experimentally by ligation of the corpus epididymidis, a procedure which prevents fresh spermatozoa from entering the cauda epididymidis, the ductus deferens and subsequently the ejaculate.

The earliest investigation of aged spermatozoa in the male tract was reported by Hammond and Asdell (1926). Complete infertility occurred 38 days after surgical ligation of the corpora epididymides in the rabbit, although spermatozoa were still motile at 60 days. Normal litter size and number of pregnancies were maintained up to 20 days, with a marked decline thereafter. No observations were made on the conceptuses.

In the guinea-pig, degenerative changes in epididymidal spermatozoa appear with the passage of time and result in the loss of fertilizing capability (Young, 1929, 1931). Complete fertility was lost after 35 days and at 20 to 25 days an increase in the incidence of abortions and resorptions occurred. Sperm retained in the male lose their ability to fertilize ova after 10 to 14 days in the mouse (Snell, 1933) and after 21 days in the rat (White, 1933). However, motility was retained for 42 days in the latter.

Increased temperatures have been shown to accelerate sperm ageing in the rabbit (Asdell and Salisbury, 1941). The testes and epididymides were anchored in the abdomen where the temperature is 3°F
greater than that in the scrotum. Spermatogenesis ceased after 24 hours. Fertilizing capacity, as judged by live-births, disappeared after eight days and motility after 14, as compared with 38 and 60 days, respectively, for spermatozoa aged in the scrotum.

Later studies assessed the early embryonic stages resulting from fertilizations with aged sperm. Igboeli and Foote (1969) reported a decrease in cleavage and kindling rates when rabbit spermatozoa had aged 28 to 35 days in the caudal epididymides. In a well-planned study, Tesh and Glover (1969) showed that fertilization rate was unaffected until spermatozoa were aged 28 days, thereafter it declined steadily reaching zero at 49 days. Pre-implantation loss increased progressively to 28 days while post-implantation loss reached a peak at 35 days. A number of congenital abnormalities, including abnormal skull sutures and the absence of gall bladders, occurred.

(iii) The in vitro ageing of spermatozoa

The various studies on the effect of storage in vitro of spermatozoa have been recently reviewed by Hart and Salisbury (1970). They indicate that decreased fertility and increased embryonic loss are direct consequences of ageing of bovine spermatozoa. A similar ageing effect has been demonstrated in swine (Dziuk and Hanshaw, 1958); First, Stratman and Casida, 1963) and in the rabbit (Miller and Blackshaw, 1968). In the latter, as in cattle, the deteriorating effects of ageing are preceded by an improvement in fertility. Salisbury and Hart (1970) suggested that this is due to a selection against abnormal spermatozoa for fertilization sites, shortly after storage before ageing occurs. Abnormal sperm, present in the ejaculate
immediately after collection, compete effectively with normal spermatozoa for fertilization sites. In the rabbit, besides decreased fertility, high pre- and post-implantation losses have been observed after storing sperm at 5°C for 24 and 48 hours (Koefoed-Johnsen, 1971).

B. Biology of Rabbit Spermatozoa and Fertilization

In the male rabbit, the ability of the spermatozoon to fertilize an ovum is developed as it passes from the caput to the caudal epididymidis (Bedford, 1963, 1966), an important storage area (Kennelly and Foote, 1964; Orgebin-Crist, 1968a) and the immediate source of most ejaculated spermatozoa (Kirton, Desjardines and Hafs, 1967). Metabolic activity of spermatozoa is considered to be low in the male tract (Salisbury and Lodge, 1962). Their transport along the tract, resulting in their appearance in the ejaculate, is facilitated by rhythmic contractions of the smooth muscles in the epididymidis and vas deferens.

In natural mating, insemination is intravaginal. Spermatozoa, transported rapidly by means of the flagellar motion of the sperm tails and the muscular activity of the female reproductive tract, reach the oviduct as early as one hour after insemination (Adams, 1956; El-Banna and Hafez, 1970). Sufficient numbers to effect fertilization are present in the oviduct after five to six hours (Adams, 1956). The average ejaculate, 0.7 ml., contains 200 to 300 million spermatozoa (Adams, 1961), a number which far exceeds that required for fertilization. Maximal levels of fertilization have been reported when $1 \times 10^6$ spermatozoa were deposited in the vagina (Chang, 1946; Wales, Martin and O'Shea, 1965). In the naturally mated rabbit,
ovulation occurs approximately 10 hours after mating (Hammond and Marshall, 1925; Walton and Hammond, 1928; Harper, 1961). At this time, it has been estimated that 2,000 to 5,000 spermatozoa are present in the fallopian tubes (Austin, 1948; Braden, 1953).

During passage through the female tract, the spermatozoon undergoes a final maturation process, capacitation, by which it attains the ability to penetrate the cumulus oophorous and the zona pellucida of the ovum. Capacitation of rabbit spermatozoa, a prerequisite for fertilization, has been reported to take place after five to six hours residence in the female tract (Chang, 1951a; Austin, 1951). However, more recent findings indicate that spermatozoa require a considerably longer residence for maximum fertilizing competence (Kirton, 1967; Cummins and Orgebin-Crist, 1971; Harper, 1970a). The changes which occur as a concomitant of capacitation are uncertain. However, there seem to be modifications in the pattern of sperm metabolism since the respiration rate of capacitated spermatozoa is greater than freshly ejaculated spermatozoa (Bedford, 1970). No morphological changes have been reported after capacitation.

The morphology and structure of the ejaculated spermatozoon have been studied extensively. Excellent descriptions of the ultrastructure have recently appeared in the literature (Fawcett, 1970; Bernstein and Teichman, 1972). Described briefly, the head which consists of the nucleus, is flattened in the dorso-ventral plane and exhibits three distinct regions in the covering. The anterior one-half to two-thirds of the nucleus is covered by the acrosome, the middle by the thin equatorial segment of the acrosome, and the remainder by the
post-acrosomal segment. The acrosome, a membrane-limited structure, seems to be responsible for the release of lytic enzymes which facilitate access of the sperm to the ovum.

The tail, the propelling force for movement of the spermatozoon, is composed consecutively of the neck, middle-piece, principal-piece and end-piece. The neck contains the basal plate, the connecting piece and the centrioles. Axial contractile filaments arise from the latter and continue throughout the tail. The middle-piece contains the mitochondrial sheath surrounding the coarse fibres and the filaments.

After capacitation, the spermatozoon becomes competent to undergo the acrosome reaction in response to chemical stimuli from the recently ovulated egg and the follicular fluid. This reaction involves breakdown of the outer membrane of the acrosome during its fusion and vesiculation with the underlying plasma membrane (Bedford, 1970). Release of the acrosomal enzymes probably facilitates the passage of sperm through the granulosa cell investment to the surface of the zona pellucida of the ovum. With the aid of a "zona lysin" in the sperm head, the penetrating sperm bounded with the inner acrosomal membrane traverses the zona pellucida. The post-acrosomal region becomes associated with the vitelline surface and fusion of egg and sperm membranes follows. The posterior part of the sperm head then sinks into the vitellus while the rostral region, incorporated later, is engulfed by the oöplasm within a "phagocytic vesicle". With great rapidity, the sperm nucleus then disintegrates into the
oöplasm followed by its separation from the sperm tail.

In the meantime, the chromosomes of the penetrated egg or secondary oöcyte are arranged on the second meiotic metaphase spindle which is oriented radially in the vitellus. The first polar body is extruded before ovulation. Observations on this later phase of fertilization have been presented (Chang and Hunt, 1962; Harper, 1970b). By the time the penetration of the vitelline membrane is completed, the chromosomes of the secondary oöcyte have started to separate gradually to the poles of the spindle. Telophase follows and one group of chromosomes from the secondary oöcyte is extruded from the vitellus in the form of the second polar body. The haploid chromatin in the secondary oöcyte gradually migrates toward the centre of the egg being transformed simultaneously into the early female pronucleus. The diffuse sperm chromatin aggregates to form an early male pronucleus which migrates toward the centre of the egg and meets the female pronucleus. Syngamy then occurs with the fusion of the two pronuclei. With the acquisition of the diploid chromosome number, the process of fertilization is completed approximately 24 hours after insemination or fourteen hours after ovulation (Pincus, 1931; Austin and Braden, 1954; Chang, 1951b). The site of fertilization is the ampulla-isthmus junction of the oviduct (Greenwald, 1961).

Immediately after fertilization, the first cleavage division of the zygote occurs. Gregory (1930) has provided a classic description of the detailed development of the rabbit zygote. The beginning of the blastocyst stage is marked by the appearance of a cleft within
the morula, 65 hours post-insemination. By 70 hours, the blastocyst enters the uterus. It begins to implant after the sixth day. By this time it has increased in diameter from 1 or 2 mm. to 4 mm. (Davies and Hesseldahl, 1971) and the trophoblast is one-cell thick except where continuous with the inner cell mass. The cell number has then multiplied to approximately 60,000 to 70,000 (Lutwak-Mann, 1971). However, considerable variability in cell number is seen in blastocysts recovered from normal rabbits of approximately the same weight. An interesting feature of the rabbit ovum is the acquisition of a thick mucin coat over the surface of the zona pellucida (Bacsich and Hamilton, 1954). Composed of acid mucopolysaccharides, it is secreted by the lining of the epithelial cells of the oviduct.

C. Karyotypic Analysis of the Domestic Rabbit

It is almost 50 years since the diploid chromosome number for the rabbit was accurately determined as 44 (Painter, 1926). Using amnion cells of embryos, Painter (1926) showed that there is a similar chromosome number and morphology in the dissimilar Flemish Giant and small Polish breeds. Although Painter (1926) confined his studies mainly to chromosome counts, he described the X chromosomes as medium-sized and the Y chromosome as the smallest element in the complement.

With improved techniques for studying mammalian chromosomes in various tissues, chromosome counts in the rabbit have been supplemented with karyotype analyses. In 1956, Melander published the first complete karyotype analysis of the rabbit chromosomes. The chromosomes were arranged in pairs according to length and centromeric position.
The sex chromosomes were described as a large submetacentric X and a small acrocentric Y while the autosomal pairs were numbered from one to 21 in decreasing order of size. Later, advances in karyological techniques led to revision of the karyotypic arrangement, although most authors agree in the general descriptions of the autosomes.

Slight to moderate revision of Melander's (1956) arrangement of the chromosomes was made by Teplitz and Ohno (1963); McMichael, Wagner, Nowell and Hungerford (1963); Pruniéras, Jacquemont and Mathivon (1965) and Ray and Williams (1966), all of whom used chromosome length as the main basis for classification.

The arrangement of the karyotype primarily on the basis of arm ratio, was employed by Nichols, Levan, Hansen-Melander and Melander (1965); Hsu and Benirschke (1967); Dave, Takagi, Oishi and Kikuchi (1965). The former two groups of investigators designated the X and Y as a medium-sized and a small submetacentric chromosome, respectively; the Y being the smallest in the complement. They arranged the chromosomes into four groups, each with a characteristic arm index. Six pairs of chromosomes with median to submedian centromeres were placed in the first group. The second group consisted of five pairs of autosomes with submedian centromeres. The third group was composed of six pairs with subterminal centromeres while the fourth group had four pairs with subterminal to terminal centromeres. The chromosomes within each group were placed according to length, relative to other members of the group. Nichols et al. (1965)
numbered consecutively all chromosome pairs, with number eight assigned to the medium-sized submetacentric X chromosome. Hsu and Benirschke (1967) separated the sex chromosomes from the autosomes. They did not number the autosomes. The karyotypic arrangement followed in the present investigation is that of Hsu and Benirschke (1967) except that the autosomal pairs are numbered consecutively from one to 21 (Figures 1 and 2).

Issa, Atherton and Blank (1968) argued that classification of chromosomes on the basis of arm ratio alone would result in overlapping between groups, with consequent difficulty in assigning chromosomes to the various groups. They, as well as Myers, O'Leary and Fox (1964) used the combined basis of the arm ratio and relative length of the chromosomes in the arrangement of the karyotype. However, these two studies differ in description and groupings of the chromosomes. Also, the arrangement proposed by Issa et al. (1968) consists of as many as 11 groups. It is cumbersome to handle so many groups in the construction of karyotypes.

