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A CLINICAL CYTOGENETICAL STUDY OF THE HORSE

A thesis presented in partial
fulfilment of the requirements for the degree
of Doctor of Philosophy in
Veterinary Science at
Massey University

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ABSTRACT

Chromosome preparations were derived from short-term cultures of blood lymphocytes and from fibroblast-type cell cultures of subcutaneous fascia obtained from 44 clinically normal horses. The quality of mitotic cells compared favourably with similar studies in other species. Some characteristic morphological features were found which aid in the recognition of certain chromosomes, but the identification of many homologous pairs by conventional techniques was difficult. The normal karyotype of the horse ($2n = 64$) was characterized following Giemsa staining and C-banding. The latter procedure was shown to be a valuable aid for the identification of specific chromosomes, in particular the sex chromosomes. After centromere staining, the X chromosome of the horse is characterized by the presence of prominent C-bands on its long arms, while the Y chromosome appears as an intensely-stained block.

Analysis of mitotic chromosomes prepared from ten phenotypically normal but infertile mares revealed, in one animal, the consistent lack of one sex chromosome in the four tissues studied. Centromere staining and sex chromatin studies confirmed that this mare had the karyotype 63XO. Examination of the genitalia of this XO mare disclosed the presence of apparently normal reproductive organs except that macroscopic follicles were absent from both ovaries. Histologically, a number of presumptive atretic Graafian follicles and a discrete area of apparently functional luteal tissue were discovered in the right ovary. The significance of these findings are discussed and compared with

the features of the analogous syndromes associated with the XO anomaly in other species.

It is suggested that disorders of the sex chromosomes may occur commonly in horses and may contribute significantly towards the low fertility of the thoroughbred mare. Attention is drawn to the important practical applications of cytogenetic studies in the investigation of infertility in broodmares, and in the certification for sale of young horses intended for use as breeding stock.

Cytogenetic studies of spontaneous abortions in women have established that a large proportion of pregnancy wastage in humans is due to chromosome aberrations. The counterparts of many of the chromosome errors initially described in humans have now been reported among domestic animals. In particular, the XO anomaly seems remarkably common among infertile mares. These findings have indicated that chromosomal abnormalities may play a significant role in the aetiology of spontaneous abortion and embryonic loss in domestic animals. To investigate an hypothesis that chromosome anomalies are an important cause of prenatal loss in the mare, an attempt was made to analyze, cytogenetically, a series of equine abortions. From a range of tissues obtained from 26 aborted foals, cell cultures were prepared but failed to grow, and chromosome analysis was therefore not possible for any of these specimens. It seems that the equine foetus is retained in the uterus of the mare for a considerable period after the foetus has died and usually consists of partially-autolyzed non-vital tissues at the time of its expulsion.

Consequently, a study was made of the metaphase

chromosomes prepared from 22 equine embryos after their surgical removal from mares' uteri. A normal karyotype was found in each specimen. Although inconclusive on their own, the results of this study form an important contribution towards cumulative studies of embryonic loss in the horse. The current findings are discussed in the light of similar studies of induced abortions in women and embryonic loss in animals, and the potential for further investigation in this field is emphasized.

From the limited number of cytogenetic studies in domestic animals, it appears that chromosome analyses, particularly with the aid of more refined techniques such as centromere staining, may be of considerable value in investigations of infertility and embryonic loss in the horse.

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PRELUDE

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PRELUDE

Spontaneous abortion has been defined as the natural termination of a pregnancy before the foetus is viable (Morison, 1970). In the horse this includes foals born before 290 days of gestation (Rossdale, 1965; Platt, 1973a). Even today, little is known of the causes of prenatal loss in domestic animals (Hanley, 1961) although it is responsible for a serious loss of potential productivity.

A prenatal death rate of about 40% has been established for the rat, rabbit and ferret (Corner, 1923) and similar values of 35-40% and 25% are quoted for the pig (Smith and Marlowe, 1971) and cow (Hanley, 1961) respectively. In these species prenatal loss accounts for at least one third of the total conceptions and the actual figure may be even greater when hitherto undetected early embryonic losses are included. From slaughter observations, Quinlivan, Martin, Taylor and Cairney (1966) reported a prenatal loss of 23% in sheep, with the greatest mortality occurring during the first 30 days of pregnancy.

The mare apparently has the lowest reproductive rate of all the domestic animals and reproductive efficiency, as measured by live foal percentage, is lower in thoroughbreds and standardbreds than in other breeds managed less intensively (Burkhardt, 1948; Hutton and Meacham, 1968).

In the United States, Bergin (1970) recorded a live foal percentage in thoroughbreds of 55.3% between 1950 and 1956. By 1970 this figure had fallen to 50%. Weatherby's statistical data reported a 50% live foal rate for the

United Kingdom and Eire in 1973, while in Australia, Osborne (1975) defined national foaling rates of 49% in 1950 and 47% in 1973, and noted an increasing disparity between conception and foaling rates in recent years. In New Zealand, Dewes (1973) found that a foaling percentage of 67% in 1903 had fallen to 49% in 1971. An even lower foaling rate of only 45% was recorded by van Niekerk (1965a) in South Africa between 1962 and 1965.

Osborne (1975) suggested that the gravity of the situation may have been exaggerated as these estimates of fertility were based on the number of thoroughbred mares registered in the stud-books of the various countries rather than on the number of mares actually served. More objective data can be obtained from surveys of mating groups.

A three year survey of two large thoroughbred studs in New Zealand disclosed that of all mares mated, only 54% produced a viable foal (Elliott, Callaghan and Smith, 1971). A similar figure was released by the New Zealand Thoroughbred Breeders' Association (Anon., 1970) for the number of live foals resulting from matings by eight commercially popular stallions in 1966. In a group of 852 thoroughbred mares, Laing and Leech (1975) found that of the 77.9% which conceived, 69.7% produced a viable foal. Productive mares again constituted only 51% of the total number of mares surveyed. In addition, these figures attached major significance to the high incidence (30.3%) of prenatal loss, of which only a small proportion was manifested as clinical abortion.

Estimates of total prenatal loss in the horse have been confused due to problems of measuring the conception rate. In most studies conception rate has been determined

by rectal palpation between 35 and 45 days after service (Sullivan, Turner, Self, Gutteridge and Bartlett, 1975). Much controversy exists over the role that manual pregnancy testing may play in embryonic loss. Voss, Pickett and Back (1973) established a deleterious effect on conception rate of rectal palpation during oestrus, and Osborne (1975) incriminated rectal palpation during early pregnancy as a cause of embryonic loss. Other workers (Allen, 1974a; Irwin, 1975) have found early manual pregnancy testing to have no effect on the incidence of foetal death.

Under current systems of intensive stud management the total conception rate of thoroughbred mares is probably between 65% and 70% (McGee, 1970; Laing and Leech, 1975). Sullivan et al., (1975) recorded a first service conception rate of 43%, a cumulative pregnancy rate of 77% and a pregnancy loss of 12% after 45 days. Figures released by the New Zealand Racing Conference reveal an annual abortion rate of 13%, similar to that reported from other studies (Day, 1957; Matassino, 1962; McGee, 1970), and a 30% incidence of so-called 'barren' mares. A proportion of the latter had almost certainly undergone embryonic loss.

The most reliable method of determining total prenatal loss is by post-mortem examination of mares at various intervals after service. In 1920, a survey in Scotland (Corner, 1923) of 28,000 matings in Clydesdales and 3,640 matings in thoroughbred horses revealed prenatal death rates of 48% and 59% respectively. A comparable result was obtained by Osborne (1975) following the inspection of 5198 uteri. She detected positive evidence of abortion in 50.3% of 765 uteri which showed evidence of pregnancy. In the study by Osborne (1975), a proportion of the loss was attri-

buted to increased stress factors, namely unsympathetic handling, long distance transport and inadequate food and water.

From a study of prenatal loss in the pig, Corner (1923) discovered an initial loss of 20% of ova before the fourth week of pregnancy. Hanley (1961) reported that 33% of pig embryos die during the first half of gestation. Dufour and Fahmy (1975) considered embryonic mortality to be the most important factor determining litter size in different breeds of pigs. They found that 19% of ova released from the ovary were lost during the first 23 days of pregnancy, while losses after this stage were very few. In his investigation of repeat-breeder cows, defined as those cows not conceiving to first service, Casida (1953) found that 59.4% of presumably normal embryos were lost during the first 34 days of gestation. Obviously early embryonic death was a major limiting factor in the reproductive performance of these animals.

From cumulative studies in humans, Rock (1940) concluded that an abortion rate of 25% was a conservative estimate, and it was later shown (Hertig, Rock, Adams and Menkin, 1959) that the greatest loss of ova occurred during preimplantation stages of pregnancy. Following the examination of ten abnormal human conceptuses obtained by hysterectomy, Hertig, Rock and Adams (1956) doubted whether the more abnormal conceptuses would have developed to a stage sufficient to cause clinical evidence of pregnancy. Combining the frequency of clinical abortions (15%) with a 30% loss of preimplantation stages, Carr (1970a) calculated that prenatal death accounted for 45% of all conceptions

in women.

As in other species, detected abortions in the horse represent only a small fraction of total prenatal loss, most of which occurs during early stages of pregnancy (Moberg, 1975). The extent of the problem of early embryonic loss in mares was emphasized by Du Plessis' (1964) discovery that 38% of mares which did not return to oestrus after service were found to be barren when examined for pregnancy after 40 days. In yearling mares Mitchell and Allen (1975) reported a high incidence (46%) of early pregnancy loss evenly distributed between 60 and 140 days; losses earlier than 60 days of gestation were unknown but losses later than 140 days were very few.

The causes of embryonic loss in domestic animals have remained largely unexplained. Consequently, treatment has often been empirical. In 1953 Casida wrote of sub-fertile cows: "Many different treatments are attempted in getting repeat breeders to conceive. Almost any treatment appears to do some good, but none appears to do a great deal of good."

Investigation of the aetiology of embryonic loss in the horse has been dominated by the fields of infection and endocrinology. This emphasis is now being questioned.

Infectious agents were shown to be of little or no importance in the aetiology of early foetal loss in yearling mares (Mitchell and Allen, 1975). Although infection of the foetus <u>in utero</u> may be a significant cause of prenatal mortality in later months of pregnancy, Platt (1973b, 1975) concluded that infection was probably seldom the primary cause of abortion. To explain the divergence between conception and foaling rates, Osborne (1975) drew attention

to the increasing stresses of modern intensive stud management. Other factors incriminated in prenatal loss have included hormone malfunction, immaturity and inadequate nutrition.

Much of the enthusiasm for various treatments, particularly hormone therapy, has derived from clinical experiences. In this context it has been shown (Malpas, 1938) that 62% of women who had aborted twice carried the third baby to term without specific treatment. Ganjam, Kenney and Flickinger (1975a) have questioned the efficacy of exogenous progesterone in the treatment of abortion and concluded that "the whole problem of habitual abortion and its treatment in mares needs re-evaluation."

For more than 50 years it has been known that many aborted conceptuses were structurally abnormal. Environmental factors were believed to be primarily responsible for congenital defects in the developing embryo until abnormalities were discovered in preimplantation stages of pig embryos (Corner, 1923). In association with this finding Corner (1923) wrote: "it seems that in the pig there is little evidence that faulty implantation is a cause of embryonic mortality and abnormality, but much to indicate that embryos may become abnormal in spite of a uterine environment which is by all the criteria at our present command, anatomically and functionally normal." Anomalies of development were present at all stages of pregnancy and he also observed defective and normal embryos in the same uterus. Corner (1923) attributed at least part of the embryonic mortality to lethal factors within the germ cells and embryos. Similarly, abnormalities in early monkey

embryos were thought to have originated from constitutional defects of the embryo itself (Corner and Bartelmez, 1953).

Hertig and Rock (1949) studied 28 early human conceptuses obtained by hysterectomy and decided that 25% of them had been destined to abort. In all cases the endometrium was normal. In a subsequent study (Hertig et al., 1956) 34 zygotes of fewer than 17 days gestation were recovered from the uteri of women of proven fertility. Ten conceptuses were abnormal, of which four would probably never have implanted.

The idea that chromosome disorders might be a significant cause of spontaneous abortion in humans received support with the finding of two children with lethal chromosome constitutions (Edwards, Harnden, Cameron, Crosse and Wolff, 1960; Patau, Smith, Therman, Inhorn and Wagner, 1960) and shortly after, two instances of foetal death were found to be associated with triploidy of the foetus (Penrose and Delhanty, 1961; Delhanty, Ellis and Rowley, 1961).

The first reports of chromosome studies of spontaneous abortions in women revealed a high incidence of chromosome aberrations (Carr, 1963 ; Clendenin and Benirschke, 1963). Many subsequent studies have established the incidence of chromosome anomalies in spontaneous abortions at approximately 36% (Carr, 1970a), a frequency remarkably similar to that found in ten day pig blastocysts (McFeely, 1967).

Almost 1% of liveborn infants have a major chromosomal abnormality (Friedrich and Nielsen, 1973; Hamerton, Canning, Ray and Smith, 1975) but this represents only a small proportion of all conceptuses with chromosome

abnormalities. The incidence of chromosome anomalies found in conceptuses decreases progressively from conception until term (Carr, 1970a; Machin, 1974).

A variety of chromosome aberrations are now known to be associated with embryonic death and prenatal loss in the pig (McFeely, 1967) and cat (Benirschke, Edwards and Low, 1974) and anomalies of the sex chromosomes have been found in infertile mares. Studies of habitual abortion in women have suggested that parental chromosome anomalies may play a role in the aetiology of recurrent abortion and one case of a chromosomal translocation in a boar has been linked with embryonic death (Äkesson and Henricson, 1972).

Thus human studies have revealed the importance of chromosomal errors in infertility and abortion, and numerous examples of chromosome anomalies in the domestic animals are now known. Due to the limited amount of research undertaken in this field, the significance of chromosome disorders in embryonic loss has not yet been established for the domestic animals. However, the preliminary reports outlined above have demonstrated remarkable similarities to the situation in man.

PART II

MATERIALS AND METHODS

PART II

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II. 1. PRELIMINARY STUDY OF ABORTED FOETUSES

A preliminary study of spontaneously aborted equine foetuses was undertaken on cases referred by field veterinarians. Twenty-six abortuses with gestational ages between five months and term were involved in this study.

When the abortus was available a routine autopsy was performed, supplemented by bacteriological and histological investigation of body organs, including the spleen, brain, lung, lymph nodes, placenta, kidney, liver and adrenal glands.

From the foetus, a sample of subcutaneous fascia was collected into a universal bottle containing HBSS (Hanks' Balanced Salt Solution; Hanks, 1948), and cell cultures were prepared according to the method described by Hyman (1968).

II. 2. METHOD FOR OBTAINING EQUINE EMBRYOS

i) Experimental animals

A group of 12 mares and one stallion was grazed on a 30 acre grassed paddock from October until March. During the intervening six months they were confined to a three acre field and fed hay. Each horse was drenched regularly with 60g Equizole and 15g Neguvon at two monthly intervals. Treatment for lice in winter and spring involved dusting each horse twice at 14 day intervals with rotenone powder (Appendix I).

Due to grazing management observation of the mating

group during the breeding season was infrequent and it was not possible to obtain accurate mating dates for individual mares. Diagnosis of pregnancy therefore relied on rectal palpation.

ii) Pregnancy diagnosis

Diagnosis of early pregnancy by rectal palpation involved the detection of changes in tone and thickness of the walls of the uterine horns and palpation of an embryonic mass. Van Niekerk's (1965a, 1965c) claim that it is "possible to make a diagnosis of pregnancy at 14 days and a definite diagnosis of pregnancy 16 to 19 days after ovulation" was disputed by Bain (1967a) who was unable to give a negative and often even a positive diagnosis of pregnancy at fewer than 25 days. Bain (1967a) considered 30 days after ovulation the earliest practical time to present mares for pregnancy diagnosis.

The reproductive tract of each mare was examined per rectum at intervals of one week throughout the breeding season. Particular attention was paid to the state of the ovaries and uterus. In view of Bain's (1967a) conclusion, mares were not presented for surgery unless a pregnancy of at least 30 days duration had been established. A positive diagnosis of pregnancy was made at approximately 30 days gestation on the basis of uterine tone and the presence of an embryonic swelling. This was confirmed immediately prior to surgery.

iii) Anaesthesia

Each mare was premedicated with a tranquillizing drug and thiopentone sodium was used to induce anaesthesia within a padded stall. This was followed by intubation, and main-

tenance of anaesthesia with oxygen and halothane supplied through a semi-closed to and fro anaesthetic apparatus incorporating a soda lime cannister to remove carbon dioxide.

iv) Surgery

The patient was positioned on the adjustable operating table in dorsal recumbancy with the hind legs passively extended, and the operation site was routinely prepared for surgery.

An incision 15 cm in length was made through the linea alba in the ventral midline between the umbilicus and the mammary gland. The uterus was located and the pregnant horn exteriorized. Until the uterine incision was completed the embryo was temporarily excluded from the site of incision in the uterine horn by manual pressure from outside the uterus. Still enclosed within its intact chorio-allantoic membrane the embryo was expressed through the incision and transferred to a sterile glass container (Figure II. 1).

Standard surgical techniques were employed to close the wound. Separate closure of the peritoneum was considered unnecessary and it was usually included in a single layer of sutures with the linea alba.

Tetanus antitoxin was administered to the patient after each operation but no additional postoperative treatment was given. The patient was returned to the mating group three days after surgery and the skin sutures were removed a week later.

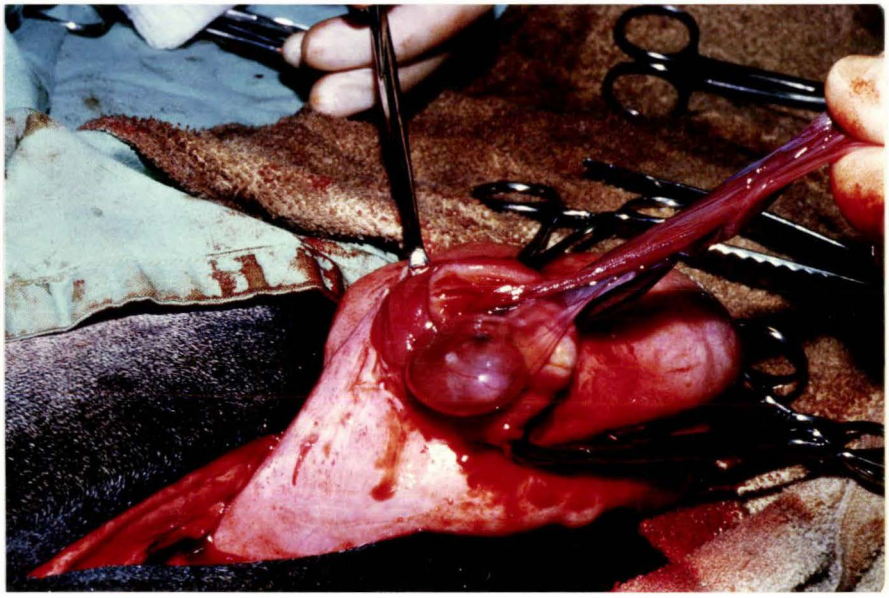


Figure II.1. Surgical removal of an equine embryo from the uterus of a mare.

II. 3. IN VITRO CULTURE OF BLOOD LEUCOCYTES

i) History

In 1960, Moorhead, Nowell, Mellman, Batipps and Hungerford introduced a procedure for the preparation of human leucocytes for chromosome analysis. This involved the short term cell culture of 10 ml of venous blood. Modifications of the basic technique (Edwards and Young, 1961; Edwards, 1962; Frøland, 1962) were designed to separate out the leucocytes and reduce the volume of blood required, and Arakaki and Sparkes (1963) obtained satisfactory chromosome preparations from blood volumes as small as 0.05 ml.

The horse lends itself to leucocyte culture more readily than do the other domestic animals due to the rapid spontaneous separation of leucocytes and erythrocytes when equine blood is allowed to stand. Payne, Ellsworth and DeGroot (1968) utilised this property in the development of a simple technique for the short-term culture of equine lymphocytes. In this study leucocyte cultures to determine the mitotic karyotype of live horses were prepared by a modification of the latter procedure. The complete leucocyte culture technique used for the horse is shown in the flow diagram, Figure II. 2. Details of the materials used are given in Appendix III.

ii) Culture of cells

Four 10 ml blood samples were collected aseptically from the jugular vein into an heparinized vacutainer. For transport to the laboratory the vacutainer was packed with ice inside an insulated flask.

Each sample was allowed to stand vertically for 20 minutes at room temperature. The erythrocytes sedimented

quickly while the leucocytes lagged behind and formed a "buffy coat". Two ml of the leucocyte-rich plasma were transferred to a universal container to which was added six ml of medium 199, 0.2 ml phytohaemagglutinin and sufficient sodium bicarbonate (10%) to adjust the pH to neutrality.

Each culture was incubated at 37°C for 48 to 72 hours.

iii) Metaphase arrest with colchicine

Alterations in the pH of the culture were visible as a change in colour due to the indicator, phenol red, which was included in the growth media. Rapid metabolism of cells resulted in a fall in pH and a colour change from red to yellow. Cultures judged to contain actively dividing cells were treated with colchicine after 48 hours incubation while colchicine was not inoculated into slower growing cultures until 72 hours of incubation had elapsed. After the addition of colchicine (0.1 µg in 0.1 ml) to each culture, incubation was continued for a further 16 hours.

Different animals and tissues vary in their response to colchicine, and the duration of treatment and concentration of colchicine must vary according to experience with individual tissues. Excessive contraction of chromosomes has been noted with colchicine treatment at high concentrations (Hyman, 1968) and for prolonged periods (Moorhead et al., 1960). In this study satisfactory results were achieved with colchicine treatment over a considerable interval but at very dilute concentrations.

iv) Treatment with hypotonic fluid

Hypotonic fluid treatment of cell suspensions to spread metaphase chromosomes was introduced by Hsu (1952). The discovery of this effect was the result of an accident

whereby cultures were washed in hypotonic Tyrode's solution instead of isotonic saline before fixation. Many solutions are now known to produce a comparable effect (Makino and Nishimura, 1952; Hsu and Pomerat, 1953; Hyman, 1968).

Rupture of cell membranes may be a consequence of prolonged exposure of cells to hypotonic solution and there is a tendency for chromosome outlines to become blurred (Tjio and Levan, 1956). The aim is to abbreviate hypotonic treatment to a minimum in the attempt to induce chromosome scattering without the unfavourable effects on the chromosome surface.

The author used a 0.075 molar solution of potassium chloride (Hungerford, 1965) for hypotonic treatment. Adequate spread and definition of metaphase chromosomes were obtained after incubating cells in hypotonic solution for 20 minutes.

v) Fixation of cells

The quality of metaphase plates is influenced by the method and duration of fixation. Rothfels and Siminovitch (1958) found that clumping of chromosomes could be reduced by the slow addition of fixative and delayed breaking up of the cell deposit. This can also be achieved by the fixation of cells suspended in considerable amounts of hypotonic fluid (Axelrad and McCullough, 1958). Ford and Hamerton (1956) reported that the quality of fixation was slightly improved by using chilled fixative.

In the present study fixation of cells for 60 minutes at 4°C produced good quality metaphase chromosomes.

Cells in suspension were fixed undisturbed for 60 minutes at 4°C in a freshly prepared mixture of methyl alcohol and glacial acetic acid (3 : 1). Spreading of

chromosomes was facilitated by dropping the cell suspension from a height of 20 cm on to clean slides covered by an even film of chilled distilled water. Slides were then allowed to air dry before each slide was labelled with a serial number, case number and the year.

vi) Staining techniques

a) General procedure for mitotic cells

Prepared dry slides were dipped in absolute alcohol and stained with 2% Giemsa for 10 minutes, rinsed under tap water and allowed to air dry (Appendix IV).

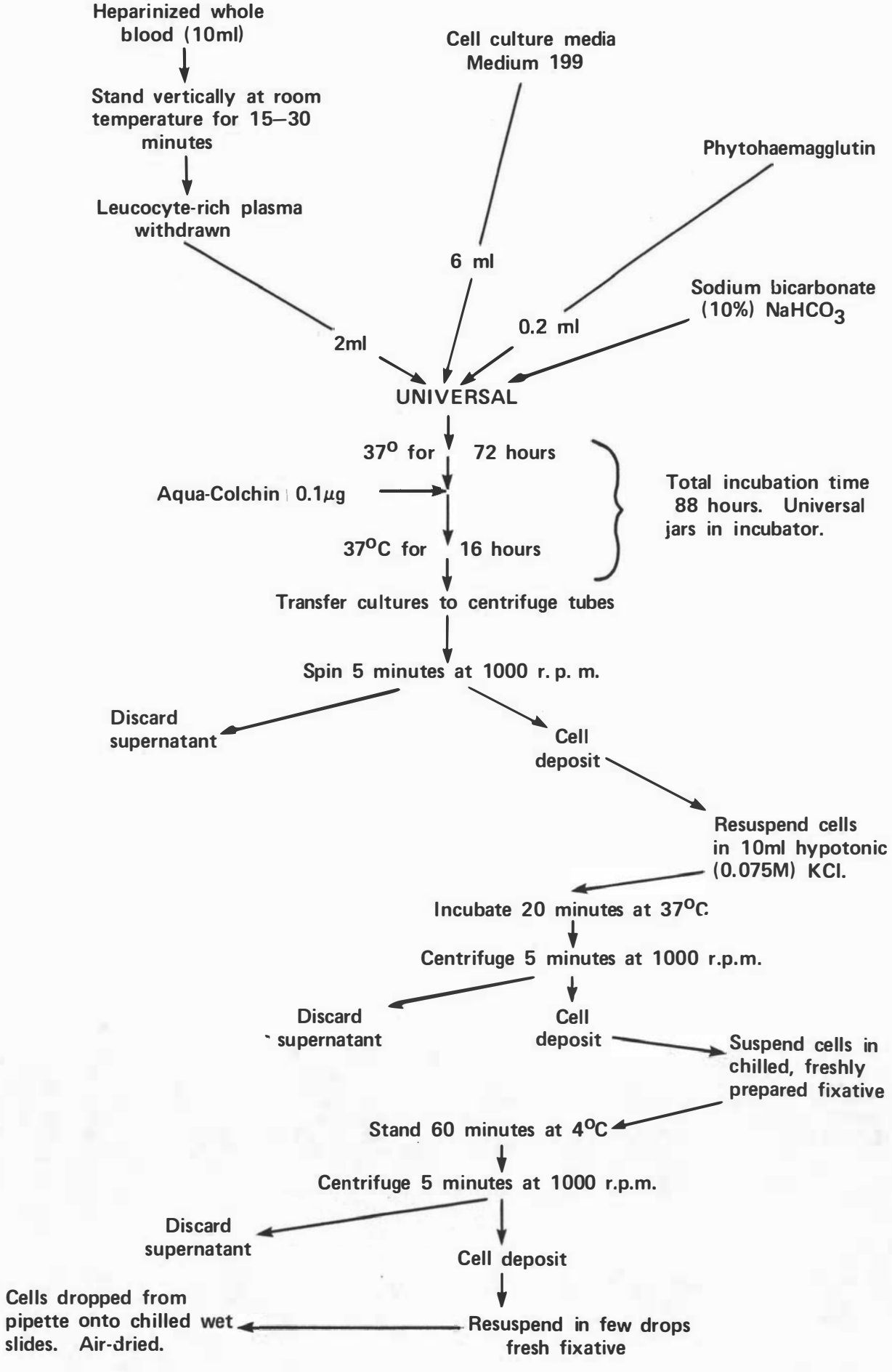
Prior to staining, a precursory examination of the slides was made with a phase contrast microscope. Those slides considered suitable for chromosome analysis were stained, soaked in xylol for five minutes, and a coverslip was affixed using DePex.