Morphological characteristics such as secondary constrictions and in particular satellites, have been helpful in the identification of chromosome pairs in the rabbit karyotype. Melander (1956) did not observe any secondary constrictions in his study. However, Pruniéras et al. (1965) and Shaver (1968) observed them on medium to small-sized metacentric chromosomes which are numbered five and 10 in the series followed in the present investigation. Issa et al. (1968) reported a maximum of seven satellited chromosomes in mitotic figures. Satellites have been observed on one of the pairs of small acrocentric
chromosomes (Pruniéras et al., 1965; Nichols et al., 1965; Issa et al., 1968), which is numbered 20 in this study. They have been reported on large submetacentric pairs (Pruniéras et al., 1965; Nichols et al., 1965; Issa et al., 1968), which are numbered 13 and 16 in this study.

There has been some discrepancy in the description of the sex chromosomes of this species. With regard to the Y chromosome, the dispute now seems to be almost settled. Most of the recent authors feel that it is the smallest in the series, although similar in length to the small acrocentric chromosomes. They state that it is a submetacentric with a characteristic arm ratio. This has been confirmed by autoradiographic techniques which demonstrate the late-replicating pattern of the Y chromosome (Issa et al., 1968). In most mammals, late replication is typical of sex chromosomes.

Several different arm ratios and sizes have been assigned to the X chromosome. In all published karyotypes, so far, there are other chromosomes similar in relative length and arm ratio to the chromosome which the authors have designated as the X. Usually, most authors select the X chromosome from among those it resembles, mainly because of the impossibility of pairing it with an autosome in male cells. This procedure has been severely criticised (Patau, 1960). Teplitz and Ohno (1963) stated that the X was a small acrocentric chromosome. Pruniéras et al. (1965) suggested that the X showed morphological variability from a large metacentric in some cases to a medium-sized submetacentric chromosome in other cases. A report by Dave et al.
(1965) stated that the X was the fifth largest chromosome in the complement. However, among many authors there is a general agreement that the X is a medium-sized submetacentric chromosome. This has been supported from autoradiographic studies (Ray and Williams, 1966; Issa et al., 1968). Issa et al. (1968) remained "convinced that the rabbit X chromosome cannot be identified by any means other than the labelling pattern in female cells".
III. MATERIALS AND METHODS

A total of 16 adult male and 55 female rabbits were used to determine the incidence of chromosome abnormalities in two populations of six-day blastocysts. There were two separate experiments. The blastocysts studied in the first experiment were recovered from females in which ova were fertilized by spermatozoa aged in utero. In the second experiment, blastocysts were recovered from females inseminated with spermatozoa aged in the male tract. The rabbits were of California and New Zealand White strains and were purchased from a commercial dealer. All animals were given constant access to drinking water and food pellets (Master Feeds Ltd.). This was especially important in the second experiment, since good nutrition is essential for lowering the chances of absorption of spermatozoa in the male tract. Artificial insemination was employed in each experiment. Its procedure was the same in both.

A. General Procedure for Artificial Insemination

Sexually mature males were trained to serve an artificial vagina which was constructed according to the model described by Bredderman, Foote and Yassen (1964). A small drop of each semen sample collected was placed on a slide in order to examine the motility and morphology of spermatozoa under the microscope. The semen samples from different males were neither pooled, nor diluted. Only samples with spermatozoa showing high motility were used for insemination.
All females were caged separately for 19 to 21 days prior to use in order to avoid pseudopregnancy. At the time of insemination each female was restrained in a supine position and the body above the hind legs was wrapped in a towel to prevent struggling. The ejaculate was then quickly drawn into the inseminating pipette. The latter was inserted carefully into the vagina, avoiding injury to the vaginal wall, beyond the pelvic brim by directing the tip of the pipette towards the backbone. When inserted to an approximate depth of four inches, the pipette was withdrawn a 1/4 inch and the semen was expelled into the anterior vagina.

B. Procedure for Ageing Spermatozoa in the Female Reproductive Tract

Ageing of spermatozoa was accomplished by inseminating females at different times in relation to ovulation.

Forty females weighing 2.9 to 4.2 kg. and nine bucks selected for their high fertility, were assigned to this experiment. Sperm counts were made using a haemocytometer on semen samples from each buck. In the control females and in does in which sperm were aged for 14 to 28 hours, 0.1 to 0.2 ml. of semen containing 50 to 100 x 10⁶ spermatozoa was used for insemination. For spermatozoa aged 29 to 31 hours, each doe was inseminated with 0.6 ml. semen with a sperm count of approximately 250 x 10⁶. All does were inseminated within five minutes of semen collection.

Ovulation, induced by an injection of 60 i.u. human chorionic gonadotrophin ('A.P.L.' Ayerst Laboratories) into the marginal ear vein, was assumed to occur 10 hours after injection (Harper, 1963). Thirteen females served as controls and were injected with HCG at
the time of insemination. In the experimental group, a delay in
the administration of human chorionic gonadotrophin (HCG) until 4 to
21 hours post-insemination resulted in a prolonged interval between
insemination and ovulation, thus allowing spermatozoa to be stored
for an estimated 14 to 31 hours. The storage period will be termed
the ageing period in this investigation. The number of animals
allotted to each of the 11 different time intervals is shown in
Table 1.

C. Procedure for Ageing Spermatozoa in the Male Genital Tract

(i) Surgical procedure

In this experiment 10 adult male rabbits, showing a high degree
of fertility, were subjected to a surgical operation. Three of these
bucks were used in the previous experiment. The corpus epididymidis
was ligated bilaterally in eight males to prevent the passage of
fresh spermatozoa from the caput epididymidis and testis. Thus,
further sperm replenishment in the caudal epididymides was avoided
and only sperm present at the time of operation passed into
subsequent ejaculates. Figure 3 illustrates the anatomical
arrangement of the testis and epididymidis.

Bucks were anaesthetized by an injection of 2.5% sodium thiopental
(Surital, Parke Davis and Co.) into the marginal ear vein. Since
there was a variation in response to the anaesthetic, an effective
dosage was administered slowly and intermittently with a saline-drip.
The dosages ranged from 6 to 16 ml. The saline-drip kept the vein
open and prevented clotting. Sterile procedure was followed
throughout the entire operation.
Sham operations were performed to ensure that the procedure did not affect the normal function of spermatozoa. In one sham-operated male, an incision of approximately 1 cm. was made through the skin of each scrotal sac on the lateral surface. A similar incision was made through the fascia and tunica vaginalis. The corpora epididymides were located but not ligated. Throughout the operation, care was taken neither to injure nor to ligate blood vessels supplying the testes or epididymides. Following careful suturing of the fascia, wound clips were used to close the incision in the skin. An intramuscular injection of 200,000 i.u. penicillin and streptomycin (Pen-di-Strep, STB) was given immediately post-operatively and the animal returned to a cage that had been sterilized. A similar injection of antibiotics was repeated on the day following the operation.

The identical procedure for the sham operation was followed in the eight males with the exception that the corpus epididymidis was ligated bilaterally. In these males two ligatures (Figure 4) of sterile non-absorbable 4-0 silk (Davis and Geck) were made, approximately 0.5 cm. apart, on the distal portion of the corpus epididymidis. The segment between the ligatures was later removed. In this approach, there was little chance of traumatizing the tissues since the testes and epididymides, except for the segments removed, were not handled. However, in two bucks the incision was made more ventrally than laterally and as a result the testes had to be removed from the scrotal sacs to locate and ligate the corpora epididymides. Therefore, a second sham operation was performed. In this operation,
the testes of one male were removed from the scrotal sacs and returned, without ligation of the corpora epididymides.

(ii) **Semen evaluation and insemination**

Immediately after collection, semen samples were examined microscopically for spermatozoan motility and density. Afterwards, spermatozoa were stained with aqueous nigrosin-eosin solution (Glover, 1960). This stain gave a good indication of the number of dead spermatozoa which stained eosinophilically. In order to examine the morphology of the spermatozoa, semen smears were stained with a 1:1 mixture of crystal violet-aniline blue for 30 minutes. Before staining, the smears were dried in air and fixed in neutral formol-saline (10% formaldehyde in 0.9% saline) for 30 minutes. The slides were examined using the high dry objective and phase contrast microscopy. Photomicrographs of selected spermatozoa were taken using Kodak Panatomic X film.

Ejaculates collected from each of the eight bucks two to four days prior to ligation of the corpora epididymides and from six other males were used to inseminate females, serving as controls. These were the same females in the control group of the first experiment. All females in this experiment were given an ovulation-inducing injection of 60 i.u. HCG intravenously at the time of insemination. Semen was collected seven days post-operatively from the first sham-operated male and 35 days from the second animal. The day of operation was considered as Day 0. It was used as the reference point for assessing the age of the sperm after ligation of the corpora epididymides. The primary aim was to collect semen at weekly
post-operative intervals. Difficulty was encountered in obtaining ejaculates at the appointed time, therefore, an attempt was made to collect semen one to two days before or after the weekly intervals. Table 5 records the number of does inseminated with semen collected at the different intervals.

One ejaculate collected post-operatively on Day 7 was divided into two equal parts to inseminate two females. Apart from these, all other females were inseminated with the full ejaculate collected at each post-operative period. No other ejaculates were collected post-operatively from males in which spermatozoa were aged for 35 days. This was to prevent depletion of available motile spermatozoa in the ejaculate. One female was mated to a male in which spermatozoa had aged 19 days. A vaginal smear, from this female after mating, revealed the presence of motile spermatozoa and the female was immediately injected with 60 i.u. HCG.

D. Recovery of Blastocysts and Chromosome Analysis

All females were sacrificed six to seven days after HCG injection by an overdose of sodium pentobarbital (Nembutal, Abbott Laboratories). An abdominal incision was made in the midline and the uterine horns were exposed. They were clamped at the utero-tubal and utero-vaginal junctions. The ovaries were removed in order to count the number of corpora lutea, as an indication of the number of ova shed. The clamped uterus was excised by cutting through the vaginal and oviducal walls adjacent to the respective clamps. It was quickly placed in a Petri dish containing physiological saline warmed to 37°C. The uterine horns were flushed with warm saline to recover the blastocysts.
To flush the horns, an incision was made below the clamp at the utero-tubal junction and a second cut above the clamp at the utero-vaginal junction. A 5-ml. syringe containing the warm saline was introduced into the first incision of each uterine horn. The blastocysts were then gently forced through the utero-vaginal junction into the Petri dish with the saline expelled from the syringe.

Chromosome spreads were prepared from each blastocyst by the method published by Shaver and Carr (1967). After removal from the tract, the blastocysts were immediately transferred from the warm saline to a bottle containing 20 ml. tissue culture medium 597 (Connaught Laboratories) and colcemid (Grand Island Biochemical Co.) in final concentration of 1/10,000. Colcemid arrests mitosis at metaphase. The medium with the colcemid was pre-warmed to 37°C and the blastocysts were incubated at this temperature for 1 1/2 to 1 3/4 hours.

Following incubation, the blastocysts were treated with a hypotonic solution. Each blastocyst was placed in a separate 10-ml. centrifuge tube containing 1 to 2 ml. of 0.9% sodium citrate warmed to 37°C. The sizes of the blastocysts were noted. They were numbered from the smallest to the largest in descending order of magnitude by placing each in a numbered centrifuge tube. Each numbered tube was provided with a Pasteur pipette which had the same number as that of the tube. With the aid of the Pasteur pipette, each medium or large-sized blastocyst was broken and made into a cell suspension in the hypotonic solution. Small blastocysts were not broken and were treated whole. All blastocysts were incubated in the hypotonic
citrate for 10 to 15 minutes.

After incubation, the tubes were then centrifuged at 800 to 1000 rpm for five minutes. The supernatant citrate was decanted carefully from each tube and was replaced by approximately 2 ml. of methanol-acetic (3:1) fixative. Tubes were then allowed to stand in the refrigerator at 4°C overnight. The fixative was decanted the next day, after centrifugation, and the cell suspension or the small whole blastocysts were treated with 45% aqueous acetic acid for two minutes. Following centrifugation most of the supernatant aqueous acetic acid was decanted. The cells of the broken blastocysts were then re-suspended in the remaining supernatant which was approximately 0.4 ml. in volume. Smears were made by placing small drops of the cell suspension on slides warmed on a hot plate at 56°C. The cells of the small unbroken blastocysts were then dissociated in a few drops of aqueous acetic acid. After five to 10 minutes the smears were dried and were ready for staining with ammoniacal Giemsa (Genest and Auger, 1963).

Metaphase plates were scored using a 100X oil-immersion objective and bright field microscopy. Chromosome morphology and the sex complement were noted. An analysis of the chromosomes in a minimum of 30 cells and the construction of four karyotypes from the cells of each blastocyst was the primary aim. When an abnormality was suspected, as many cells as possible were analysed and additional karyotypes were made. Photomicrographs of metaphases for karyotypes were taken with Kodak High Contrast Copy film and were printed on F5 Kodabromide paper. The karyotypes were assembled by cutting out
individual chromosomes from photomicrographs and arranging them as previously outlined.
IV. RESULTS

A. New Observations on the Sex Chromosomes of the Rabbit

(i) The X chromosome

In a proportion of metaphase plates from blastocysts with an XX chromosomal complement, one chromosome was differentially stained and was readily distinguished under the microscope (Figure 5). This chromosome, which stained more intensely than all others, was medium-sized with a submedian centromere. It had the size and centromeric position of the X chromosome and invariably was assigned as an X, without any conscious effort, when the karyotypes were being assembled. It tended, however, to be longer than its homologue. A karyotype of Figure 5b may be seen in Figure 2. Apart from the more intense staining, this chromosome characteristically demonstrated (a) a peripheral or near-peripheral location on the metaphase plate, although on a few occasions it was seen in the centre; (b) a smooth outline with no evidence of quaternary coiling when other chromosomes in the complement showed this coiling, and (c) chromatid arms which were thinner than those of other chromosomes in the complement. Sometimes a sharp kink was seen midway along the long arm(s) and sometimes the chromosome was bent upon itself. Such a chromosome with the features described above was first observed after the study had been in progress for some time. It was afterwards seen in at least 50
blastocysts. These blastocysts were recovered from females of both strains. The distinctive X chromosome was demonstrated in 10 to 90% of cells with a female complement in the different blastocysts. It was never observed in metaphase plates with an XY sex complement. In some tetraploid cells with a female sex chromosome complement, two such X chromosomes were obvious in metaphase spreads. The evidence outlined above strongly suggests that this chromosome which demonstrates positive heteropycnosis, is the late-labelling X chromosome.

(ii) The Y chromosome

During this investigation, a minute chromosome was observed in a number of blastocysts with an apparent male chromosomal complement. All these blastocysts had a modal count of 44 chromosomes including the minute chromosome. The normal complement of autosomes and a normal X chromosome were present in each blastocyst; the minute chromosome was therefore assumed to be of Y origin. In most cells, the unusually small Y chromosome had the appearance of a centric fragment (Figure 6). These blastocysts with the presumptive male chromosomal complement accounted for 60% of all the blastocysts recovered from five females, three Californians and two New Zealand Whites. The remaining 40% of blastocysts recovered from these females all had a normal XX complement.

The five females were inseminated with ejaculates from either of two bucks which were of Californian strain. In these two bucks, an identical minute Y chromosome was detected in metaphase plates from leucocytes after peripheral blood cultures. Both bucks had normal gonadal and genital development. On the basis of corpora lutea counts and percentage of blastocysts recovered, these bucks demonstrated
normal fertility. A full account, including autoradiographic techniques, of this finding in one of the bucks has been reported elsewhere (Martin and Shaver, 1972). These observations indicate that there is polymorphism of the Y chromosome in the rabbit.

B. Blastocysts Recovered from Females in which Sperm were Aged

_In Utero prior to Fertilization_

The percentage of ova released, as judged by the number of corpora lutea in the ovaries, and recovered as blastocysts six to seven days later is given in Table 1. A recovery rate of 82.6% was obtained in the control population, where insemination of females was immediately followed by the administration of HCG. The influence of ageing in spermatozoa on the rate of blastocyst recovery is presented graphically in Figure 7. Ageing of sperm for 14 and 16 hours had no effect on the recovery rate of blastocysts as compared with the control group. The greatest recovery rate, 93.8%, was observed when spermatozoa were aged 18 hours before the estimated time of ovulation. Two hours later, the percentage of blastocysts recovered decreased suddenly to 60.9%. The decline continued with the most pronounced effect noticeable when sperm were stored longer than 28 hours. At 30 hours only 22.7% of the ova released were recovered as blastocysts.

Although there was a total of 27 ovulation points in the ovaries of three females in which spermatozoa had aged for an estimated 31 hours, no blastocysts were recovered from these females. At autopsy these females were found to be sexually mature with apparently normal genital and gonadal anatomy. There was no evidence of infection in the reproductive tract. It was therefore concluded that the spermatozoa had reached the end point of vitality at 31 hours.
Every female in this study ovulated resulting in a total of 381 corpora lutea with 251 blastocysts recovered six to seven days later. There was a great variation in size of blastocysts recovered from each female in which ova were fertilized by aged spermatozoa.

C. Chromosome Abnormalities in Blastocysts Recovered from Females in which Spermatozoa had Aged In Utero

The chromosomal complement was examined in 239 of the 251 or 95% of the blastocysts. The analysable blastocysts ranged in size from large to very small. Those which could not be analysed were generally small and after processing were seen to have pycnotic nuclei, indicating degeneration. A total of 14 blastocysts with detectable chromosomal defects emerged from this study. The cytogenetic data for these heteroploid blastocysts is presented in Table 2.

(i) Blastocysts recovered in the control series

Of 109 blastocysts recovered from 13 females, 105 were suitable for chromosome analysis. A single heteroploid blastocyst was obtained in this group (Table 2, la). It was small in size with a 2N/4N mixoploid constitution. In 35 cells studied, 60 and 40 percent had chromosome counts in the diploid and tetraploid range, respectively. A typical karyotype of the tetraploid cell line, which had an XXXX gonosomal complement, may be seen in Figure 8. The incidence of chromosomal anomalies in this group was 0.95%.

(ii) Blastocysts recovered in the experimental series

The remaining 13 heteroploid blastocysts, five mixoploids, five chromosome mosaics, and three sex chromosome chimaeras, were observed in the experimental series giving a 9.7% incidence of chromosomal
anomalies. This incidence in the experimental series is significantly different from that in the control series ($\chi^2 = 6.7; P < 0.01$).

These anomalies were scattered amongst the various ageing intervals with no increased frequency at any specific time. The heteroploid blastocysts observed at the different ageing periods will be described in groups according to the type of anomaly.

(a) **Mixoploidy**

The five mixoploid blastocysts in the experimental series were very small in size. There was a marked contrast in size between these blastocysts and others recovered from the same female. In fact, mixoploids were by far the smallest blastocysts seen in the entire study.

One of two chromosomally abnormal blastocysts recovered from a female in which spermatozoa had aged for 18 hours was a 2N/4N mixoploid. In 31 cells studied, nine had 88 chromosomes or a count in the tetraploid range, and 22 had counts in the diploid range (Table 2, 5b). Thus, the percentage of cells in the two lines was 30 and 70, respectively. The sex chromosome complement of the tetraploid population was XXY.

Two other mixoploids were observed in another female in which spermatozoa had also aged for 18 hours (Table 2, 6a and b). Tetraploidy and diploidy were observed in 22% and 78% respectively, of the 27 cells examined in blastocyst 6a. Karyotypic analysis revealed the sex chromosome complement in the tetraploid line, to be XXXX. An interesting finding in three of these tetraploid cells was the presence of duplicated heteropycnotic X chromosomes. The other
mixoploid blastocyst from this female had a 2N/4N/8N constitution. This blastocyst had cells with abnormally large nuclei and others with pycnotic nuclei. In the 25 cells available for analysis, 56, 28, and 16 percent were present in the diploid, tetraploid, and octoploid range, respectively. The gonosomal complement was XX in the diploid and XXXX in the tetraploid cells karyotyped.

The remaining two mixoploid blastocysts were obtained from a female in which spermatozoa had aged for 28 hours (Table 2, 10a and b). One of these blastocysts had a 43/44/88 chromosome constitution. Among 32 cells studied, 13 had 43 chromosomes, nine had 44, and eight had 88 or a count in the tetraploid range. The missing element in the 43-cell line was a submetacentric chromosome, possibly a homologue of pair 9 (Figure 9). Karyotypes representing the two remaining populations of cells may be seen in Figures 10 and 11. The sex chromosome complement in the tetraploid population was XXXY. The other mixoploid blastocyst had a similar sex chromosomal complement. Of the 25 cells analysed in this blastocyst, 41 and 59 percent had counts in the tetraploid and diploid range, respectively. Some in the diploid range had extra or missing chromosomes at random. The polyploid cells in all mixoploids ranged from 22 to 44% as compared to 0.27% of total metaphases in all other blastocysts.

(b) Mosaicism

All five of the chromosome mosaic blastocysts had two cell lines, a diploid and a hypo- or hyper-diploid one.

Two mosaics, a 43/44 and 44/45, arose from ova fertilized by sperm aged 44 hours before ovulation and were found amongst nine
blastocysts recovered from one rabbit (Table 2, 2a and b). In the first blastocyst, 40 cells were examined. Of these 26 had 44 chromosomes and 12 had 43. Only four small acrocentric chromosomes were counted in the 43-cell line, while five were present in the diploid line giving an XY sex complement (Figure 12). Karyotypes from cells in the 43-cell line (Figure 13) revealed that the missing chromosome was possibly the Y, since pairs 20 and 21 were easily identified. The sex chromosome constitution of this blastocyst may be represented as X0/XY. Thirteen of 30 cells studied in the second blastocyst had 45 chromosomes while 15 had 44. Six small acrocentrics were observed in all cells in the 45-cell line. In the 15 karyotypes studied, the sex complement was established as XY in the 44-cell line and in the 45-cell line the additional small acrocentric chromosome was similar to the Y (Figures 14 and 15).

Another 43/44 mosaic (Table 2, 4a) was found amongst six blastocysts recovered from a female in which spermatozoa had aged 16 hours. Ten cells had 43 chromosomes, 15 had 44 and the remainder had 42 or less. Sixteen karyotypes prepared from both cell lines indicated that the sex complement was XX and that a homologue of pair 1 was consistently missing in the 43-cell line (Figures 16 and 17).

The third 43/44 mosaic blastocyst was one of two abnormal ones among 10 recovered from a female in which spermatozoa had aged for an estimated 18 hours (Table 2, 5a). Fifty-six and 40% of the 59 cells studied had 44 and 43 chromosomes, respectively. The remaining two cells had 42 chromosomes. Seventeen karyotypes were established. From these, the missing element in the 43-line was found to be
consistently a large metacentric chromosome, a homologue of pair 3. A typical karyotype from this cell line is shown in Figure 18. The sex complement was XY. Figure 19 is a karyotype representing cells in the 44-cell line.

All four chromosome mosaic blastocysts described previously were either large or medium-sized. However, the fifth one (Table 2, 7a) was a very small blastocyst recovered from a female in which spermatozoa were aged 20 hours. It was the second mosaic with 44/45 cell lines. The extra chromosome in the 45-cell line was consistently a large acrocentric which was similar to number 18 (Figure 20). A karyotype of the diploid cell line may be seen in Figure 21. The presence of two tetraploid cells indicates that this blastocyst was possibly a hyperdiploid mosaic mixoploid. All the cells demonstrated a minute Y chromosome. This blastocyst arose when a female was inseminated with semen from one of two rabbits, described earlier, with a minute Y chromosome.

(c) **Chimaerism**

The three blastocysts showing sex chromosome chimaerism were recovered from three females in which spermatozoa had aged 14, 22, and 28 hours. They were the only heteroploid blastocysts obtained from these females. The normal complement of autosomes and either an XX or an XY sex chromosome constitution was present in each blastocyst. The distribution of cells with the XX and XY sex chromosome complement for each chimaera is given in Table 3. The chimaeric blastocysts recovered after spermatozoa were aged 14 and 28 hours were large in size, the other was medium. All were of normal morphological appearance. Figures 22 and 23 represent both cell populations in one of the chimaeric blastocysts.
D. Sex Ratio of Blastocysts Resulting from Sperm Aged In Utero

With the exception of the chimaeras, the sex chromosome complement of all blastocysts analysed in this experiment is given in Table 4. When insemination was immediately followed by HCG injection, in the control series, 51 of 105 blastocysts had an XY and 54 an XX sex chromosome complement. This gave a sex ratio of 94 males to 100 females.