b) Centromere staining

Since the initial observations of the differential staining of the centromeric heterochromatin of mitotic chromosomes from mice after treatment with sodium hydroxide and Giemsa stain (Pardue and Gall, 1970; Yunis, Roldan, Yasmineh and Lee, 1971), centromeric staining as a means of chromosome identification has been recognized in several species including man (Chen and Ruddle, 1971), the mouse (Hsu, Cooper, Mace and Brinkley, 1971), ox (Hansen, 1973) and the goat and sheep (Evans, Buckland and Sumner, 1973; Bruère, Zartman and Chapman, 1974).

The method described by Chandley and Fletcher (1973) was used for centromeric staining of mitotic chromosomes in this study. This procedure was in turn based on that of Sumner (1972) with the variation that incubation with $Ba(OH)_2$ lasted 10 minutes rather than 10 seconds (Appendix V).

FIGURE II. 2. FLOW DIAGRAM OF LEUCOCYTE CULTURE TECHNIQUE FROM HORSE BLOOD.



II. 4. FIBROBLAST CULTURE

i) Introduction

Many methods of cell culture have been used for the preparation of metaphase chromosomes from mammalian cells. The cell culture techniques commonly used involve (1) the culture of tissue explants with the aid of adherents, and (2) monolayer cell cultures employing enzymes for cell dispersion.

Although enzymic disaggregation for small explants of solid tissue has been described (Puck, Cieciura and Robinson, 1958), this technique is often inadequate for fibrous tissues and small biopsies. For these tissues the explant culture methods using adherents are effective.

Since the introduction of the cellulose sponge to hold explants against the growing surface (Leighton, 1951), a wide range of adherents performing a similar task has been described. Hsu and Kellogg (1960) used a cellophane sheet to hold tissue explants against the growing surface, while Basrur, Basrur and Gilman (1963) described an explant culture technique allowing chromosome characterization of cells growing on coverslips. Probably the most popular method of fixing tissue fragments to the growing surface has been the use of a plasma clot (Harnden, 1960). Cells from the primary explant may be dispersed with trypsin and subcultured, or they may be studied directly. The procedure used in this study was adapted from the plasma clot embedding technique described by Hyman (1968).

The monolayer technique of cell culture developed by Evans and Earle (1947) has proved very popular and its use has been described by a large number of workers (Rinaldini,

1954; Hinz and Syverton, 1959). The cells are dispersed from living tissues by the action of enzymes, most commonly trypsin, often supplemented by mechanical agitation. Although most suitable for the cell culture of embryonic tissues (Moscona, 1952), the culture of trypsin-dispersed cells as a monolayer has been successfully applied to a wide range of tissues and extended even to placental tissue (Thiede, 1960) and trophoblast (Valenti, 1965). A similar procedure was followed by the author for the monolayer cell culture of equine embryonic tissues.

All cell culture procedures require complete sterility and a high degree of cleanliness of apparatus and glassware. Details of the materials and solutions used are given in Appendices VII and VIII.

ii) Preparation of cell monolayer from tissue explants

Metaphase spreads of cells from solid tissues were obtained following cell culture by a plasma clot embedding technique (Hyman, 1968). This procedure was employed for tissue samples from aborted equine foetuses and living adult horses.

a) Preparation of tissue explants

Prior to sampling, the biopsy site was given a routine surgical preparation. A sample of subcutaneous fascia was excised from the neck of a standing horse with the aid of a local anaesthetic (Xylocaine 2%). This was infiltrated in the form of an L-block to exclude toxic effects of the local anaesthetic solution from the tissue sample. A similar procedure but without local anaesthetic was followed for the sampling of tissues from non-living specimens. Tissues selected for culture included muscle,

tendon, cartilage, liver, lung, mucous membrane, amnionic membrane, tunica albuginea and skin.

A tissue sample one cubic centimetre in size was collected into a 25 ml universal bottle containing ten ml of HBSS. Under a laminar flow cabinet the tissue was divided into fragments about 1 mm square. These were transferred to a universal container, ten ml of growth medium were added, and the culture placed in an incubator at 37°C.

After two days incubation the floating explants were replaced from the universal into 50 ml culture flasks. Fourteen explants, each to be embedded beneath a plasma clot, were equidistantly positioned on the floor of each flask. At least 6 cultures were prepared for each specimen.

b) Method for obtaining plasma

Fresh plasma was obtained from a normal donor or, when convenient, from the patient under investigation. Blood was collected aseptically into a sterile vacutainer containing three ml of Alsever's solution, centrifuged at 2000 r.p.m. for ten minutes, and the calcium-free plasma withdrawn into a sterile bijou bottle. Plasma was best used immediately but in some cases was stored at 4°C for as long as two days.

c) Explant attachment for primary culture

The equine plasma was reconstituted by mixing 0.2 ml of sterile calcium chloride solution (2% $CaCl_2$) with one ml of the prepared plasma solution. Clotting began after approximately five minutes. One drop of reconstituted plasma was superimposed over each explant and the excess removed with a Pasteur pipette, leaving the tissue fragment surrounded by a thin film of plasma. Firm clot formation

was enhanced by incubating the culture flasks for 15 minutes at 37°C before growth medium was added.

Explants in primary cultures remain attached to the flask while cellular growth radiates outwards (Figure II. 3). Fibroblast-like cells most commonly proliferate from explants obtained from almost any organ (Puck, Cieciura and Robinson, 1958).

Before the outgrowth of living cells commences, proteolytic digestion of the clot by enzymic action within the explant may result in the explant breaking loose from the clot (Hyman, 1968). The period of incubation with explants floating in growth medium appeared to reduce enzyme activity and the problem was overcome.

d) Development of cell monolayer

When adequate cell growth around the explants had developed, the primary culture was rinsed with two ml of phosphate buffered saline (PBS) and incubated at 37°C for five minutes with one ml of 0.05% trypsin solution (ATV). Trypsin acts on the interfibrillary substance (Day, 1949) and the plasma clot, dislodging both growing cells and explants.

After cell detachment was complete, the remaining trypsin was inactivated by the addition of one ml of foetal bovine serum to each culture flask. Growth medium was replenished and the culture incubated for six hours before the media containing cellular debris and floating explants was aspirated and replaced with fresh media. Living cells remain attached to the floor of the flask and multiply to form a monolayer.

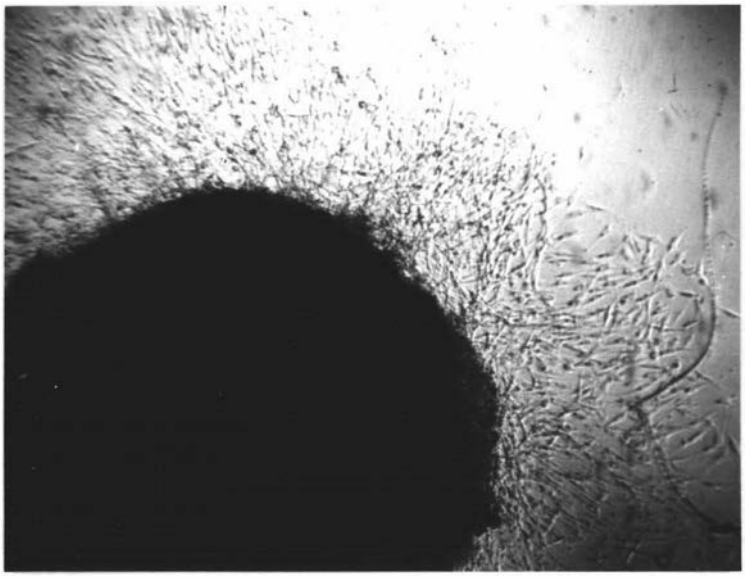


Figure II.3. Primary cell culture showing outgrowth of cells from a tissue explant. (Magnification 5x).

iii) Trypsin disaggregation technique for cell culture

a) Treatment of embryos

Equine embryos aged between 35 and 65 days of gestation were removed aseptically from the uteri of pregnant mares and transported separately to the laboratory in 500 ml screw-top jars containing a sterile transport medium (Appendix VIII a). The intact embryo was photographed and a measure of crown-rump length assessed with a flexible rule. Due to varying criteria of measurement, it was difficult to obtain a consistent standard for aging horse embryos. The method employed was comparable to the "curved crown-rump length" used by Joubert (1956), taken as "the greatest distance from the tip of the forehead along the dorsum to the tail head". The age of the embryo was then calculated from known standards (Bergin, Gier, Frey, and Marion, 1967; Evans and Sack, 1973).

Under sterile conditions the embryo was separated from its membranes, transferred to a petri dish containing two ml of an antibiotic solution (Appendix VIII c) and chopped as finely as possible with crossed scalpel blades. The resulting mass of cubes of edge less than two mm floating in antibiotic solution was transferred to a 125 ml flask and immersed in trypsin.

Problems of cellular contamination were considered to be minimal because equine embryos were obtained within their intact placental membranes before implantation was advanced. For cell culture only the embryo itself was used and the surrounding membranes were discarded.

b) Primary culture

Fifty ml of 0.25% trypsin solution were introduced to the flask and the mixture agitated with a magnetic stirrer. After 20 minutes agitation at 37°C the supernatant was filtered through a stainless steel mesh into several universal containers for centrifugation. Occasionally cell yield was insufficient at this stage and a further 50 ml of trypsin solution were added and the flask contents agitated again for 20 minutes. In some cases this process was repeated several times.

Following centrifugation the supernatant was removed and the cells resuspended in ten ml of foetal bovine serum, transferred to a graduated centrifuge tube and spun at 1500 r.p.m. for five minutes.

The cell concentration was estimated from packed cell volume in the graduated centrifuge tube. To inoculate primary cultures, cells were resuspended in foetal bovine serum to a concentration of 2% packed cell volume, and 0.5 ml of the resultant suspension introduced into each 50 ml culture flask. Each culture was fed with five ml of growth medium (Appendix VII b) and incubated at 37°C. A minimum number of 7 primary culture flasks were prepared from each specimen.

The lag period of growth following the explantation of a suspension of cells after treatment with trypsin (Levan and Biesele, 1958) was not evident in this study. Normal and abnormal cell populations apparently have a similar initial growth rate (Hyman, 1968) but the possibility that chromosome anomalies may be caused or concealed by effects of cell culture cannot be ignored.

iv) Replenishment of growth medium

Requirements of growth media and growth limiting factors have been well documented (Harris, 1955; Hyman, 1968). Details of the growth medium employed in this study are listed in Appendix VII.

Harris (1955) reported that growth rate is independent of serum concentration above a minimal concentration (10-15%). Hsu and Kellogg (1960) recommended 20% serum in growth media for primary cultures and an atmosphere of 5% carbon dioxide to prevent a rapid rise in pH. Although high levels of serum have been found to lead to increased lipid content of cells and acidification of media (Hanks, 1948), a concentration of 40% serum was found to be suitable for this study.

Five ml of growth medium was used to sustain cell growth in 50 ml culture flasks. To remove unattached cells and debris, the growth medium was renewed six hours after the inoculation of primary and secondary cell cultures. Otherwise the growth medium remained unchanged for as long as one week unless there was a marked change in pH as indicated by a colour change due to the phenol red contained in the growth medium.

Actively growing cultures of embryonic origin provide carbon dioxide from cell metabolism and adjustment of culture atmosphere with 5% CO_2 is unnecessary for such tissues. However, tricine buffer (Appendix VII. c) was included in the growth medium to stabilize pH.