At all ageing periods, except 20 and 29 hours, there was a greater percentage of blastocysts with an XY complement. This was most noticeable at 28 and 18 hours with 82% and 70%, of the blastocysts, respectively, showing an XY complement. When sperm were aged for 18 hours, all 30 resulting blastocysts were analysed. This was the largest number recovered from females in which sperm were aged at a period greater than 10 hours. The total number of blastocysts with a male sex complement in the experimental series was 80, while the female sex complement was seen in 51. This gives a sex ratio of 157 males to 100 females. However, this excess of blastocysts with a male complement in the experimental series was not significantly different from the number of those with the same complement in the control series ($\chi^2 = 3.20; .10 > P > .05$). Also, there was no significant difference between the sex ratio of the experimental group and the hypothetical sex ratio of 100, ($\chi^2 = 3.41; .10 > P > .05$).

E. Evaluation of Semen and Sperm Morphology Following Surgery

The semen collected before and after the sham operation showed no difference in sperm density, motility and morphology. The first
of these two criteria were also unaffected in ejaculates recovered up to 15 days after ligation of the corpora epididymides. From 21 to 40 days, ejaculates had a low sperm density and noticeably large quantities of inactive spermatozoa. An accumulation of dead spermatozoa, as judged by the vital staining with aqueous nigrosin-eosin, was seen in those collected 27 or more days after ligation. Sperm motility was essentially zero in those recovered at 40 days. Consequently, the latter were not used to inseminate females.

Abnormal morphology was noted among spermatozoa recovered before and after surgery. Before ligation, the main abnormality was curved or partially coiled tails. Seven days post-operatively, the ejaculates contained spermatozoa with head cap abnormalities (Figure 24a) as well as those with curved or partially coiled tails. A noticeable large number of decapitated spermatozoa, head cap and tail defects were present in semen collected after 14 days. The detachment of the head cap from the sperm head ranged from slight to complete in the semen collected at these later times. Completely detached acrosome caps did not always remain in situ, many were dislocated (Figure 24b). In conclusion, although no definite counts on abnormal forms were made, it was very apparent, from light microscopical observations, that an increase in the degenerative changes of spermatozoa occurred with the passage of time.

F. The Recovery of Blastocysts from Females Inseminated with Spermatozoa Aged in the Male Tract

Details of the numbers of corpora lutea and of blastocysts recovered are given in Table 5. Judging from the corpora lutea counts,
all ova released from females inseminated with sperm from males in
the sham-operated group were fertilized and progressed to six-day
blastocysts. Thus, it appears that the surgical procedures had no
adverse effect on the ability of spermatozoa, at least those re-
covered up to 35 days post-operatively, to fertilize ova and support
embryogenesis to the blastocyst stage. The 13 females inseminated
with control ejaculates, including those collected before ligation,
gave rise to 109 blastocysts. With a total of 132 corpora lutea from
these females, the recovery rate of blastocysts in the control group
was 82.6%.

In the experimental series, blastocysts were recovered from
females inseminated with spermatozoa aged in five of the eight males.
There was no decrease in the recovery rate of blastocysts resulting
from spermatozoa aged for seven and 12 to 15 days, as compared with
the control group. At 19 to 21 and 27 days ageing of spermatozoa the
rate was 47.4 and 75%, respectively. However, the numbers of females
inseminated at these later times are less than those at the earlier
ageing periods. Thus, the lower rates might not be a true reflection
of the length of time sperm were aged. No blastocysts were recovered
from the three females inseminated with 35-day-old spermatozoa from
three different males. All of these females ovulated with a mean
ovulation rate of 10.3 ± 1.5. At autopsy they were found to be
sexually mature with apparently normal genital and gonadal development.
Failure to recover blastocysts from these females was attributed to
functional deterioration of the aged spermatozoa.
G. Chromosome Abnormalities and the Sex Ratio in Blastocysts

Recovered from Females after Insemination with Spermatozoa Aged in the Male Tract

One hundred and ninety-seven of 207 or 95.2% of the blastocysts recovered were suitable for cytogenetic analysis.

(i) Blastocysts recovered in the control group

Chromosomes were examined in 105 of the 109 blastocysts obtained from 13 females in the control group. A single heteroploid blastocyst, a small 2N/4N mixoploid, was recovered in this group and is recorded in Table 6. Details of this blastocyst are presented in the results of the previous experiment.

In females inseminated with ejaculates from the sham-operated males, 20 of the 24 blastocysts recovered were analysed. All had normal chromosomes. The remaining blastocysts which could not be analysed were among four of six obtained from one female. In two of the unanalyisable blastocysts, there were very few mitoses, while in the others the mitoses were of poor quality. However, the results from the analysable blastocysts indicate that the sham operations did not affect the chromosome complement of the blastocysts, the main criterion measured. Consequently, data from these rabbits were combined with data from the control group. This gave a count of one heteroploid blastocyst in a total of 125 analysed, an incidence of 0.8% anomalies.

(ii) Blastocysts recovered in the experimental population

All except two of the 74 blastocysts recovered in this group were analysed. Eight of the 72 or 11.1% of these were chromosomally
abnormal. There was a statistically significant increase in the incidence of abnormalities in the experimental series when compared with the control group ($\chi^2 = 8.9; P < 0.01$). These findings are listed in Table 6. The abnormalities, which were of a varied nature, arose when ova from five females were fertilized by spermatozoa aged in three males.

(a) **Blastocysts recovered when females were inseminated with spermatozoa aged for seven days**

Twenty-three of the 24 blastocysts from three females were studied cytogenetically. Anomalies were present in three. Two blastocysts from two females were 2N/4N mixoploids (Table 6, 2a and 3b). After examining 40 cells in one of these, 65 and 35% were found to be diploid and tetraploid, respectively. In the other, the respective percentages of cells were 60 and 40. Karyotyping showed the sex complement to be XXXX in the tetraploid population of both blastocysts, which were small in size.

The third abnormal blastocyst was small in size and had a triploid chromosome complement. In the 21 cells examined 20 had 66 chromosomes. One cell which was apparently broken had 45 chromosomes. The sex complement as revealed by karyotyping was XXY (Figure 25). This triploid was one of two abnormal blastocysts recovered from the same female (Table 6, 3a).

(b) **Blastocysts recovered from females inseminated with spermatozoa aged 12-15 days**

All 32 blastocysts recovered from four females were analysed cytogenetically. Three different types of anomalies occurred in
blastocysts from two females. One blastocyst obtained from a female inseminated with 15-day-old spermatozoa was trisomic. Of the 30 cells examined in this blastocyst, 26 had 45 chromosomes (Table 6, 4a). Arrangement of the karyotypes revealed that the extra chromosome was similar to pair 1 and that an XY sex complement was present (Figure 26). The blastocyst was large-sized with normal morphological appearance. It was the only chromosomally abnormal blastocyst of nine recovered from the female.

A chromosome mosaic blastocyst resulting from an ovum fertilized by a spermatozoon aged for 14 days was medium in size. Of the 34 cells analysed, 10 had 45 chromosomes and 21 had 44. After assembling 10 karyotypes, the extra chromosome in the 45-cell line was seen to be similar to pair 17 (Figure 27). A karyotype representing the diploid population may be seen in Figure 28. This 44/45 mosaic had an XY sex complement and was one of two abnormal blastocysts from one female.

The third abnormality was detected in a small blastocyst which was very granular in appearance and seemed either to have been developing abnormally or degenerating. Only 16 metaphase plates were suitable for analysis. The modal chromosome count was 44 and under the microscope there appeared to have been six small acrocentric chromosomes in the complement. However, when the karyotypes were assembled, one member of pair 5 was missing and one of the apparently small acrocentric chromosomes paired with the long arms of the remaining member of pair 5 (Figure 29; Table 6, 5b). The sex complement was XY. The abnormal chromosome may have been produced by a deletion of the short arms of pair 5 or a translocation. The former
seems the more likely since no other detectable morphological change was observed in the karyotypes.

(c) **Blastocysts recovered from females inseminated with spermatozoa aged 19–21 days**

Nine blastocysts were recovered from two females that were inseminated. One of three blastocysts obtained from one female could not be analysed, due to paucity of cells and the poor quality of the few mitoses. The two abnormalities in this group (Table 6, 6a and b) occurred in blastocysts recovered from a female inseminated with 19-day-old spermatozoa. All 31 cells analysed in one of these blastocysts had a chromosome count of 45. The extra element was a small metacentric chromosome. Karyotypes of five cells showed three chromosomes to be similar to pair 6 (Figure 30). This blastocyst, medium in size, had an XX sex complement.

The second abnormal blastocyst was also trisomic and medium-sized. Of the 41 cells analysed, 36 had a chromosome count of 45. The extra chromosome in all cells was a small acrocentric. The presence of satellites was helpful in identifying the extra chromosome. Many cells showed three satellite small acrocentric chromosomes, an indication that the extra chromosome was possibly a member of pair 20. The sex complement was XY and a typical karyotype is shown in Figure 31.

(iii) **The sex ratio in blastocysts recovered**

The sex complement was determined in all blastocysts analysed. The results are summarized in Table 7 which does not include the
triploid blastocyst with the XXY sex complement. In the combined control and sham-operated group, 61 of 125 blastocysts had an XY and 64 an XX sex chromosome complement. This gave a sex ratio of 95 males to 100 females. In the experimental series, 35 of 71 blastocysts demonstrated an XY and 36 an XX sex complement. Here, the sex ratio was 97 males to 100 females. No significant difference was found between the two groups.
V. DISCUSSION

A. The Morphological Differences Between the Two X Chromosomes in the Normal Female Rabbit Karyotype

The morphologically distinct chromosome, designated as an X, seen in metaphase plates was a medium-sized submetacentric chromosome. Several authors have agreed that the X is submetacentric and medium in size (Painter, 1926; Melander, 1956; Sarkar, Basu and Miller, 1962; McMichael et al., 1963; Prunieras et al., 1965; Issa et al., 1968).

In the human species, there have been reports of morphological differences between the two X chromosomes in female cells (German, 1964; Mukherjee, Miller, Breg and Bager, 1964). Mukherjee et al. (1964), studying polysomic X individuals, demonstrated that the late-replicating X chromosome is shorter than its homologue. In the present study, the finding of positive heteropycnosis of a chromosome in cells with a female complement is similar to the observation of Ohno and Makino (1961). While studying human somatic cells, these authors noted that in females one of the chromosomes showed positive heteropycnosis during prophase. They reported that the heteropycnotic chromosome, which was not observed in males, was often bent acutely at its centre.

It cannot be said with certainty that the heteropycnotic chromosome
seen in this study is late-replicating. Conclusive proof of this could be obtained from autoradiographic techniques, which were not possible in this experiment. This chromosome, however, has the same characteristics as those of the late-labelling X in the human female. Like the heteropycnotic chromosome seen in the rabbit female complement, the human late-replicating X has been observed to have a peripheral location on the metaphase plate (Morishima, Grumbach and Taylor, 1962), a bent configuration and to be absent from male cells (Gilbert, Muldal, Lajtha and Rowley, 1962).

The relatively thinner arms and smoother outline of the presumptive late-replicating X, in this study, may be accounted for on the basis of differential coiling. There is a greater degree of supercoiling of the chromatin in the repressed X, as in the case in other heterochromatin. Supercoiling of the chromatin is a reflection of the relative genetic inactivity. It is interesting to note that Melander (1962), studying anaphase and interphase stages, concluded that anaphase tension of the longer arm of one of the rabbit X's causes the transformation of this arm into the sex chromatin. This chromatid tension and stretching may account for the increased length of the heteropycnotic X at metaphase.

It is easy to associate the kink, the bending, and the peripheral location of the positively heteropycnotic X with the condensation of one X and the appearance of sex chromatin on the periphery of the nucleus. The conclusion that the late-replicating X chromosome represents the heterochromatic X which participates in the formation
of sex chromatin has been strongly supported (Morishima et al., 1962). In the rabbit sex chromatin begins to appear in the embryo 84 hours post-fertilization. It reaches a high frequency between 100 - 200 hours (Plotnick, Klinger and Kosseff, 1971).

Issa et al. (1968) declared that the X chromosome in the rabbit cannot be identified by any means other than its labelling pattern in female cells. Its detection by the morphological characteristics described here is a simple and straight-forward procedure. The ease with which such an X chromosome can be identified in the metaphase plate facilitates the identification of the sex complement. It supplements the usual method of counting the number of very small chromosomes. Five very small chromosomes are present in the normal male complement, while only four are present in the normal female complement. The detection of a positively heteropycnotic, medium-sized submetacentric chromosome on the periphery would immediately indicate the presence of an XX complement.

Secondly, the ease with which an X chromosome can be identified in the rabbit female complement can be useful in recognizing numerical X chromosome abnormalities. In humans, it has been shown that when more than one X chromosome is present in a cell, the supernumerary X's become condensed forming distinct sex chromatin bodies (Barr and Carr, 1960). Assuming that a similar condensation occurs in the rabbit where the submetacentric X is similar to some autosomes, supernumerary X's will be manifested as heteropycnotic chromosomes. Hence, these chromosomes may be easily detected.
Although several investigators have studied the rabbit karyotype from various adult tissues and cells of the blastocyst, the observation of morphological differences between the X chromosomes in cells with a female complement has not been previously reported. This may be due to the selection of cells for chromosome analysis. After 110 hours post-fertilization, the incidence of a condensed X chromosome in female embryos may be as low as 10% (Plotnick et al. 1971). Since most authors analyse the chromosomes in only 20 to 30 cells from a possible 60,000 to 70,000 present in the blastocyst, there is a definite possibility that cells showing the condensed X may not be detected. Secondly, the specific stain may have enhanced the appearance of this condensed X chromosome. Stains other than Giemsa have been used by most of the investigators studying the rabbit karyotype.

B. The Recovery Rate of Blastocysts from Females in which Sperm Were Aged In Utero

The greatest recovery rate of blastocysts, 94%, was observed when spermatozoa were aged for an estimated 18 hours. At this period of ageing, the difference between the recovery rate and that in the control was statistically significant. A similar improvement in fertility accompanying prolonged storage of sperm has been reported previously (Chang, 1955; Dzuik, 1965; Kirton, 1967; Harper, 1970a; Roche, Dzuik and Lodge, 1968; Brackett, 1970; Cummins and Orgebin-Crist, 1971). These authors suggested that the development of maximum fertilizing competence of rabbit sperm within the female tract requires considerably longer than the six to eight hours considered.
adequate for capacitation. In any ejaculate, spermatozoa have different degrees of physiological maturity and hence different capacitation rates. Harper (1970a) provided an additional explanation to account for an increased penetration of rabbit eggs which entered the oviduct at the end of the ovulatory period and up to 19.5 to 20 hours after insemination. He attributed the increase in fertilization rate partly to increased numbers of spermatozoa at the fertilization site at the later times. In this connection, Braden (1953) has shown that the mean number of sperm found in the ovarian third of the oviduct increases progressively from 84 at six hours after mating to 343 at 20 hours after mating.

The decrease in the rate of recovery of blastocysts when sperm were aged for 20 hours, as compared with the rate in the control series, was statistically significant. This result in vivo is in accordance with the finding of reduced blastocyst development in vitro when ova were fertilized by spermatozoa stored for 20 hours in the female tract (Maurer et al. 1969). Studies by Hammond and Asdell (1926) and Tesh (1969) indicated that the first adverse effect of ageing on the function of spermatozoa appeared when the storage period in utero was 22 hours. The discrepancy between the finding in the present investigation and those in these studies may be explained by the type of insemination procedure employed. Hammond and Asdell (1926) and Tesh (1969) used mixed semen from different bucks for insemination. This procedure, heterospermic insemination, has been shown to result in improved fertility when compared with homospermic insemination (Beatty, 1960; Napier, 1961). In this study, as in that of Maurer et al.
(1969), semen samples from different males were not pooled. The discrepancy may also be due to the variation in the time of ovulation between animals and among the follicles within an animal. Harper (1963) reported that in the rabbit, ovulation occurs between 9.5 and 13 hours with a mean of 10 hours after an injection of HCG. In addition, strain differences must be considered.

The first adverse effect of sperm age on the rate of blastocyst recovery in this study was seen when sperm were only two hours older than the age at which they resulted in a peak recovery of blastocysts. Noyes and Thibault (1962) suggested that some hours after sperm reach the capacitated state deterioration rapidly occurs producing a loss of fertilizing ability. This may probably explain the sudden decrease in recovery of blastocysts.

No blastocysts were recovered when spermatozoa were aged for 31 hours. This finding is in accordance with previous investigators regarding a 30-hour fertile life-span for rabbit sperm in utero (Hammond and Asdell, 1926; Tesh, 1969).

C. Chromosome Abnormalities in Blastocysts Resulting from Females in which Spermatozoa were Aged In Utero

A few general considerations must be made concerning the detection of chromosomal abnormalities. The technique of analysing chromosomes at metaphase, after colchicine treatment, is limited in detecting structural aberrations of a size less than half a micron. Small deletions or duplications are therefore not seen. Numerical changes in the karyotype will, however, be detected without difficulty.
Nevertheless, mosaicism, the presence of more than one cell line, may remain cryptic unless many cells are critically examined. Therefore, it is instructive to note that estimates of the occurrence of chromosomal aberrations are probably minimal estimates.

The incidence of chromosomal anomalies in blastocysts resulting from sperm stored longer than 10 hours was significantly different from the incidence in the control series where spermatozoa were stored for 10 hours. These results indicate that ageing of sperm in the female tract has an adverse effect on the chromosome complement of resulting blastocysts. Two of the types of anomalies observed, namely mixoploidy and mosaicism, implicate cleavage errors as a consequence of in utero aged spermatozoa on ovum development. The third type of anomaly, chimaerism, may occur as a result of fertilization errors.

The mixoploids observed were mainly of the 2N/4N constitution. Conceptuses with this cytogenetic constitution have been reported to a limited extent in spontaneously-aborted human specimens (Inhorn, Therman and Patau, 1964; Thiede and Salm, 1964). To date, only one human neonate with mixoploidy of the 2N/4N variety has been reported in the literature (Kohn, Mayall, Miller and Mellman, 1967). In mice, this condition has been induced experimentally by the application of colchicine (Edwards, 1958) and heat shock (Beatty, 1957).

Beatty and Fischberg (1952) proposed that inhibition of the cleavage spindle is a mechanism responsible for the formation of tetraploid cells. Tetraploid cells in mixoploid zygotes originate
from the failure of cytokinesis when karyokinesis occurs at any
division subsequent to first cleavage. In tissue culture, tetra-
ploid cells have been seen after prolonged periods of proliferation.
However, the frequency with which they occur under these conditions
has been shown to be less than 3 percent (Tijo and Puck, 1958;
Hayflick and Moorhead, 1961).

While the significance of tetraploid cells in culture is doubt-
ful the occurrence of these cells in direct preparations, as employed
in this study, reflects a true in vivo situation. The increased in-
cidence of mixoploidy resulting from this investigation may be
correlated with the observation of Maurer et al. (1969). They re-
ported that ageing of rabbit spermatozoa in utero was responsible for
a significant suppression of cleavage rates in the resulting embryos
cultured in vitro.

The five mosaics seen in this study involved three acrocentric
and two large metacentric chromosomes. Mosaicism was also found in
conjunction with mixoploidy in one blastocyst, here a submetacentric
chromosome was involved in aneuploidy. Aneuploidy within the different
groups of acrocentric chromosomes is well established in man. Large
metacentric chromosomes have been associated previously with aneu-
plody in rabbit blastocysts (Shaver and Carr, 1967; Hofsaess and
Meacham, 1971). One has also been seen in the trisomic condition in
the second experiment in the present investigation. Mosaicism is not
common in human abortions although numerous types have been found in
living persons (Inhorn, 1967).
Chromosome mosaics arise as a result of cleavage errors, either by non-disjunction or anaphase lagging. Non-disjunction is the failure of separation of paired homologous chromosomes or sister chromatids during anaphase. This phenomenon may occur in a meiotic or a mitotic division. When non-disjunction occurs after the first mitotic division, three cell lines each containing 45, 44 and 43 chromosomes will arise in the rabbit karyotype. Non-viability of the 43-cell-line would lead to a hyperdiploid mosaic with a 44/45 constitution. If the 45-cell-line were non-viable, a hypodiploid mosaic with a 43/44 constitution would result.

Anaphase lagging results from failure of one member of a chromatid to move with sufficient speed at anaphase to reach either pole of the nucleus before cytoplasmic division. As a result, the chromatid becomes excluded from either daughter cell and is subsequently lost. If this event occurred in a normal rabbit a hypodiploid mosaic with a 43/44 constitution would result. In a trisomic zygote, loss of one of the three homologous chromosomes at anaphase would lead to a 44/45 mosaic. In man, the sex chromosomes, especially the Y, are prone to lag behind autosomes in their polar migration at mitotic anaphase. It is of interest that two of the five mosaics seen involved chromosomes that were likely the Y.

The finding of sex chromosome chimaerism in blastocysts resulting from spermatozoa aged in utero is of special interest. Sex chromosome chimaerism with a 44,XX/44,XY constitution has not been reported previously in the rabbit. However, this chromosome constitution is usually associated with intersexuality, a phenomenon observed in this
species (Koch, 1963; Beatty, 1964; Shaver, 1967). The only reported chromosome study of intersex rabbits, revealed the presence of a normal XY chromosome complement (Shaver, 1967). However, in this report only bone marrow was studied and chimaerism cannot be excluded. On the other hand, sex chromosome chimaerism is well documented in man (Oversizer, 1964; Waxman, Gartler and Burt, 1962), cattle (Dunn, Kenny and Lein, 1968), swine (Basrur and Kanagawa, 1971; Vogt, 1968), goats (Padeh, Wysoki, Ayalon and Soller, 1965), cats (Thuline, 1964) and sheep (Alexander and Williams, 1964) where different forms of intersexuality have been exhibited.

The mode of origin of the XX/XY chromosome constitution has been fully reviewed by Ford (1969). He outlined nine distinct aetiological mechanisms, which may be divided into the following groups: (a) dispermic fertilization with two separate acts of syngamy and (b) the contribution of two independent zygotes. The latter may occur early in the pre-implantation stages or later in development after the placenta has been established. A third mechanism involving a series of early mitotic errors in an XY zygote has been proposed (Forteza, Bonilla, Báguena, Monmeneneu, Galbis and Zaragoza, 1963). Lennox (1966) considered this mechanism to be very remote since it requires an unlikely situation in which three events occur in succession: loss of a Y chromosome by anaphase lag, non-disjunction of an X chromosome in the new XO cell line and loss of the XO cell line as well as the non-viable cell line without an X.

Chimaeras which originate at the later stage of embryonic development are termed post-zygotic or blood chimaeras. They result
from the mutual exchange of circulating cells between dizygotic twins via placental anastomosis (Goodfellow, Strong and Stewart, 1965; Woodruff, Fox, Buckton and Jacobs, 1962). Clearly, this mechanism would not be responsible for chimaerism detected in the blastocyst stage.

Chimaerism resulting from contributions of two independent zygotes early in embryogenesis has been produced experimentally and presumably may occur spontaneously. In mice, Tarkowski (1961), Mintz (1962) and Mystkowska and Tarkowski (1968) have developed different methods for fusion of two separate morulae at the eight-cell stage after removal of the zona pellucida. The resulting 16-cell masses are incubated until they develop into blastocysts. These blastocysts are later injected into pseudopregnant foster mothers where they implant, grow to term and result into live-births. Random fusion of morulae with XX and XY chromosome complements would lead to a proportion of offspring with an XX/XY constitution. Spontaneous or experimental fusion of morulae would not be very feasible in the rabbit where the embryos have a thick mucin coat (Bacsich and Hamilton, 1954). Surface properties, for example stickiness, are modified by this coat.

The evidence favouring dispermic fertilization was first provided by Gartler, Waxman and Giblet (1962). The authors postulated that double fertilization with dispermy and participation of two haploid nuclei, daughters of the ovum nucleus, is a likely mode of origin of chimaeras. Convincing evidence (Zuelzer, Beattie and Reisman, 1964; Bain and Scott, 1965; Russel and Woodiel, 1966; McFeely, Hare and
Biggers, 1967) favour the participation of the ovum pronucleus and the second polar body in double fertilization with dispermy.