Disturbances of pH can cause aneuploidy, as noted by Ford (1973), who also observed chromosome abnormalities in actively dividing human foetal fibroblasts cultured in an alkaline medium. Fluctuations in pH have been suggested

(Ford, 1973; Ingalls and Shimada, 1974) as a mechanism for inducing chromosomal errors in the zygote.

v) Assessment of cell growth

An inverted microscope was used to assess cell growth in culture flasks. A culture was subdivided when cell multiplication had formed a complete monolayer on the floor of the flask.

All cells adopt an elongated configuration when grown for sufficiently long periods in culture (Puck, Cieciura and Robinson, 1958). Fibroblasts in the process of mitotic division are characterized by a change in morphology from spindle-shaped to spherical. Thus the proportion of dividing cells can be estimated by microscopic examination to give an indication of the timing and effect of colchicine treatment (Figures II. 4 and II. 5). A culture was considered suitable for colchicine treatment to arrest mitotic division in metaphase when the cell monolayer covered approximately 50% of the available surface.

Figure II.4. Fibroblast cell culture which has formed a complete monolayer. (Magnification 5x).

Figure II.5. Monolayer cell culture after 16 hours exposure to colchicine. Note the large number of spherical cells which have been arrested in metaphase. (Magnification 5x).

vi) Preparation of cultures for subculture or harvest

Monolayer cell cultures growing in 50 ml flasks were either divided into secondary cultures, frozen for storage, or treated with colchicine and harvested. A standard technique was used to provide a cell suspension.

Growth medium was aspirated from the culture flask and the monolayer rinsed with two ml of PBS. Under the action of one ml of 0.05% trypsin solution (ATV), living fibroblasts became spherical and lost their attachment to the flask. Depending on cell type and the age of the culture, detachment was complete after five to 15 minutes at 37°C. Rapidly growing cells and those arrested in metaphase by colchicine were dislodged more easily than older cells in slow growing cultures. An inverted microscope was used to check that all cells were detached.

Susceptibility to trypsin varies according to cell type (Harris, 1955) but trypsin is very slow to damage live cells (Medawar, 1941; Moscona, 1952). Trypsin selectively digests dead cells due to changes in permeability (Rinaldini, 1954). Whether or not the trypsin treatment of cell cultures results in an increased frequency of chromosome breaks is a matter of dispute (Levan and Biesele, 1958; Hsu and Kellogg, 1960).

a) Subcultures

When cell detachment was complete, surplus trypsin was inactivated by the addition of one ml of foetal bovine serum (F.B.S.) to each culture flask.

Subcultures were prepared by inoculating half of the cell suspension in each culture (one ml) into a fresh flask. The original flask and new subculture were both fed with five ml of growth medium and incubated at 37°C. This process

could be repeated many times and a maximum of eight passages was used for one case (19B) in this study.

b) Harvest of fibroblast cultures

Colchicine was inoculated into each culture to a final concentration of 0.02 µg/ml and incubation continued for 16 hours before harvest. Each culture was treated with trypsin as described previously and transferred to a graduated centrifuge tube together with the growth medium and PBS washings from the same flask.

Subsequent harvesting procedures were identical to those used for leucocyte cultures.

vii) Storage of cells

Suspensions of cells were stored at -70°C. Embryonic cells not required for the inoculation of primary cell cultures were frozen after disaggregation and concentration. Primary and secondary cell cultures surplus to requirements were also concentrated and frozen at -70°C.

Cells destined for frozen storage were suspended in FBS to a packed cell concentration of 20%, to which was added 10% by volume of dimethyl sulphoxide. The suspension was dispensed in one ml aliquots into glass ampoules, wrapped in several layers of cotton wool, packed in a plastic bag and placed at -70°C for at least 12 hours. The glass ampoules were then unwrapped and stored in a plastic container.

When required to inoculate fresh cultures, an ampoule was thawed rapidly at 37°C, diluted with FBS and inoculated into culture flasks to a final concentration of 2% packed cell volume.

II. 5. SEX CHROMATIN STUDIES

i) Sex chromatin of nerve tissue

During the autopsies of three horses, sections of nerve tissue were obtained within one hour of death from the following regions:

(1) Cerebellum.

(2) Cervical region of spinal cord.

(3) Thoracic region of spinal cord.

(4) Lumbar region of spinal cord.

(5) Spinal cord from the region of the sacral vertebrae.

These histological sections were fixed in 4% formaldehyde for 24 hours before undergoing a routine embedding process prior to sectioning. Sections of thickness 7μ were transferred to slides and stained with cresyl echt violet (Coleman and Bell, 2%) for five seconds. The gray matter was examined microscopically for the presence of sex chromatin. Approximately 100 cells from each region of the spinal cord were scored for the presence or absence of a sex chromatin body. For each cell examined a sketch was drawn, showing the shape of the cell and the relative positions of the nucleus, nucleolus and sex chromatin.

ii) Sex chromatin of polymorphonuclear neutrophils

Peripheral blood smears from three horses were routinely prepared, air dried, and stained with MacNeal's stain. Slides were scanned under oil immersion (500x magnification) and neutrophils scored for the presence of nuclear appendages ("drumsticks").

II. 6. ANALYSIS OF RESULTS

i) Microscopy

All slides were scanned with the low power (10x) objective. Suitable metaphase spreads were selected for examination under the 20x objective. Vernier readings of the relative position of the microscope stage were recorded for each selected cell. The selection and number of metaphases analyzed are important factors in assessing the bias against the detection of possible mosaics. To avoid selection of nuclei, every metaphase selected under low power was counted under high power.

Chromosome spreads of satisfactory quality were studied under oil immersion at 500x or 1000x magnification. The chromosomes in each cell were counted and recorded, and any abnormalities noted. From each case a number of cells were chosen to be photographed and karyotyped. A similar procedure was adopted with the sex chromatin studies.

The number of cells counted and analyzed varied between cases. Hyman (1968) considered the analysis of 20 cells selected at random from at least two cultures to be sufficient except in the case of mosaicism. McCreanor (1974) judged the counting of 30 selected cells sufficient to exclude mosaicism. Mosaicism is suspected if a consistent abnormality is found in some but not all cells.

ii) Photographic techniques

Photographs were taken at 500x magnification with a green Balzers K-4 filter placed over the light source to reduce glare. The wide field (10x) eyepieces were exchanged for one ordinary eyepiece (10x) and one photographic eyepiece with a focussing frame.

A constant exposure time of one second was adhered to with Copex-pan rapid film 37 A.S.A. (Agfa-Gevaert). Each film was developed, and enlarged and printed on Ilfobrom (Ilford, Australia Proprietary Ltd.) Number 2 or 3 paper, depending on whether the negative was dark or light. (For further details refer to Appendix IX). For the printing of negatives two scales of enlargement were used: 6x and 9x magnification.

iii) Construction of karyotypes.

Ademco dry mounting tissue was fixed to the back of a print with a heated iron. Individual chromosomes were carefully cut out, paired, and arranged in groups on a ruled cardboard sheet. The loose chromosomes, together with a photograph of the entire cell, were pressed onto the cardboard sheet (70-80°C for 15 seconds) to complete the karyotype.

PART III

GESTATION AND ABORTION IN THE MARE

PART III

GESTATION AND ABORTION IN THE MARE

III. 1. LITERATURE REVIEW

i) Normal Gestation

The duration of pregnancy in the mare is influenced by many factors (Trum, 1950) and varies widely around a mean of 336 days (Bergin, 1970). Differentiation of the embryo and organogenesis are completed during the first 30 days of gestation.

Individual mares are most irregular with respect to oestrous behaviour and the timing of ovulation, but most mares ovulate about 24 hours before the end of behavioural oestrus (Hughes, Stabenfeldt and Evans, 1975). During each oestrous cycle many follicles recommence development but only a few ovulate while the remainder undergo atresia. After rupture of the Graafian follicle into the ovulation fossa (Witherspoon, 1975), the ovum is transferred to the fallopian tube by the fimbriated infundibulum. Transport of the ovum to the site of fertilization, the ampulla, is accomplished by cilial currents and muscular contractions under the control of sex hormones.

In most mammals the fertile life of the egg is probably about 24 hours, but deterioration commences after a few hours and may have important effects on subsequent development. Sperm remain viable in the female reproductive tract for considerably longer than the ovum and in most species sperm must undergo a period of capacitation in the female genital tract before they are capable of fertilization. Mating should therefore occur slightly before ovulation to ensure that

capacitated sperm are present in the fallopian tube when the ovum is liberated. However, the necessity for a period of sperm capacitation has not been established in the horse.

The entry of a sperm into the egg stimulates both structural and functional changes: most important are the initiation of development of the fertilized ovum and the induction of the polyspermy block. Successive cleavage divisions convert the fertilized ovum into a ball of cells, the morula, and accumulation of fluid within the morula leads to the formation of the blastocyst. The blastocyst consists of a hollow sphere containing an inner cell mass which gives rise to the embryo, and the peripheral trophoblast which forms the placenta and extra-embryonic membranes.

Tubal transport is again achieved by ciliary action and muscular peristalsis, and the early blastocyst reaches the uterine lumen usually between four and six days after ovulation (Oguri and Tsutsumi, 1972). In the mare, only fertilized eggs ever reach the uterus; unfertilized ova remain trapped at the isthmus of the fallopian tube for as long as seven months and may undergo some degree of parthenogenetic cleavage before degenerating (van Niekerk and Gerneke, 1966).

Like other mammalian embryos, the equine embryo has limited nutrient reserves and must rely on uterine milk for nourishment before implantation. The uptake of uterine milk is enhanced by specialized groups of cells on the trophoblast which form a transient attachment to the uterine epithelium after about 20 days of gestation (McLaren, 1972).

The equine placenta has been classified as diffuse, microcotyledonary and epitheliochorial (Steven and Samuel, 1975). Since there is no erosion of maternal or foetal layers

it is difficult to decide exactly when attachment of the embryo occurs. Zietchmann and Krölling (1955) considered that the embryo remained unattached in the uterine horn for up to five weeks, but van Niekerk (1965c) noticed the first signs of allantoic development 21 days after ovulation. Not until the eighth week do chorionic villi grow into the folds of uterine wall (Austin, 1969) and by the fourteenth week implantation is complete (McLaren, 1972).

As the primitive yolk-sac placenta is converted into a true allantochorionic placenta, growth of the allantois is accompanied by the formation of endometrial cups, first visible as a band around the circumference of the pregnant uterine horn after about 40 days of gestation (Clegg, Boda and Cole, 1954). Specialised chorionic girdle cells invade the endometrium on the 36th day (Moor, Allen and Hamilton, 1975) to produce the endometrial cups responsible for the secretion of pregnant mare's serum gonadotrophin (PMSG). The precise function of the endometrial cups has remained obscure, but they are the source of high levels of gonadotrophin in the mare's blood between 40 and 130 days of pregnancy (Allen, 1969). Use is made of this in the haemagglutination inhibition assay for pregnancy diagnosis. Recent studies have indicated that the primary corpus luteum does not regress completely until mid-gestation (Squires, Garcia and Ginther, 1974) and ovarian activity during pregnancy is most marked before day 40 (Bain, 1967b), thereby preceding the production of PMSG by the endometrial cups. Secretion of PMSG is halted as the mare's defence mechanisms recognize the cups as foreign tissue and mount an immunological rejection. The foetal genotype has a profound influence on PMSG production and the lifespan of the endometrial cups (Allen,

1969), and persistence of cups was prolonged for more than 200 days in an immunological tolerance situation (Spincemaille, Bouters, Vandeplassche and Bonte, 1975).

Endocrinology of pregnancy

The pattern of ovarian activity until mid-gestation is suggestive of surges of activity at ten or multiples of ten day intervals (Allen, 1974b), but from mid-gestation until term the mare's ovaries remain small and inactive, and ovariectomy from the fourth month onwards does not result in abortion (Lovell, Stabenfeldt, Hughes and Evans, 1975). Although the primary corpus luteum is not essential as a source of progesterone throughout pregnancy it remains active for at least 160 days (Squires and Ginther, 1975) and when fully functional it secretes up to ten times the amount of progesterone necessary to maintain pregnancy (Amoroso, 1955). Secondary follicles and corpora lutea develop in one or both ovaries and both primary and secondary corpora lutea continue to function until approximately 220 days of gestation (Squires and Ginther, 1975). Plasma progestagens after mid-gestation are assumed to be of placental or foetal origin (Squires et al., 1974).

The concentration of plasma progestagens exhibits two peaks: one during the third month of pregnancy which coincides with high levels of PMSG and is probably induced by the endometrial cups or secondary corpora lutea, and the second in the eleventh month which probably represents the secretion of placental progestagens (Ganjam, Kenney and Flickinger, 1975b). Plasma progesterone levels fall markedly after parturition but higher levels remain in the newborn foal (Lovell et al., 1975), suggesting that the foetal gonads are the source of progestagens in late pregnancy (Ganjam

et al., 1975b).