Dispermy is normally prevented in mammals by the vitelline block and the zona reaction mechanisms. Either or both mechanisms may be effective in a particular species. In the rabbit, dispermy is prevented by the vitelline block. After entry of a spermatozoon into the vitellus a change in the surface of the vitelline membrane prevents attachment of other spermatozoa while 100 or more may penetrate the perivitelline space (Braden, Austin and David, 1954; Dickmann, 1964; Austin and Walton, 1960). The zona reaction is highly efficient in the mouse (Chang and Hunt, 1956) and hamster (Austin, 1956). In the rabbit, Harper (1970b) has reported an increased incidence of dispermy when eggs enter the oviduct 14 hours or later after insemination. He explained that the increase was due to the presence of more fully capacitated sperm at the site of fertilization at the later times and thus a greater chance of two sperm entering the vitellus simultaneously.

Similarly, digyny, the retention of the second polar body, has been seen with an increased incidence in rabbit ova fertilized by spermatozoa aged in utero (Thibault, 1967). The simultaneous occurrence of digyny and dispermy with an X- and Y-bearing sperm, after sperm ageing could result in double fertilization giving rise to XX/XY chimaeric embryos with contributions from both fertilized polar body and ovum. This is likely to be the mode of origin of the chimaeric blastocysts observed in this study.
D. The Sex Ratio in the Population of Blastocysts Arising from In Utero Aged Spermatozoa

On the basis of Mendelian principles, it is expected that equal numbers of X- and Y-bearing sperm are produced during meiosis. Thus, it is assumed that the ratio of male:female at conception, the primary sex ratio, is unity. On practical grounds, the primary sex ratio cannot be established with certainty. However, chromosome studies in the pre-implantation stages of development in the pig (McFeely, 1967) and sex chromatin studies in the rabbit (Melander, 1962; Edwards and Gardner, 1967) have shown that there is a sex ratio of approximately 100 males to 100 females. This is in good correlation with the theoretical value.

The sex ratio in the population of blastocysts resulting from spermatozoa aged in utero showed a slight preponderance of the male chromosome complement (157:100) though this excess was not significantly different from the hypothetical sex ratio (100:100). This finding of an excess of males might be more than a chance occurrence. There have been other reports of a non-significant increase of males in the offspring resulting from in utero aged spermatozoa in the rabbit (Hammond and Asdell, 1926) and in the human (Marshall, 1968). In studies of the sex ratio large populations are required in order to make definite conclusions. Perhaps if the sample size in the above studies and in the present one had been larger, a more decisive effect of sperm ageing on sex ratio might have emerged. Interestingly enough, James (1971) with the use of demographic data, hypothesised that the sex of the conceptus is associated with the cycle day of insemination:
male zygotes being formed earlier in the cycle, on the average, than females.

The primary sex ratio may be influenced by differences in the longevity, transport or fertilizing capability of the X- and Y-bearing spermatozoa. In an excellent review of the genetics of the mammalian gamete, Beatty (1970) indicated that the X- and Y-bearing sperm showed differences in their sedimentation behaviour and other physical attributes. It is conceivable that if prolonged storage influences any of these properties, the sex ratio might consequently be affected.

E. Spermatozoan Characteristics and Recovery of Blastocysts Resulting from Sperm Aged in the Male Tract

The most pronounced effect of ageing on spermatozoa, as judged by complete failure to recover blastocysts, was seen when sperm were aged for 35 days. However, Hammond and Asdell (1926) obtained litters and Tesh and Glover (1969) observed 29-day-old foetuses resulting from sperm aged for 40 to 42 days. Similarly, these respective authors reported that motility of spermatozoa was maintained for 60 and 42 days. In this study the limit of motility was 35 days. The discrepancy in the findings may be accounted for on the basis of the type of insemination, individual variation among bucks, as well as strain differences. Homospermic insemination was used in this experiment whereas in the other investigations insemination was heterospermic.

The morphological changes observed in spermatozoa after ageing
are similar to those reported by other investigators (Igboeli and Foote, 1969; Paufler and Foote, 1968; Tesh and Glover, 1969). Coiled tails, seen in most semen samples, are associated with abnormal locomotion patterns. Damaged acrosomes or head membranes have more severe effects. Spermatozoa thus afflicted lose their ability to establish contact at the surface of the ovum and consequently do not effect fertilization. The ejaculates which contained an apparent increase of dead and decapitated spermatozoa were responsible for low recovery rates of blastocysts. It is conceivable that the presence of these abnormal spermatozoa indicates a generalized deterioration of the semen quality. Thus, intact spermatozoa in these semen samples might have been altered metabolically and consequently lost the ability to fertilize ova.

F. Chromosome Abnormalities in Blastocysts resulting from Sperm Aged in the Male Genital Tract

The 14-fold increase in the incidence of chromosome anomalies in blastocysts resulting from sperm aged in the male tract was statistically significant, when compared with the incidence in the control series. The numerical anomalies included trisomy, triploidy, mixoploidy and mosaicism. There was one structural anomaly.

The commonest abnormality seen was trisomy. There were three cases, all involving autosomes which included the largest and the smallest in the series. In mammals trisomy for a large autosome ordinarily results in embryonic death (Fechheimer, 1968). In unselected spontaneously-aborted human specimens autosomal trisomies form the largest group of anomalies (Inhorn, 1967). However,
trisomies for small autosomes are well documented among newborns producing characteristic stigmata. These have been extensively re-
viewed by Polani (1969). Apart from man, the mouse is the only other species in which viable autosomal trisomics have been reported.
Griffen and Bunker (1964) and Cattanach (1964) reported pure trisomy while partial trisomics have been reported by Lyon and Meredith (1966). The mice were sterile or semi-sterile but had no gross deformities.

The most frequent cause of trisomy and other forms of aneuploidy is non-disjunction. The occurrence of non-disjunction at either the first or second meiotic division of gametogenesis could lead to a disomic gamete bearing one chromosome more than the haploid number. Such a gamete would possess 23 chromosomes in the rabbit. When a disomic gamete engages in syngamy with a normal haploid gamete possessing 22 chromosomes, a trisomic zygote will result. Dis-
junction in an existing trisomic parent, secondary non-disjunction, will also lead to disomic gametes and ultimately trisomic offspring. However, trisomic adult rabbits have not been reported. Apart from this, a trisomic parent would be likely to have other blastocysts with the exact trisomic state. This was not the case in the 36 blastocysts obtained from gametes of the two males and females from which the trisomics arose. Therefore, secondary non-disjunction is an unlikely origin of the trisomic blastocysts.

In the rabbit, non-disjunction in the mitotic division of first cleavage would result in cells with 43 and 45 chromosomes instead of the normal 44 chromosomes. Termination of the 43-cell line would give
rise to a trisomic embryo with 45 chromosomes.

There is a striking association between the incidence of non-disjunction and advanced maternal age in man. These events have also been observed in experimental animals following intrafollicular ovum ageing (Witschi and Laguens, 1963; Mikamo, 1968a, 1968b; Butcher and Fugo, 1967) and post-ovulatory ovum ageing (Austin, 1967; Mikamo, 1968b). Their occurrence has been shown to be related to degeneration of the spindle fibres and to a decrease in oöplasm viscosity (Witschi, 1952; Mikamo, 1968a). The trisomics observed in the present study may have arisen as a consequence of ovum ageing.

On the other hand, the spermatozoa could be directly responsible for the trisomics. Disomic spermatozoa produced at spermatogenesis and which are normally at a selective disadvantage (Thompson, 1965) might, in the absence of fresh spermatozoa, be at an advantage in effecting fertilization. The prevention of fresh spermatozoa, normal and abnormal, entering the ejaculate would increase the changes of old normal and abnormal ones reaching available fertilizable ova. Roche et al. (1968) showed that fresh rabbit sperm had a competitive advantage over aged ones.

One case of triploidy, which is a result of a fertilization error, was seen in this population of blastocysts. The spontaneous incidence of triploidy in the rabbit, as assessed by chromosome counts, (Shaver and Carr, 1967, 1969; Hofsaess and Meacham, 1971) and the presence of trinucleate ova (Austin and Braden, 1953; Austin, 1960; Adams and Chang, 1962; Thibault, 1967; Orgebin-Crist, 1968b) approaches zero. The data from the present control series is in agreement with
this finding. Triploidy is lethal in the mammalian conceptus. These foetuses are almost invariably lost during the first half of gestation in rats (Piko and Bomsel-Helmreich, 1960), pigs (Bomsel-Helmreich, 1965), mice (Vickers, 1969) and man (Edwards, Yuncken, Rushton, Richards and Mittwoch, 1967; Carr, 1971). In the latter, however, cases have been described in which triploidy in all cells of the body was compatible with extra-uterine life (Conen, 1970; Mikamo, 1970). In the rabbit, Bomsel-Helmreich (1965) found triploid embryos at midterm, 15 days after conception, although Ekins and Shaver (1969) reported a significant reduction in triploid conceptuses by day 10.

Triploid embryos may be induced experimentally by hyperthermia and colcemid treatment. In mice, Fischberg and Beatty (1952) found that a period of hyperthermia 2½ to 4½ hours post-coitus resulted in 15% heteroploidy, mostly triploids, in 3½-day-old blastocysts. Bomsel-Helmreich (1965) obtained 97% trinuclear rabbit ova, presumably triploids, after in vitro fertilization and colcemid treatment.

The evidence for the several modes of origin of triploidy has been extensively reviewed by Austin (1960). The possible mechanism for syngamy include a) one female and two male pronuclei or dispermy; b) one male and two female pronuclei or digyny; and c) one haploid and one diploid pronucleus or aneugamy.

There is very little information on the incidence of diploid sperm or ova. Beatty (1970) reported that their formation is under
the influence of genetic control. Esnault and Ortavant (1967) suggested suppression of the second meiotic division as a cause of diploidy in bull spermatozoa. Patau (1963) confirmed the presence of diploid sperm in man by Feulgen-microspectrophotometry. In the rabbit, one spermatozoon among 3200 was the size expected of a diploid one (Beatty, 1961). Diploid spermatozoa have larger heads. They are usually associated with infertility (Beatty, 1970), thus it is very unlikely that such a spermatozoon was the cause of triploidy in this study. Diploid ova can arise as binucleate oocytes and are usually the consequence of nuclear fusion of two oogonia or duplication of oogonial chromosomes without cytokinesis. Binucleate oocytes are seen primarily in immature animals and are extremely rare in adults (Kent, 1959).

The factors associated with dispermy, a common form of polyspermy, have been discussed previously. Polyspermy may be induced by delayed fertilization in the pig (Day and Polge, 1968).

The main cause of digyny is the failure of extrusion of the second polar body. Studies have revealed an increased incidence of digyny with delayed fertilization in the golden hamster (Yanagimachi and Chang, 1961) and the pig (Thibault, 1959). A similar finding has also been reported in the rabbit (Thibault, 1967; Chang and Hunt, 1968; Orgebin-Crist, 1968b). Chromosome studies showed that 12.8% of rabbit blastocysts from matings delayed for six to nine hours were triploids (Shaver and Carr, 1967, 1969). None of 10 triploids obtained had an XXY sex complement. This probably further supports the finding that delayed fertilization is responsible for an increased
incidence of digyny in the rabbit. Triploid mouse blastocysts resulting from delayed fertilization were also shown to have a sex chromosome complement which implicated digyny as the aetiological mechanism (Vickers, 1969).

The sex complement of the triploid blastocyst in this study was XXY, implicating either dispermy or digyny as the causative factor. This blastocyst might have been the consequence of delayed fertilization caused as a result of an alteration in ovum maturation or sperm transport. It is conceivable that when spermatozoa are aged their motility is reduced impairing their ability to reach the fertilization site and to activate and penetrate the egg within the normal time. In this same species evidence from epididymidal spermatozoa (Orgebin-Crist, 1968b) and from in vitro aged sperm (Miller, 1968) has suggested that embryological anomalies have been due to reduced motility of spermatozoa, resulting in delayed fertilization and thus egg senescence.