Nett, Holtan and Estergreen (1975) recorded plasma oestrogens present in low concentrations early in gestation, rising to a peak between 210 and 240 days, then gradually declining towards parturition. A similar pattern was observed for urinary oestrogens, with oestrone being the major compound excreted (Raeside and Liptrap, 1975).

ii) Causes of abortion in the mare

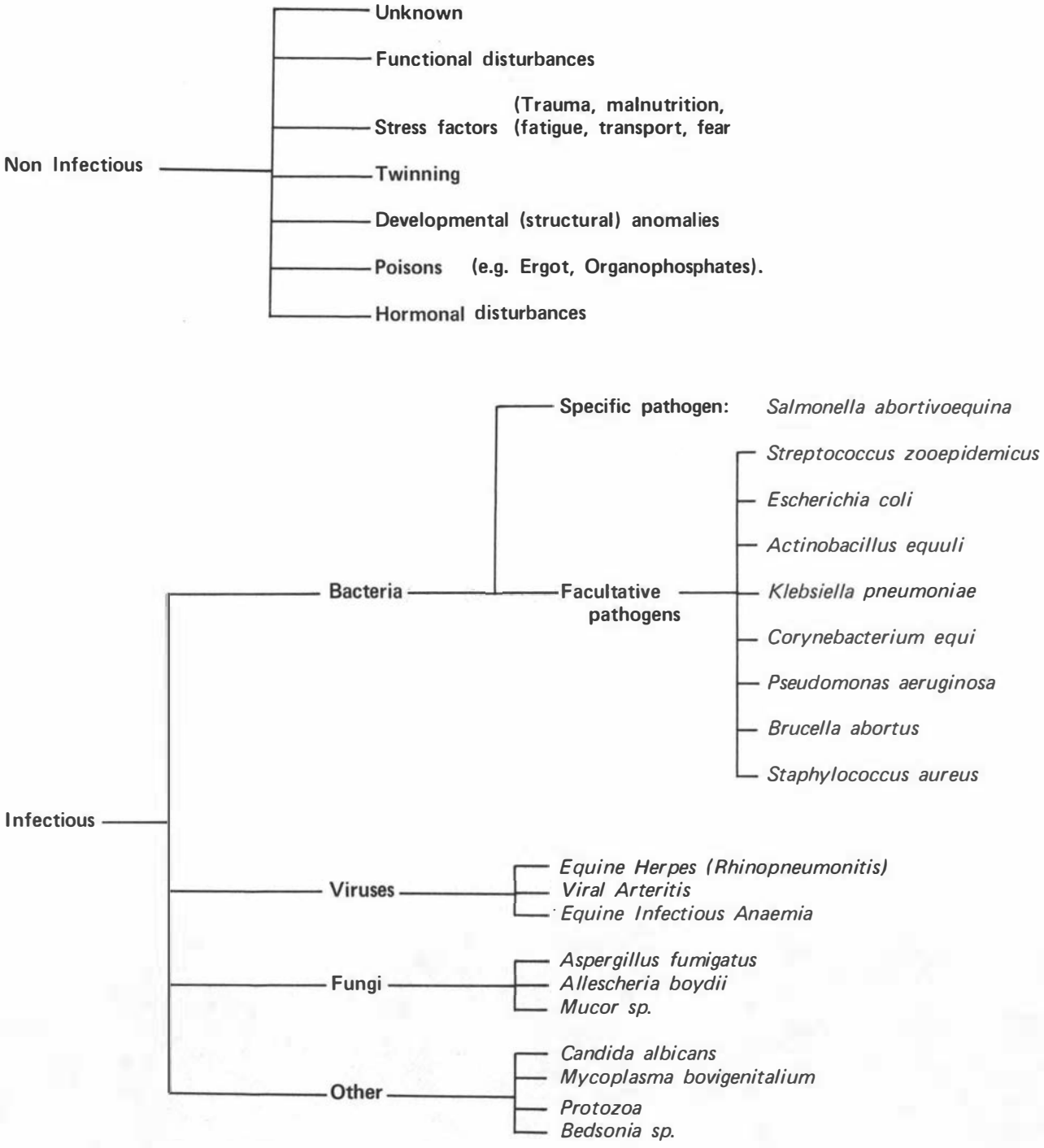
A list of the majority of the factors that have been associated with equine abortion is shown in Table III. 1. In many instances the relation between the designated cause and the abortion itself has not been defined. The substantiation of many causes, particularly functional and stress factors, has been difficult, and evidence has often been conflicting. The following are brief comments on some of the possible causes.

a) Functional factors

Orderly transport is vital in order that the blastocyst enters the uterus at the appropriate stage of development. Some of the embryonic mortality near implantation may have been due to functional factors such as faulty synchronization of the stages of development of the uterus and blastocyst (Platt, 1973a).

Platt (1973a) recorded that foals which were abnormal at birth constituted 68% of the total perinatal mortality. Indications of foetal anoxia were observed in some abortions attributed to premature labour while others were considered secondary to functional failures of the foetus and placenta.

TABLE III. 1. FACTORS ASSOCIATED WITH ABORTION IN THE MARE



b) Stress factors

Abortion in the mare has often been attributed to stress factors, namely fear (Mahaffey, 1968), trauma, fatigue, transport, and inadequate nutrition (Osborne, 1975), but information in this area is conflicting (Tesio, 1958). In the absence of significant lesions, diagnosis has usually relied on the case history.

c) Embryonic resorption

The expulsion of foetal products has occasionally been recognized during early stages of pregnancy but the death of an early conceptus has frequently been followed by its slow resorption back into the tissues of the mare. Detected abortions have, therefore, represented only a small proportion of the total prenatal loss in mares.

Van Niekirk (1965b) established the occurrence of embryonic resorption and demonstrated a correlation between resorption and poor nutrition.

d) Development anomalies of the conceptus

Anatomical defects of the foetus sufficiently severe to result in abortion have been recorded infrequently (Platt, 1973b). In New Zealand, such abnormalities have included, cephalocoele, cerebellar hypoplasia and arthrogryposis (O'Hara, 1976). Placental anomalies have been more numerous (Prickett, 1970), and Whitwell (1975) described seven pathological conditions involving the umbilical cord.

e) Hormonal disturbances

The use of progesterone to treat habitual abortion in women probably led to its use for a similar purpose in mares. Several workers have claimed success in preventing abortion by implanting progesterone pellets in mares but evidence supporting the efficacy of hormone therapy in equine

abortion has been largely circumstantial (Ganjam et al., 1975a).

Rossdale (1968) considered that the use of luteinizing hormone up to 120 days of gestation to promote the development of follicles and subsequent corpora lutea was more logical, while Nishikawa (1954) claimed success at reducing the abortion rate with implants of stilboestrol.

f) Twinning as a cause of equine abortion

Probably more than 2% of all pregnancies begin as twins (Vandeplassche, Podliachouk and Beaud, 1970) of which the vast majority (64.5%) are aborted, usually in the last three months of pregnancy (Jeffcott and Whitwell, 1973).

The thoroughbred mare seems incapable of increasing placental or uterine size sufficiently to nourish two foetuses and the villous chorionic area provided for twins is only slightly greater than for a singleton foal. Jeffcott and Whitwell (1973) found that the type of placentation has an important influence on the survival of twin foetuses. However, placental insufficiency has not explained fully the losses of twin pregnancies and the abortion of twins in the mare may have an immunological basis (Jeffcott and Whitwell, 1973).

g) Bacteria

Facultative pathogens

Uterine infection in susceptible mares has long been considered a major cause of impaired fertility (Collins, 1964; Brooksby, 1965) and foetal loss (Boyd, 1969). Considerable emphasis has been placed on bacterial culture results from cervical swabs (Lieux, Baker, DeGroot, Laskey, Raynor, Simpson, and Tobler, 1970) but Mahaffey (1968) noted that the presence of bacterial infection does not necessarily

preclude a successful gestation and the production of a normal foal.

Foetal death has been attributed to bacterial placentitis but the infection was often suspected to be secondary to some functional or anatomical disorder of the uterus (Dimock, Edwards and Bruner, 1947; Platt, 1973b), and the role of infection in causing embryonic loss in early pregnancy is unknown.

Group C streptococci (Streptococcus zooepidemicus, β haemolytic) are considered the most serious pathogens commonly found in the reproductive tract of the mare, and infection with this organism has been associated with infertility and abortion (Dimock and Edwards, 1936; Dimock, 1939; Moran and Cronin, 1957; Bain, 1966; Elliott et al., 1971). However, from a study of equine abortuses over two breeding seasons, Dimock (1939) found that those with no significant findings contributed by far the largest group. Most abortions caused by β haemolytic streptococci or other types of bacteria have occurred later than the seventh month of gestation (Bain, 1963).

Other bacteria incriminated in equine infertility and abortion are listed in Table III. 1. These have included Klebsiella pneumoniae, Escherichia coli, a common secondary invader, Actinobacillus equuli, Staphylococci, Corynebacteria (Bain, 1963; Bain, 1969; Platt, 1973b), and Pseudomonas aeruginosa (Hughes, Loy, Asbury and Bard, 1966).

Salmonella abortivoequina

Salmonella abortivoequina causes a specific infectious disease characterised by late abortions in mares, septicaemia in newborn foals, and testicular lesions in males. This organism has not been isolated from equine abortions in

New Zealand.

Mating at the foal heat

Much controversy exists concerning the wisdom of mating mares at the foal heat but it is often unavoidable due to the rigid limits of the imposed breeding season. Several authors have recorded low conception rates (Elliott et al., 1971) and increased numbers of abortions and malformed foals (Jennings, 1941, 1950), but evidence is conflicting.

h) Mycotic abortion

Three types of fungi have been implicated in equine abortion; namely Mucor sp., Aspergillus fumigatus and Allescheria boydii (Mahaffey, 1967). Infection, usually through the cervix (Platt, 1973b), has been followed by abortion at later than eight months of gestation (Mahaffey and Adam, 1964). Excessive or prolonged antibiotic therapy for chronic endometritis has predisposed to the establishment of fungi or yeasts, particularly Candida sp. (Zafracas, 1975), in the genital tract.

i) Viral abortion

Equine viral rhinopneumonitis (EVR)

Equine rhinopneumonitis virus, a member of the herpes group, has caused epizootics of abortion in horses in the U.S.A. (Dimock and Edwards, 1936) and on one stud farm in New Zealand (O'Hara, 1976). The distinctive syndrome was that of a mild respiratory tract infection frequently succeeded some months later by abortion. Abortion usually occurred between eight months and term and has not been recorded earlier than the fifth month of pregnancy (Doll and Bryans, 1963).

Viral arteritis

A severe disease characterised by acute infection of the upper respiratory tract and abortion in mares has been attributed to the arteritis virus, a viral agent provisionally classified as a Togavirus (Doll, Bryans, McCollum and Crowe, 1957). This disease has not been reported in New Zealand.

III. 2. RESULTS FROM ABORTED FOETUSES

In a preliminary study of equine abortions, 26 foetuses were examined over a 12 month period (Series B). Specimens ranged in gestational ages between five months and term and included five twin foetuses. Each abortion was subjected to a complete post-mortem examination, supplemented for the less autolyzed specimens with bacteriology (aerobic culture on blood agar) and histological examination of various tissues including placenta, lung, liver, kidney, spleen, brain, lymph node and adrenal. The possible diagnoses of the abortions, based on autopsy findings, are shown in Table III. 2.

Prior to the period of this study a further 18 aborted foals had been autopsied at Massey University between 1968 and 1973. The results for these specimens (Series A) are also included in Table III. 2. Details of the gestational ages of all abortuses are given in Figure III. 1. There appeared to be an increase in foetal deaths towards the end of pregnancy, partly accounted for by problems at parturition.

The presumptive cause of abortion was diagnosed in only 13 cases (30%). Six foetal deaths were attributed to twinning, two to dystocia, one to arthrogryposis, and four abortions were possibly caused by foetal infection.

With the exception of one seven month foetus showing advanced autolysis, explant cultures of subcutaneous fascia were prepared (Part II. 4. ii) from each abortus of Series B. Where long distances of transport were involved, a sample of subcutaneous fascia was obtained aseptically and carried in a universal bottle containing a sterile transport medium (Appendix VIIIa).

The cultures from three abortions, including one twin pregnancy, were discarded due to bacterial contamination. All other cultures were maintained for at least one week (average 12 days) with regular changes of medium and daily observation for evidence of cell growth. Minimal growth occurred in only one culture from a foetus aborted after 10½ months gestation, but died out before sufficient dividing cells were present for harvesting. Cell cultures from the remaining specimens failed to show any evidence of cell division.

TABLE III. 2. AUTOPSY DETAILS OF ABORTUSES.

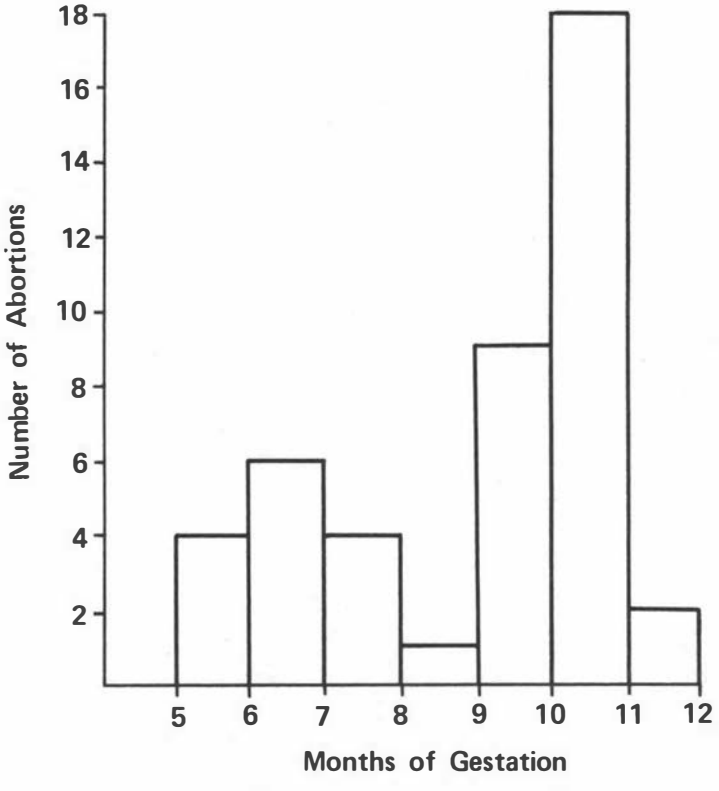
	Twinning	Developmental anomaly	Dystocia	Possible bacterial abortion	Possible viral abortion	Unknown	Total	% diagnosed
Series A	1	1 (Arthrogryposis)	2	3*	1†	10	18	?44
Series B	5					21	26	?19
TOTAL	6	1	2	3	1	31	44	?30

* Group C streptococci isolated from two specimens.

Bacterial placentitis detected histologically in third specimen.

† Possible EVR abortion (Macroscopic diagnosis).

FIGURE III. 1. GESTATIONAL AGES OF ABORTUSES



III. 3. DISCUSSION

In the majority of foetuses studied, the cause of death was not determined, and in others the observed pathological findings could not be related directly to the foetal death. As in other studies (Platt, 1973b), abortions of unknown causes constituted the largest group, and a "definite" diagnosis was achieved for only 9 abortions (20%), of which 6 were twin pregnancies. Twinning was responsible usually for late abortion in 14% of this series. In the light of pathological findings from other abortions, this may have been an overestimate, as not all twin pregnancies are aborted. These figures underline the dearth of knowledge of the aetiology of abortions in mares.

The mare's uterus provides an ideal environment for the rapid autolysis of a dead foetus. That foetuses were usually dead for some time before expulsion from the uterus was indicated by the lack of success in culturing foetal tissue. In the present study, the problem of foetal autolysis was exacerbated by transport difficulties. Advanced autolysis was especially evident in earlier abortions and for this reason Platt (1973b) concluded that "pathological examination of foetuses only becomes applicable in the middle and later stages of pregnancy."

Most embryos from spontaneous abortions in women have been dead for some time before abortion. During this time there has been breakdown of tissue and maceration, and abortuses have been expelled in various stages of maceration. In contrast to the mare, placental tissue often remains alive in the human uterus for weeks after the death of the embryo and is able to be grown in cell culture. Because of

the long period of uterine retention after foetal death in women, the age of the conceptus is estimated more accurately from its stage of development than from the apparent duration of pregnancy (Boué, Boué, Lazar and Gueguen, 1973). For the reasons outlined, it would appear that in the mare unless aborted material can be obtained soon after the death of the conceptus, there is little chance of chromosome studies proving very successful in this area.

PART IV

CYTOGENETIC STUDIES IN THE HORSE

PART IV

CYTOGENETIC STUDIES OF THE HORSE

1. THE KARYOTYPE OF THE HORSE

i) Literature review

a) Chromosomes of the horse: Equus caballus

Although cytogenetic techniques have been in use since 1916 when Bridges (1916) first correlated alterations in chromosome structure with phenotypical changes in Drosophila, technical difficulties hindered their application to mammalian tissues. That the in vitro techniques allowing visualization of chromosomes are a comparatively recent development is borne out by the fact that the human chromosome number was not finally established as 46 until as recently as 1956 (Tjio and Levan, 1956). The normal karyotypes of all the domesticated mammals have now been well established (Hsu and Benirschke, 1967).

As early as 1912, the haploid chromosome number of the horse was reported between ten and 16 (Kirillow, 1912). Wodsedalek (1914, 1916) placed the chromosome numbers of the horse and mule at 37 and 51 respectively, and from these figures he inferred a diploid number of 65 chromosomes for the donkey. Subsequent studies reported diploid chromosome numbers for the horse of 38 (Masui, 1919) and 60 (Painter, 1924; Ranquini, 1934), while a figure of 38 was proposed for the mule (Leon, 1938)(Table IV.1.).

From a study of spermatogenesis, Makino (1943) recorded a chromosome number of 66 for the horse. His complement included at least 12 chromosomes with submedian

centromeres with the X probably the largest element present and the Y the smallest. A similar study of the donkey and mule (Makino, 1955) yielded a diploid chromosome number of 66 for each species, and revealed a remarkable similarity in appearance between the chromosomes of the horse, donkey and mule.

The diploid chromosome number of the domestic horse was first correctly reported as 64 in 1958. The initial discovery by Rothfels, Axelrad, Siminovitch, McCulloch and Parker (1958) has since been confirmed by many other workers (Trujillo, Stenius, Christian and Ohno, 1962; Benirschkė, Brownhill and Beath, 1962; Sasaki and Makino, 1962; Bruère, Sutton and Davis, 1970; Marx, Melnyk, Persinger, Ohno, McGee, Kaufman, Pessin and Gillespie, 1973). Details of the various culture methods that have been used in confirming the chromosome number of the horse are listed in Table IV.2.

In a total of 64 chromosomes, Rothfels et al. (1958) found the female to possess 28 and the male 27 metacentric chromosomes, with the remainder acrocentrics. In the male, one member of the second longest pair of metacentric chromosomes, presumably the X, was replaced by a rod, although Benirschke et al. (1962) ranked the X chromosome third largest in size. It is generally agreed that the acrocentric Y is probably the smallest in the complement.

Only a few chromosomes could be identified by size and shape. Notable features included the remarkable length of the first autosomal pair of metacentric chromosomes (Sasaki and Makino, 1962), the short arms of which possessed delicate satellites, and the frequent occurrence of secondary constrictions of the long arms near the centromere on one pair

of small acrocentric chromosomes (Trujillo et al., 1962).

On the basis of chromosome size and position of the centromere, Trujillo, et al. (1962) and Benirschke et al. (1962) arranged the chromosomes of the horse into eight and four groups respectively. In contrast to the 13 pairs acknowledged by most authors (Hsu and Benirschke, 1967), Benirschke et al. (1962) recognized at least 14 pairs of metacentric chromosomes. To explain this discrepancy, Benirschke et al. (1962) submitted that differences in interpretation may have arisen due to varying degrees of colchicine-induced contraction and borderline resolution under the microscope.

Table IV.1. Establishment of the chromosome numbers of the horse, donkey and mule

Author	Date	Chromosome Number		
		Horse *E. caballus*	Donkey *E. asinus*	Mule*
Kirillow	1912	10 - 16		
Wodsedalek	1914, 1916	37	65	51
Masui	1919	38		
Painter	1924	60		
Ranquini	1934	60		
Leon	1938			38
Makino	1943	66		
Makino	1955		66	66
Rothfels *et al.*	1958	64		
Sasaki and Makino	1962	64		
Trujillo *et al.*	1962	64	62	63
Benirschke *et al.*	1962	64	62	63

* The mule is an equine hybrid produced by mating a male donkey with a mare.

TABLE IV. 2. CULTURE METHODS USED FOR OBTAINING HORSE KARYOTYPE

AUTHOR	TISSUE	CULTURE METHOD
Rothfels *et al.*, 1958	Kidney	Cell culture from tissue explants
Trujillo *et al.*, 1962	Blood leucocytes	Short term culture (< 72 hours)
Benirschke *et al.*, 1962	Blood leucocytes	Short term culture
Sasaki and Makino, 1962	Kidney and skin	Monolayer cell culture from tissue samples
Bruère, Sutton and Davis, 1970	Bone marrow	Direct examination
Marx *et al.*, 1973	Blood leucocytes	Short term culture

b) Chromosomes of the donkey. Equus asinus

A diploid chromosome number of 66 for the donkey was accepted until 1962 (White, 1961) when Trujillo et al. (1962) and Benirschke et al. (1962) established the correct values of 62 for the donkey and 63 for the mule and hinny. Both authors noted marked structural differences between species and ten more metacentric chromosomes in the donkey than the horse.

Trujillo et al. (1962) classified the chromosomes of donkey into six groups. He considered the X to be the third or fourth largest metacentric chromosome and the Y the smallest in the complement. Benirschke et al. (1962) recognized four groups with the X as the third largest metacentric and the Y the smallest acrocentric chromosome. No characteristic J-shaped elements (a pair of large metacentric chromosomes with subterminal centromeres) as described by Makino (1955) were found. However, Makino (1943, 1955) examined histological sections of testicular tissue whereas Benirschke et al. (1962) and Trujillo et al. (1962) studied metaphases from leucocyte cultures.

ii) Results and Discussion

Materials and methods have been detailed previously (Part II). The terminology and nomenclature used in this study were as defined by the Geneva Conference (1966) on the standardization of procedures for chromosome studies in abortion.

a) Chromosome number

Many variables have yet to be defined concerning the chromosome number and the morphology of individual chromo-

somes in the horse. Particularly in cases of suspected mosaicism it is important to know the expected distribution of chromosome counts from normal horses.

The chromosome counts of metaphase cells from leucocyte and fibroblast cultures from normal horses and horse embryos are summarized in Table IV.3; details of individual cases are given in Appendices X and XI. The modal number of 64 chromosomes was counted in 76% of 853 leucocytes, in 78% of 2693 mitotic figures from monolayer fibroblast cell cultures, and in 77% of all 3546 cells examined. By excluding specimens with fewer than 30 counted cells the proportion of modal cells becomes 75% for leucocyte cultures, 79% for fibroblast cultures, and 78% of 3125 cells for the combined total. (Table IV.4). These specimens involved a much smaller number of animals, but the proportion of aneuploid cells was unchanged.

From human bone marrow preparations, Court-Brown, Jacobs and Doll (1960) showed the distribution of cell counts negatively skewed around a modal number of 46, with 12% of hypomodal cells. A higher proportion (94%) of modal cells was obtained from human leucocyte cultures (Court-Brown, Harnden, Jacobs, MacLean and Mantle, 1964). When cells with fewer then 63 chromosomes were excluded, the modal number of 64 chromosomes was counted in 82% of horse leucocytes. Considering the higher chromosome number of the horse, these figures are comparable to those obtained from leucocyte cultures in humans.

Considerable variation is evident in the chromosome counts from cultured cells, a large proportion probably due to chromosome loss from cells broken during their preparation. Technical factors during slide preparation and difficulties

in counting individual metaphases were responsible for the majority of aneuploid cells, particularly in earlier stages of this study when selection of metaphases to be counted was less critical. This applied especially to chromosome counts from cell cultures of tissue explants.

However, cell breakage is not the only explanation for the presence of aneuploid cells. Jacobs, Court-Brown and Doll (1961) found that aneuploid cells were more numerous among the leucocytes of older humans and there was shown to be a sex difference associated with the increasing frequency of aneuploidy with advancing age (Jacobs, Brunton, Court-Brown, Doll and Goldstein, 1963). In addition, it appeared that the Y chromosome was preferentially lost from leucocytes in older men while in women it was the X chromosome that was involved. Thus cells lacking a sex chromosome may occur in leucocyte cultures from normal females, and the possibility of finding such cells increases with age. These findings raised problems with regard to the diagnosis of sex chromosome disorders, particularly where mosaicism was involved.

Using a cell culture method involving the growth of tissue explants, Kerr and Rashad (1966) recorded 21% of aneuploid cells from chromosomally normal human abortions. Metaphases examined after cell growth from explants of subcutaneous fascia from horses had 32% of aneuploid cells but the number of mitoses was small (301, Appendix XI). Among seven chromosomally normal rabbit blastocysts (2n = 44), Austin (1967) found hypodiploid counts (38 - 43) in 26% of cells, while the hypodiploid counts from horse embryos in this study accounted for only 16% of cells. When all metaphases counted from horse fibroblast cultures are included,

the quality of mitotic figures examined compares favourably with similar studies in other species (Table IV.5).

Polyploid cells

The fusion of two or more diploid cells results in the formation of a polyploid cell. More than one spindle may form during subsequent mitoses and depending on the behaviour of the chromosomes and spindles, a wide variety of heteroploid states may be found among daughter cells. The majority of polyploid cells for which an accurate count was made were tetraploid (Figure IV.1) with a lesser number of triploid cells. Triploid cells may arise by the malsegregation of one haploid set of chromosomes from a diploid cell line or by tripolar division of tetraploid cells which have been found in 0.5% of human diploid fibroblast cultures (Citoler, Pera and Schwarzacher, 1969).