A short arm deletion, partial monosomy, was seen in the karyotype of a very small blastocyst. Loss of genetic material appears to have a much more adverse effect on embryonic development than does the presence of extra genetic material. However, in man, short arm deletions of chromosome 5 (Lejeune, Lafourcade, Berger, Vialatte, Boeswillwald, Seringe and Turpin, 1963) and 18 (Van Dyke, Valemanis and Mann, 1964) are compatible with life and are recognized as well defined clinical entities. The deletions in these two human chromosomes have been shown to involve late DNA-replicating segments (Schmid, 1963). Such segments are heterochromatic in nature.
A deletion is preceded by a break in the chromosome and may occur at any time in the life cycle of the organism. Centromeric regions, secondary constrictions and other heterochromatic areas are more susceptible to breaks than other areas of the chromosome. Breaks at the centromeric region are very common in man where they precede centric fusion (Hamerton, 1968). The involvement of chromosome 5 in a deletion of the rabbit karyotype is interesting. This chromosome has been shown to demonstrate a secondary constriction near and above the centromere (Pruniéras et al., 1965; Issa et al., 1968). It is therefore possible that the segment deleted contained mainly late-replicating DNA with inactivated genes. This might explain the viability of the embryo to the blastocyst stage of development. The proportion of cells that will contain a deletion depends upon the time the latter occurs in the development of the embryo and the viability of the aberration. The deletion seen in this study was found in all cells of the blastocysts indicating that the aberration was carried in a gamete or arose \textit{de novo} immediately after fertilization. If the deletion originated in the germ cells of either male or female parent, one might expect to see a similar deletion in other blastocysts resulting from gametes of either of these parents. This was not the case; neither was any other structural change detected in an additional 38 blastocysts resulting from gametes of either parent involved in this situation.

The deletion could have arisen \textit{de novo} in either the genome of the spermatozoon or the secondary oöcyte after its release from the gonad. In the rabbit, a decrease in DNA content, as judged by Feulgen
staining, has been shown to accompany the \textit{in vivo} ageing of epididymal spermatozoa (Bouters, Esnault, Salisbury and Ortavant, 1967). Also, \textit{in vitro} ageing of bovine spermatozoa has been reported to evoke changes in the chromatin material (Salisbury, Birge, De La Torne and Lodge, 1961; Salisbury, 1967). Thus, there is evidence that could implicate a change in the genome of the spermatozoa as the direct cause of the chromosome alteration in the blastocyst.

In the egg, the deletion could have occurred during the final maturation of the ovum at fertilization. Fragmentation of the nucleus has been reported to occur as a result of delayed fertilization and consequently ovum ageing (Beatty, 1957). Vickers (1969) also observed chromosome deletion in an embryo resulting from an aged ovum. It is possible that the blastocyst in the present study could have been a result of delayed fertilization for the reasons previously outlined.

Mixoploidy and mosaicism were seen in blastocysts resulting from spermatozoa aged in the male tract. Both of these anomalies are the consequence of cleavage errors. The mechanism for their mode of origin has been previously discussed. An increased proportion of cleavage errors was noted in mouse foetuses recovered 9\frac{1}{2} to 11\frac{1}{2} days after delayed fertilization (Vickers, 1969). It is therefore not unreasonable to relate the latter with the cleavage errors in the blastocysts in the present study.

In conclusion, all the different types of anomalies in blastocysts resulting from spermatozoa aged in the male tract can be associated with delayed fertilization. Aged spermatozoa might be
impaired in their progression into the oviduct or in their ability to initiate normal activation of the egg. Half the number of the anomalies, the three trisomics and the deletion, may have arisen due to a chromosome aberration in the genome of aged spermatozoa. These abnormal spermatozoa may be normally present in the ejaculate but do not reach the fertilization site due to the competitive advantage of fresh spermatozoa. The deletion could have occurred in the spermatozoa as a direct result of ageing.

The anomalies arose when ova were fertilized by sperm aged for an estimated seven to 19 days after ligation of the corpora epididymides. It has been established that the age of the spermatozoa in any semen sample is not synchronized, spermatozoa may be at different absolute ages. This would account for the occurrence of anomalies from spermatozoa obtained at the various ageing periods.

6. **General Remarks**

The population of blastocysts, studied cytogenetically, resulting from spermatozoa aged in the male tract was almost half the number of that studied when sperm were aged *in utero*. Nevertheless, in the relatively small sample of the former, there was a statistically significant increase in the incidence of chromosome anomalies compared with controls. This indicates that spermatozoa aged in the male tract may play a decisive role in the production of chromosome anomalies. Blastocysts resulting from sperm aged *in utero* also showed a significant increase in anomalies compared with controls although the results were not as striking as those seen in blastocysts resulting from sperm aged in the male tract.
There was a difference in the types of chromosome abnormalities produced when ova were fertilized by sperm aged in the two environments. The qualitative aspects of chromosome anomalies are important when considering the fate of the embryos. Although any heteroploid condition will be an obstacle to normal development some conditions have more severe consequences.

In general, the types of abnormalities produced by spermatozoa aged in the male tract have been shown to have extremely severe effects on embryogenesis. Triploidy, trisomy and deletions are all associated with abortions and gross malformations. A correlation can therefore be made between findings in this study and those of Tesh and Glover (1966, 1969) and Young (1929, 1931) who reported increased embryonic mortality and morbidity when spermatozoa were aged in the male tract.

The abnormalities, with the exception of mixoploidy, observed in blastocysts resulting from sperm aged in the female tract, are generally compatible with extra-uterine life. They contribute very little to the load of chromosome abnormalities seen in abortions. In chimaeras, developmental effects are mainly confined to the reproductive system, with variable phenotypic sex expression. In experimentally-produced mouse chimaeras, the phenotype may be female, hermaphrodite, or male with a preponderance of the latter (Mystkowska and Tarkowski, 1968). The mosaic blastocysts were medium or large in size with the exception of one. These blastocysts might have continued to develop normally since there was a high proportion of diploid cells present. Selective proliferation of normal cells in mosaics, with the passage of time, has been demonstrated in several
cases in the human, (Ford, 1969) where numerous types of mosaics have been found in living persons with varying phenotypic effects.

Some of the mixoploid blastocysts seemed to have been undergoing degeneration as evidenced by the presence of pycnotic nuclei. Consequently, they might have contributed to pre-implantation loss. Tesh (1969) observed that the latter contributed to a decline in fertility when sperm were aged in *utero*, although the effect of ageing was concerned mainly with fertilization rate. On the other hand, mixoploids with a high proportion of diploid cells might progress in development due to selective proliferation of the diploid cells and suppression or elimination of the tetraploid ones. Thus, the chromosome changes in blastocysts resulting from *in utero* aged spermatozoa might be different from those associated with gross developmental anomalies. Tesh (1969) did not observe any increased incidence of foetal abnormalities when spermatozoa were aged in the female tract.
VI. SUMMARY

(1) Ovulation was induced in 40 female rabbits by an injection of HCG at various intervals from 0 to 21 hours after artificial insemination. Thus, spermatozoa were stored or aged in the female reproductive tract for 10 to 31 hours before ovulation. A total of 251 blastocysts were recovered. There was a peak in the recovery rate at 18 hours and a decline thereafter. No blastocysts were recovered when spermatozoa were aged for 31 hours.

(2) The chromosome complement was examined in 239 of the blastocysts of which 105 were obtained from 13 females in the control group. In the latter, sperm were aged for 10 hours. Only one chromosomally abnormal blastocyst, which was a 2N/4N mixoploid, was seen in the control group. The incidence of chromosome anomalies in this group was 0.95%.

(3) In the experimental series, spermatozoa aged for 14-30 hours resulted in 13 chromosomally abnormal blastocysts from a total of 134 examined. The anomalies were scattered amongst the various ageing intervals. There were five 2N/4N mixoploids, five chromosome mosaics and three blastocysts with a 44,XX/44,XY or a chimaeric constitution. The 9.7% incidence of chromosome anomalies in the experimental group
was significantly different from that in the control series.

(4) In the control series, 51 blastocysts had an XY and 54 had an XX sex chromosome complement, giving a sex ratio of 94 males to 100 females. In the experimental series, 80 blastocysts had an XY and 51 had an XX complement. The sex ratio in the experimental series was therefore 157 males:100 females. There was no significant difference between the sex ratios of the control and experimental groups.

(5) In a second experiment to age spermatozoa in the male tract, the corpus epididymidis of eight bucks was ligated bilaterally, to prevent the passage of fresh spermatozoa from the testis to the cauda epididymidis. Sham operations in which the epididymidis was exposed but not ligated were done in two additional males. The day of the operation, Day 0, was used as the reference point for assessing sperm age.

(6) Ejaculates collected from the eight males, two to four days prior to ligation and from six other males were used to inseminate females serving as controls. This was the identical control group in the previous experiment. Immediately after insemination all females in this experiment were given an ovulation-inducing injection of HCG. Semen collected post-operatively on Day 7 and 35 from the sham-operated males, and at various intervals from Day 7 to 35 after ligation, was used to inseminate females. Microscopic examination of semen samples showed deteriorative changes of spermatozoa with the
(7) A total of 98 blastocysts was recovered from 15 females inseminated with ejaculates collected post-operatively. No blastocysts arose from 35-day-old spermatozoa. Females inseminated with ejaculates from males after the sham operation gave rise to 24 blastocysts, 20 of which were analysed cytogenetically and shown to have normal chromosomes. The data for the combined control and sham-operated group gave one heteroploid blastocyst in 125, an incidence of 0.8% anomalies.

(8) Blastocysts resulting from sperm aged in the male tract showed an incidence of 11.1% chromosomal anomalies. This was significantly different from the incidence in the combined control and sham-operated group.

(9) Three females inseminated with ejaculates collected seven days post-operatively gave rise to 24 blastocysts, three of which had abnormal chromosomes. Two were 2N/4N mixoploids and the other was a triploid with an XXY sex chromosome complement.

(10) Three chromosome abnormalities were found among 32 blastocysts recovered from females inseminated with sperm aged 12 to 15 days. One blastocyst was a 44/45 mosaic, one had trisomy for pair 1 and the third had a deletion of the short arm of chromosome 5.

(11) Two heteroploid blastocysts were recovered from two females inseminated with ejaculates collected 19 to 21 days post-operatively.
One blastocyst had a trisomy for pair 6 and the other had trisomy for pair 20.

(12) In the combined control and sham-operated group, the sex ratio was 95 males to 100 females. The sex ratio in blastocysts recovered from all females inseminated with spermatozoa aged in the male tract was 97 males:100 females. No significant difference was found between the two sex ratios.
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TABLE 1

BLASTOCYSTS RECOVERED FROM RABBITS IN WHICH SPERMATOZOA WERE AGED IN UTERO

<table>
<thead>
<tr>
<th>Ageing Period (hr)</th>
<th>No. of Females Inseminated</th>
<th>Corpora Lutea</th>
<th>Blastocysts Recovered Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 (control)</td>
<td>13</td>
<td>132</td>
<td>109</td>
<td>82.6</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>29</td>
<td>24</td>
<td>82.6</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>31</td>
<td>25</td>
<td>80.7</td>
</tr>
<tr>
<td>18</td>
<td>3</td>
<td>32</td>
<td>30</td>
<td>93.8*</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>23</td>
<td>14</td>
<td>60.9**</td>
</tr>
<tr>
<td>22</td>
<td>3</td>
<td>20</td>
<td>11</td>
<td>55.0**</td>
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<td>25</td>
<td>3</td>
<td>25</td>
<td>13</td>
<td>52.0**</td>
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<tr>
<td>28</td>
<td>3</td>
<td>32</td>
<td>19</td>
<td>59.4**</td>
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<td>29</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>12.5**</td>
</tr>
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<td>30</td>
<td>2</td>
<td>22</td>
<td>5</td>
<td>22.7**</td>
</tr>
<tr>
<td>31</td>
<td>3</td>
<td>27</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>40</strong></td>
<td><strong>381</strong></td>
<td><strong>251</strong></td>
<td></td>
</tr>
</tbody>
</table>

* Difference between 10 and 18 hr.: $\chi^2 = 5.0$ (P < 0.03) statistically significant increase.