TABLE IV. 3. DISTRIBUTION OF CHROMOSOME COUNTS FROM HORSES WITH NORMAL KARYOTYPES

Cell type	Number of animals	DISTRIBUTION OF CELL COUNTS						Cells counted
		< 63	63	64	65	> 65	Polyploid	
Leucocytes	23	69 (8%)	85 (10%)	644 (76%)	37 (4%)	7 (1%)	11 (1%)	853
Fibroblasts	45	224 (8%)	223 (8%)	2096 (78%)	92 (3.5%)	14 (0.5%)	44 (2%)	2693
TOTALS	68	293 (8%)	308 (9.5%)	2740 (77%)	129 (3.5%)	21 (0.5%)	55 (1.5%)	3546

TABLE IV. 4. DISTRIBUTION OF CHROMOSOME COUNTS FROM HORSES WITH NORMAL KARYOTYPES AND AT LEAST 30 CELLS COUNTED

Cell type	Number of animals	DISTRIBUTION OF CELL COUNTS						Cells counted
		< 63	63	64	65	> 65	Polyploid	
Leucocytes	*8	57 (9%)	63 (10%)	477 (75%)	21 (3%)	7 (1%)	11 (2%)	636
Fibroblasts	†23	205 (8%)	199 (8%)	1963 (79%)	69 (2.5%)	11 (0.5%)	42 (2%)	2489
TOTALS	32	262 (8.5%)	262 (8.5%)	2440 (78%)	90 (3%)	18 (0.5%)	53 (1.5%)	3125

* Individual cases are shown in Appendix XII.

† Individual cases are shown in Appendix XIII.

TABLE IV. 5. SPECIES COMPARISON OF ANEUPLOIDY FROM EMBRYO CELLS COUNTS

AUTHOR	SPECIES	TISSUE SOURCE	CULTURE METHOD	CELLS COUNTED	ANEUPLOID CELLS	% ANEUPLOIDY
Kerr and Rashad, 1966	Human	Chromosomally normal induced abortions	Monolayer cultures from tissue explants	560	96	17
	Human	Chromosomally normal spontaneous abortions		859	197	23
TOTAL				1419	293	21
Austin, 1967	Rabbit	7 blastocysts	"Modified blood leucocyte technique"	8–36 per blastocyst		26 (hypodiploid)
Present study	Horse	Subcutaneous fascia	Tissue explants	301	97	32
	Horse	Embryos	Trypsinized monolayer	2392	456	19*
TOTAL				2693	553	20.5

* 16% hypodiploid

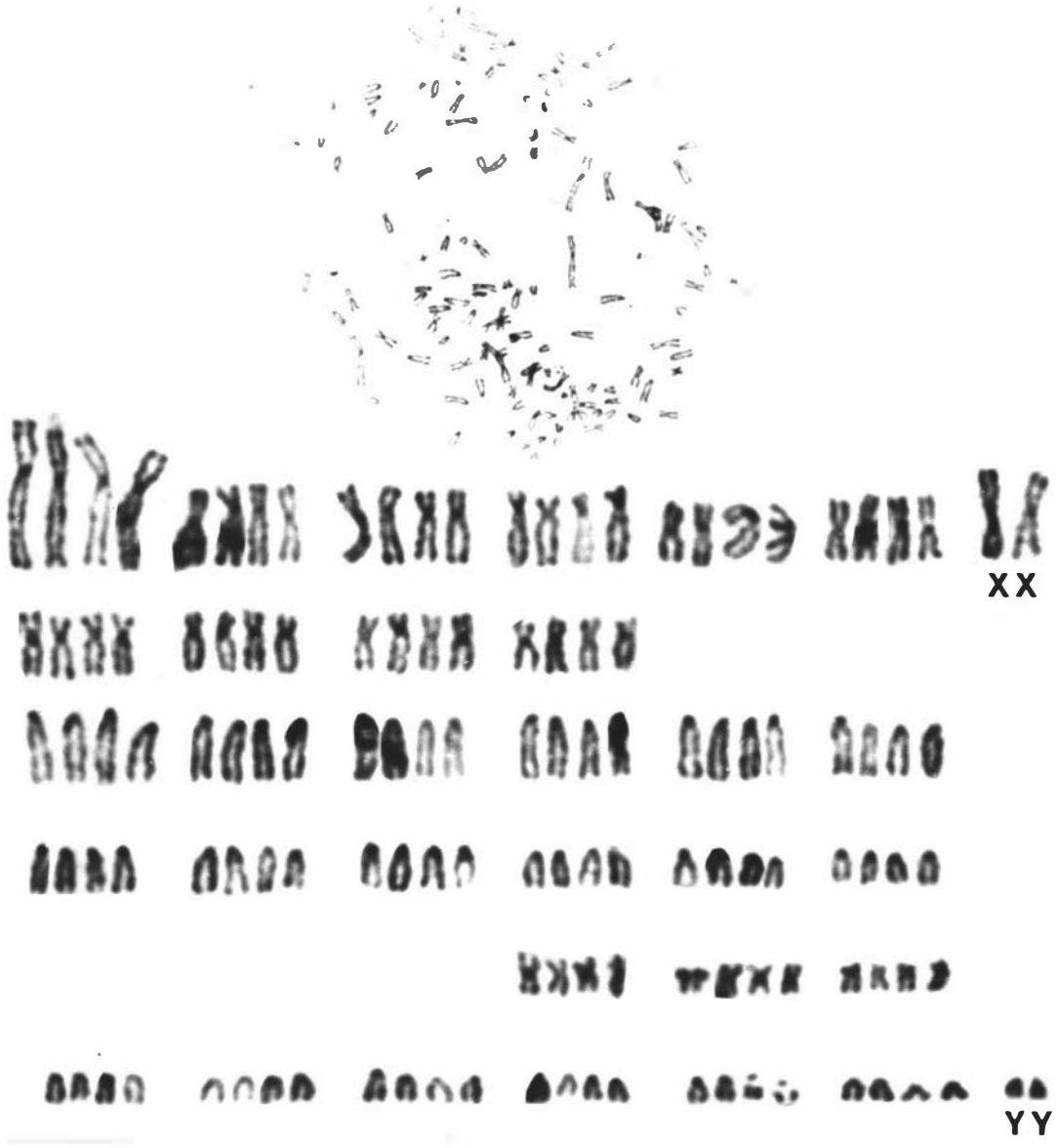


Figure IV.1. Karyotype of a tetraploid cell in a cell culture from an equine embryo. (Magnification 4500x).

b) Chromosome morphology

For the construction of the normal photographic karyotype the most useful morphological features of chromosomes are their length and centromeric position. Nomenclature for the classification of chromosomes on the basis of arm ratios and the position of the centromere was detailed by Levan, Fredga and Sandberg (1964). They suggested the classification of chromosomes into six categories depending on centromeric position. However, the identification of individual chromosomes by the estimation of their relative lengths and arm ratios is of limited value as size is affected by the degree of colchicine-induced contraction. A metacentric chromosome has its centromere at or near the median position; a chromosome is described as acrocentric when the centromere is subterminal in position, and submetacentric when it is positioned between these extremes.

The centromere, where the spindle fibre attaches to the chromosome, is often referred to as the primary constriction. An additional constricted region of the chromatid not connected with the spindle fibre constitutes a secondary constriction which may appear as an achromatic area of the chromatid (Aula, 1965). A secondary constriction near the end of a chromosome arm produces a satellite stalk. Satellite size on a particular chromosome may be a characteristic of the individual's karyotype (Ferguson-Smith and Handmaker, 1961) and in human cells satellites appear to be involved in the formation of the nucleolus. The 64 chromosomes of the normal karyotype of the domestic horse consist of 31 homologous pairs of autosomes and two sex chromosomes.

For the purposes of this study, the chromosomes of

the horse were arranged into six rows to form the routine karyotype (Figures IV.2 and 3).

Row A. This group of large metacentric chromosomes consists of 14 chromosomes in the female and 13 in the male. Pair 1 is easily distinguished by its large size but there are only slight differences in length between the other members of this group. The centromere is located submedially on the first, second and fifth autosomes, subterminally on the third and fourth, and medially on the sixth autosome.

On morphological grounds the X chromosome is also included in this row. It is the second largest chromosome in the complement with a centromere in the submedial position.

Row B. Included in Row B are four pairs of large metacentric chromosomes. Although very similar in size, they can be distinguished by the position of the centromere. Pair 7 has submedially-located centromeres, on Pair 8 the centromeres are submedial, and the remaining two pairs have subterminal centromeres.

Row C. Six pairs of large acrocentric chromosomes differing only slightly in length.

Row D. A further six pairs of acrocentric chromosomes progressively decreasing in size.

Row E. Three pairs of small metacentric chromosomes differing in size and centromeric position. The largest pair has medially-placed centromeres, in the second they are submedial, and in the third, subterminal.

Row F. Six pairs of small acrocentric chromosomes of similar appearance. Two pairs of chromosomes in this group possess characteristic paracentric secondary constrictions (Figure IV.4).

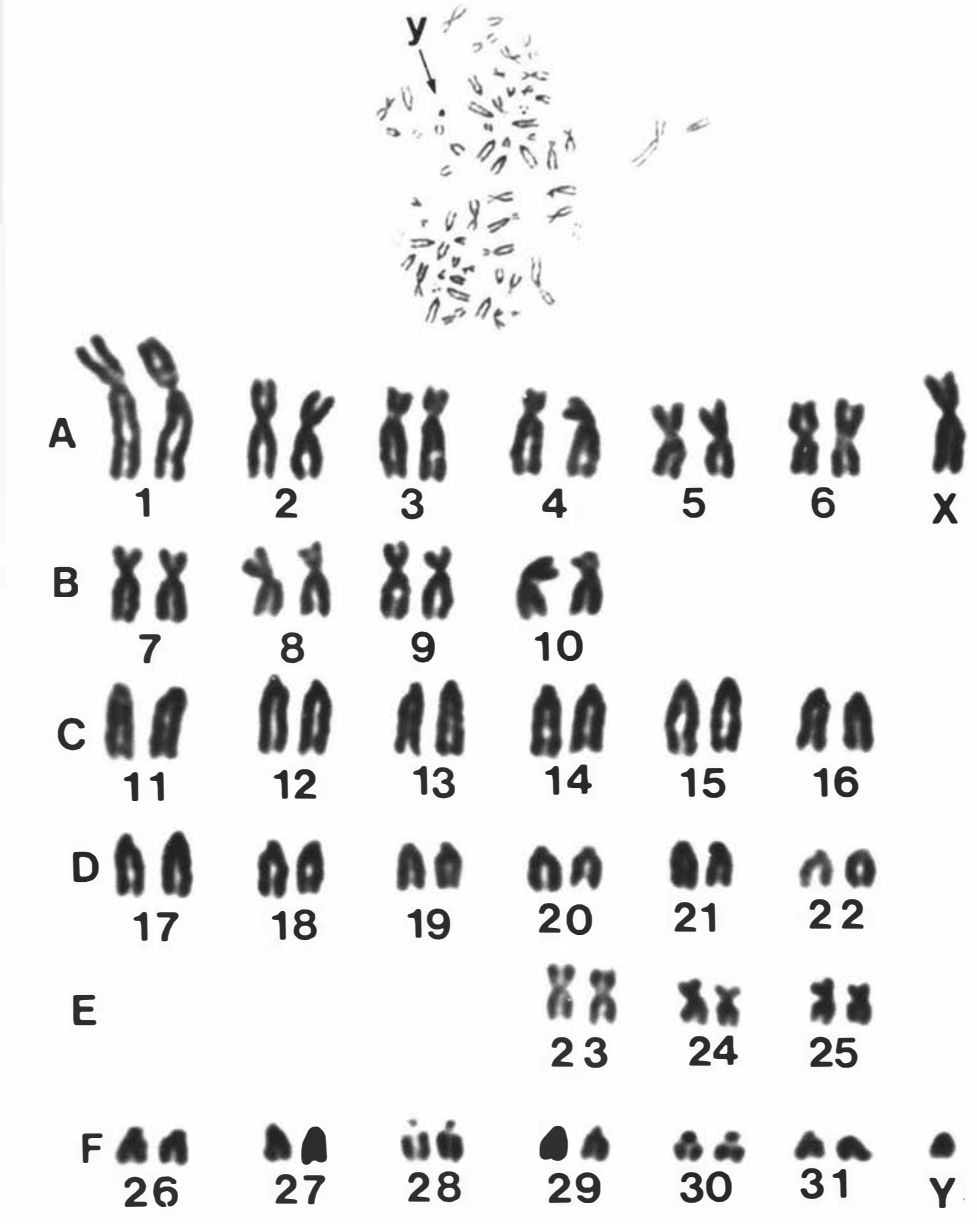


Figure IV.2. Karyotype of a normal stallion, Equus caballus. (2n = 64). (Magnification 4500x).