** Significant decrease when compared with the value in the control series.

Difference between 10 and 20 hr.: $\chi^2 = 11.0$ (P < 0.001) statistically significant decrease.
**TABLE**

**CYTOGENETIC FINDINGS IN**

**AFTER AGEING OF**

<table>
<thead>
<tr>
<th>Sperm Ageing Periods (hr)</th>
<th>Female</th>
<th>Blastocyst</th>
<th>Chromosome Count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diploid and Near Diploid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;42</td>
</tr>
<tr>
<td>10 (control)</td>
<td>1</td>
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</tr>
<tr>
<td>14</td>
<td>2</td>
<td>2 a)</td>
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<td></td>
<td></td>
<td>b)</td>
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<td>3</td>
<td>3 a)</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>4 a)</td>
<td>1</td>
</tr>
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<td>5</td>
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<td>2</td>
</tr>
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<td></td>
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<td>b)</td>
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</tr>
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<td></td>
<td>6</td>
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<td></td>
<td>b)</td>
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</tr>
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<td>7</td>
<td>7 a)</td>
<td>1</td>
</tr>
<tr>
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<td>9 a)</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>b)</td>
<td>3</td>
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HETEROPLOID BLASTOCYSTS
SPERMATOZOA IN UTERO

<table>
<thead>
<tr>
<th>Distribution Exact or Near Polyploid</th>
<th>No. Cells Analysed</th>
<th>No. Cells Karyotyped</th>
<th>Sex Chromosome Complement</th>
<th>Anomaly</th>
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<tbody>
<tr>
<td>4N/8N</td>
<td>35</td>
<td>8</td>
<td>XX</td>
<td>2N/4N Mixoploidy</td>
</tr>
<tr>
<td>14</td>
<td>40</td>
<td>8</td>
<td>XO/XY</td>
<td>Mosaicism</td>
</tr>
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<td>9</td>
<td>15</td>
<td>XY</td>
<td>Mosaicism</td>
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<tr>
<td>50</td>
<td>9</td>
<td>16</td>
<td>XX/XY</td>
<td>Chimaerism</td>
</tr>
<tr>
<td>30</td>
<td>17</td>
<td>16</td>
<td>XX</td>
<td>Mosaicism</td>
</tr>
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<td>59</td>
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<td>11</td>
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<td>Chimaerism</td>
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<tr>
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<td>12</td>
<td></td>
<td>XX/XY</td>
<td>Chimaerism</td>
</tr>
<tr>
<td>8</td>
<td>32</td>
<td>9</td>
<td>XY</td>
<td>Hypodiploid Mosaic/ Mixoploidy 43/44/88</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>9</td>
<td>XY</td>
<td>2N/4N Mixoploidy</td>
</tr>
</tbody>
</table>
## TABLE 3

**DISTRIBUTION OF CELLS IN THE CHIMAERIC BLASTOCYSTS**

<table>
<thead>
<tr>
<th>Chimaera</th>
<th>Sperm Ageing Period (hr)</th>
<th>No. of Cells with Sex Chromosomes Identified</th>
<th>Sex Chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>XY (%)</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>48</td>
<td>47.9</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>38</td>
<td>42.1</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>51</td>
<td>60</td>
</tr>
</tbody>
</table>
**TABLE 4**

THE CHROMOSOMAL SEX OF BLASTOCYSTS RECOVERED FROM FEMALES IN WHICH SPERM WERE AGED *IN UTERO* *

<table>
<thead>
<tr>
<th>Series</th>
<th>Sperm Ageing Period (hr)</th>
<th>No. of Blastocysts Analysed Cytogenetically</th>
<th>Sex Complement XY</th>
<th>Sex Complement XX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>105/109</td>
<td>51</td>
<td>54</td>
</tr>
<tr>
<td>Experimental</td>
<td>14*</td>
<td>24/24</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>21/25</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>30/30</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>12/14</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>22*</td>
<td>11/11</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>12/13</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>28*</td>
<td>18/19</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>1/1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5/5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>134/142</td>
<td>80</td>
<td>51</td>
</tr>
<tr>
<td>GRAND TOTAL</td>
<td></td>
<td>239/251</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The three chimaeric blastocysts are omitted from this Table.
TABLE 5

BLASTOCYSTS RECOVERED FROM FEMALE RABBITS INSEMINATED
WITH SPERMATOZOA AGED IN THE MALE GENITAL TRACT

<table>
<thead>
<tr>
<th>Ageing Period (day)</th>
<th>No. of Females Inseminated</th>
<th>Corpora Lutea</th>
<th>Blastocysts Recovered Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td>2</td>
<td>24</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>0**</td>
<td>13</td>
<td>132</td>
<td>109</td>
<td>82.6</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>27</td>
<td>24</td>
<td>88.9</td>
</tr>
<tr>
<td>12-15</td>
<td>4</td>
<td>39</td>
<td>32</td>
<td>82.1</td>
</tr>
<tr>
<td>19-21</td>
<td>2</td>
<td>19</td>
<td>9</td>
<td>47.4</td>
</tr>
<tr>
<td>27</td>
<td>1</td>
<td>12</td>
<td>9</td>
<td>75.0</td>
</tr>
<tr>
<td>35</td>
<td>3</td>
<td>31</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

28  284  207

* Sham operation
** Control
HETEROPLOID BLASTOCYSTS RESULTING
IN THE MALE TRACT

<table>
<thead>
<tr>
<th>Distribution</th>
<th>No. Cells Analysed</th>
<th>No. Cells Karyotyped</th>
<th>Sex Chromosome Complement</th>
<th>Anomaly</th>
</tr>
</thead>
<tbody>
<tr>
<td>3N</td>
<td>14</td>
<td>35</td>
<td>8</td>
<td>XX</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>40</td>
<td>3</td>
<td>XX</td>
<td>2N/4N Mixoploidy</td>
</tr>
<tr>
<td>20</td>
<td>14</td>
<td>40</td>
<td>3</td>
<td>XX</td>
</tr>
<tr>
<td>21</td>
<td>12</td>
<td>21</td>
<td>3</td>
<td>XXY</td>
</tr>
<tr>
<td>30</td>
<td>12</td>
<td>30</td>
<td>2</td>
<td>XX</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>30</td>
<td>XX</td>
<td>Trisomy 1</td>
</tr>
<tr>
<td>10</td>
<td>34</td>
<td>34</td>
<td>XY</td>
<td>Mosaicism</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>16</td>
<td>XY</td>
<td>No. 5 Deletion</td>
</tr>
<tr>
<td>10</td>
<td>31</td>
<td>31</td>
<td>XX</td>
<td>Trisomy 6</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>41</td>
<td>XY</td>
<td>Trisomy 20</td>
</tr>
</tbody>
</table>
# TABLE 7

**THE SEX COMPLEMENT OF BLASTOCYSTS RESULTING FROM**

**SPERMATOZOA AGED IN THE MALE TRACT**

<table>
<thead>
<tr>
<th>Series</th>
<th>Sperm Ageing Period (day)</th>
<th>No. of Blastocysts Analysed Cytogenetically</th>
<th>Sex Complement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>0</td>
<td>20/24</td>
<td>10 10</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>105/109</td>
<td>51 54</td>
</tr>
<tr>
<td>Experimental</td>
<td>7</td>
<td>*23/24</td>
<td>14 8</td>
</tr>
<tr>
<td></td>
<td>12-15</td>
<td>32/32</td>
<td>16 16</td>
</tr>
<tr>
<td></td>
<td>19-21</td>
<td>8/9</td>
<td>3 5</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>9/9</td>
<td>2 7</td>
</tr>
</tbody>
</table>

|               | 72/74                      | 35 36                                      |

* One triploid blastocyst had a sex chromosome complement of XXY.
A normal male rabbit karyotype from a six-day-old blastocyst (44,XY)
Figure 2

A normal female rabbit karyotype from a six-day-old blastocyst (44,XX)
Figure 3

Diagram of the rabbit testis and epididymidis to illustrate the anatomical arrangement of the distal regions of the tubule and the location of ligatures.

1. Caput epididymidis
2. Corpus epididymidis
3. Testis
4. Ligatures
5. Vas deferens
6. Cauda epididymidis
Figure 4

Photograph taken during surgical operation to show the incision in the scrotal skin and the application of ligatures to the corpus epididymidis, without removing the testis from the scrotal sac. The arrow indicates the segment removed.
Figure 5

Photomicrographs of metaphase spreads from blastocysts with cells in which a heteropycnotic X chromosome was detected. Complete metaphase plates are seen in (a) and (b) while (c) is a partial metaphase plate showing a kink in the long arm of the X chromosome. Arrows indicate the heteropycnotic X's; note their peripheral or near-peripheral location.
Figure 6

Karyotype of a rabbit blastocyst with an unusually small Y chromosome
Figure 7

Graph showing the effect of ageing of rabbit spermatozoa on blastocyst recovery
PERCENT RECOVERY = \frac{\text{Total No. of blastocysts} \times 100}{\text{Total No. of Corpora Lutea}}
**Figure 8**

Karyotype of a tetraploid cell with a XXXX sex complement
Figure 9

Hypodiploid karyotype with 43 chromosomes in a 43/44/88 mixoploid blastocyst recovered from a female after 28 hours in utero sperm ageing
Diploid karyotype from the 43/44/88 mixoploid blastocyst recovered from a female after 28 hours in utero sperm ageing
Figure 11

Tetraploid karyotype from the 43/44/88 mixoploid blastocyst recovered from a female after 28 hours in utero sperm ageing
Figure 12

Diploid karyotype from a 43/44 chromosome mosaic blastocyst arising from sperm aged 14 hours in utero
Karyotype from the 43 chromosome population of the 43/44 mosaic blastocyst arising from sperm aged 14 hours in utero
Diploid karyotype from a 44/45 chromosome mosaic blastocyst recovered from a female in which sperm had aged 14 hours
Figure 15

Karyotype from the 45 chromosome population of the 44/45 mosaic blastocyst recovered from a female in which sperm had aged 14 hours
Figure 16

Karyotype from the 43 chromosome population of a 43/44 mosaic blastocyst resulting from a spermatozoon aged 16 hours in utero
Figure 17

Diploid karyotype from the 43/44 mosaic blastocyst resulting from a spermatozoon aged 16 hours in utero
Figure 18

Karyotype from the 43 chromosome population of a 43/44 mosaic blastocyst from a female after 18 hours ageing of sperm *in utero*
Figure 19

Diploid karyotype from the 43/44 mosaic blastocyst from a female after 18 hours ageing of sperm in utero
Figure 20

Karyotype from the 45 chromosome population of a mosaic blastocyst resulting from a spermatozoon aged 20 hours in the female
Figure 21

Diploid karyotype of a 44/45 mosaic blastocyst resulting from a spermatozoon aged 20 hours in the female.
Figure 22

Karyotype with an XY sex complement in a cell from a chimaeric blastocyst with a 44,XX/44,XY constitution, resulting from sperm aged 14 hours in utero
Figure 23

Karyotype with an XX sex complement in a cell from a chimaeric blastocyst with a 44,XX/44,XY constitution, resulting from sperm aged 14 hours in utero
Figure 24a

Photograph showing lifting of the acrosome or head cap in spermatozoa recovered in ejaculate seven days after ligation of the corpus epididymidis. The asterisk shows a spermatozoon with an intact acrosome cap.

Figure 24b

Photograph showing gross head cap and tail defects in spermatozoa from semen collected 15 days after ligation of the corpus epididymidis.

1. Coiled sperm tail
2. Lifting of the acrosome cap
3. Completely detached and displaced acrosome cap
4. Tailless spermatozoa
Figure 25

Triploid karyotype seen in blastocyst resulting from a female inseminated with spermatozoa aged seven days in the male tract. The sex complement is XXY.
Figure 26

Karyotype showing trisomy for chromosome No. 1 in blastocyst resulting from a spermatozoon aged 15 days in the male
Figure 27

Karyotype of the 45 chromosome population from a 44/45 mosaic blastocyst recovered from a female inseminated with sperm aged 14 days in the male
Figure 28

Diploid karyotype from the 44/45 mosaic blastocyst recovered from a female inseminated with sperm aged 14 days in the male
Figure 29

Karyotype showing deletion of the short arm of chromosome No. 5. This karyotype was seen in a blastocyst arising from a spermatozoon aged 14 days in the male.
Figure 30

Karyotype showing trisomy for chromosome No. 6 in a blastocyst from a female inseminated with spermatozoa aged 19 days in the male.
Figure 31

Karyotype showing trisomy for chromosome No. 20 in a blastocyst from a female inseminated with spermatozoa aged 19 days in the male.
END OF REEL