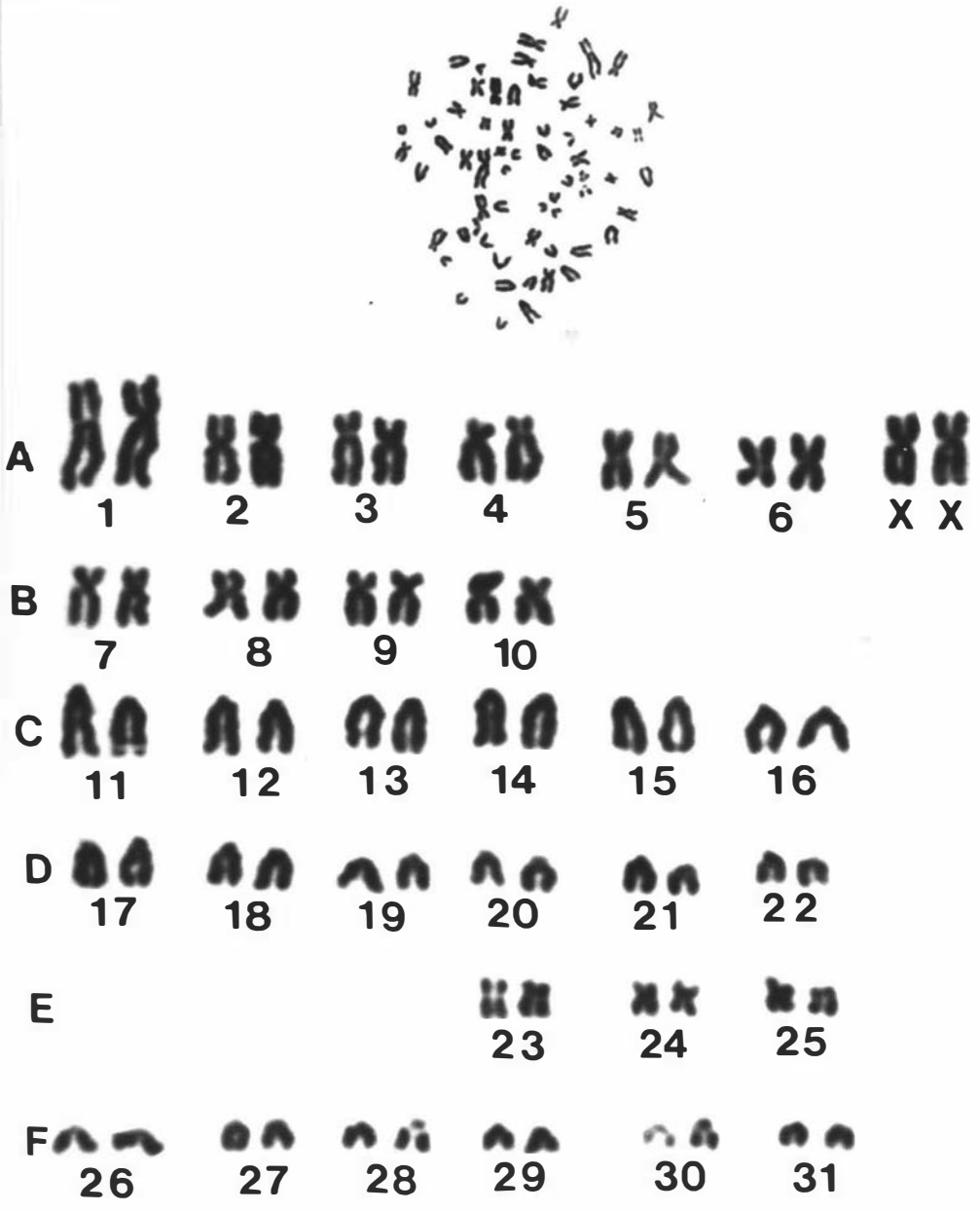


Figure IV.3. Karyotype of a normal mare, Equus caballus. (2n = 64). (Magnification 4500x).

In the male the Y chromosome, which is the smallest acrocentric in the complement, is included in Row F.

Sex chromosomes. The X chromosome, a large submetacentric included at the end of Row A, ranks second in size among the chromosomes of the horse. The Y chromosome, as stated above, is the smallest.

With the exception of the first autosomal pair, the identification of individual chromosomes was found to be very difficult. While it was possible to recognize the metacentrics in most Giemsa-stained preparations, individual acrocentric autosomes could not be positively identified without the aid of banding techniques. Other morphological features including secondary constrictions facilitate the recognition of chromosome pairs and new techniques such as C-banding have further increased the certainty with which chromosomes of the horse can be identified.

Secondary constrictions are constant in position and are useful markers for the identification of individual chromosomes in humans (Ferguson-Smith, Ellis and Dickson, 1962). Other workers have commented on the occurrence of secondary constrictions on one pair of small acrocentric chromosomes in the karyotype of the horse (Trujillo et al., 1962; Benirschke et al., 1962). In the present study, prominent secondary constrictions were consistently found on two pairs of small (Row F) acrocentric autosomes (Figure IV.4). These constrictions were positioned adjacent to the centromere on each chromosome, but were markedly different in appearance between pairs and enabled definite identification of each of these autosomal pairs. Secondary constrictions were sometimes found on other chromosomes (Figure IV.5) but were not a constant feature of use in chromosome identification.

Figure IV.4. Karyotype of a normal stallion showing characteristic secondary constrictions on acrocentric chromosomes (indicated by arrows). (Magnification 4500x).

Figure IV.5. Presumed secondary constrictions on the short arms of a large metacentric chromosome. (Magnification 4500x).

Occasional cells contained structurally abnormal chromosomes. From a fibroblast culture of subcutaneous fascia, the three cells able to be analyzed each had a presumptive chromosomal translocation involving a large metacentric chromosome (Figure IV.6). Subsequent fibroblast cultures from this mare revealed a normal female karyotype and the anomaly was considered to have arisen during cell culture, as described by Ford (1973) in cultures of human foetal fibroblasts.

One metaphase from a cell culture of an equine embryo showed an abnormal chromosome arrangement (Figure IV.7). After karyotyping, this was considered to represent an association between two homologous metacentric chromosomes, the largest pair of Row E (Pair 23). That this was observed in only one of 207 cells examined probably indicates that it arose as a mitotic error with premature separation of the chromatids.

The cytogenetical significance of secondary constrictions, and these sporadic structural aberrations remains in doubt. Ferguson-Smith and Handmaker (1961) described satellite associations in 60% of mitoses from human leucocytes and they and others (Ferrier, Ferrier, Stalder, Bühler, Bamatter and Klein, 1964; Miller and Mukherjee, 1962) indicated their possible role in the aetiology of chromosome aberrations. However, Nielsen, Friedrich and Hreidarsson (1974) found that certain families lacking short-arm satellite material from an acrocentric chromosome showed the same frequencies of abortion and nondisjunction as the general population.

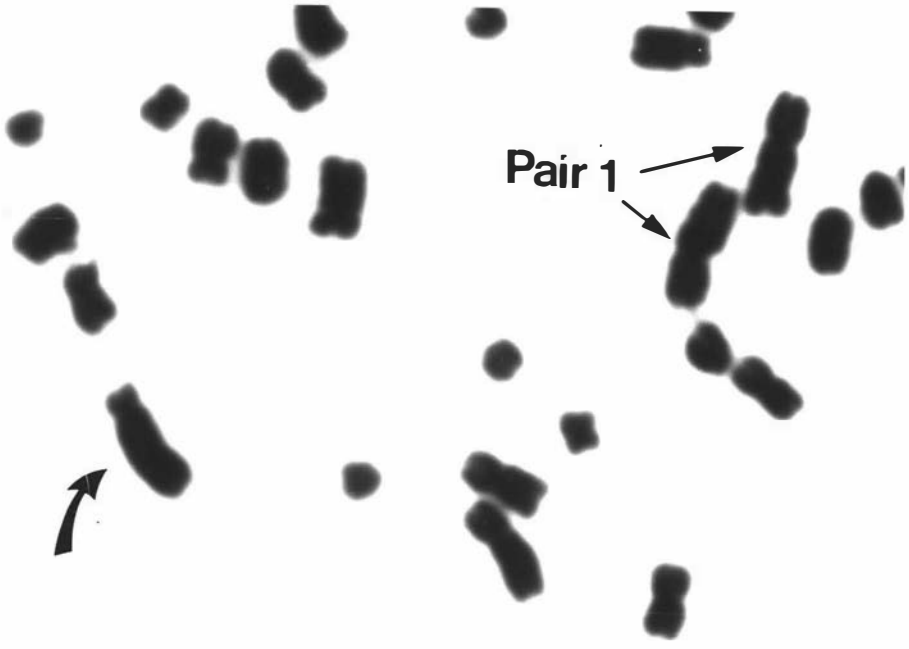


Figure IV.6. Presumptive chromosomal translocation involving a large metacentric chromosome from a normal mare (2n = 64). Note the size of the abnormal chromosome in comparison with Pair 1. (Magnification 6000x).

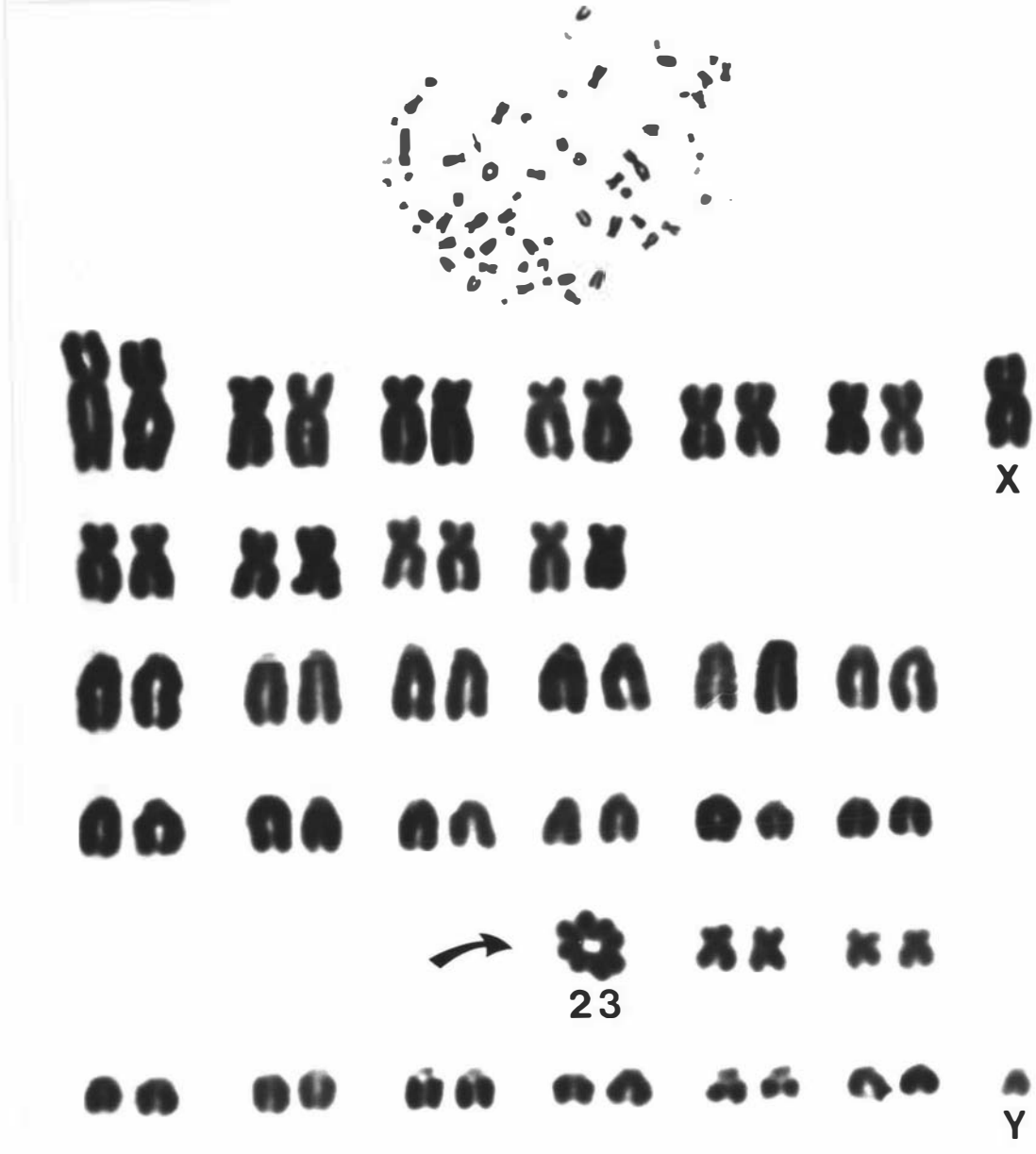


Figure IV.7. Karyotype of a cell from an equine embryo showing presumptive premature chromatid separation and association of two homologous metacentric chromosomes. (Magnification 6000x).

c) Centromere staining of horse chromosomes

C-banding of the karyotype of the horse has not been previously reported. The technique of centromere staining reveals additional morphological features which facilitate the identification of individual chromosomes. A characteristic C-banded karyotype of the horse is displayed in Figure IV.8.

This procedure is particularly valuable in the horse for the identification of the sex chromosomes. Characteristic additional C-bands are found as reliable markers on the long arms of the X chromosome while the Y chromosome appears as an intensely stained block (Figure IV.9).

A further feature is the lack of centromere staining on the first pair of metacentric chromosomes in Row E. The fact that this was the chromosome pair involved in the structural abnormality mentioned previously (Figure IV.7) suggests that this centromere may have a different structure or function from the rest.

Chromosome pairs of the horse karyotype rarely showed dimorphism after C-banding, and the size of the C-band on homologous chromosomes was usually very similar. However, one acrocentric autosome of Row D frequently stained more intensely than the other members of the same group (Figure IV.8). Some chromosome pairs, particularly Pair 3 and the X chromosome, featured exceptionally large C-bands and appeared to be almost dicentric.

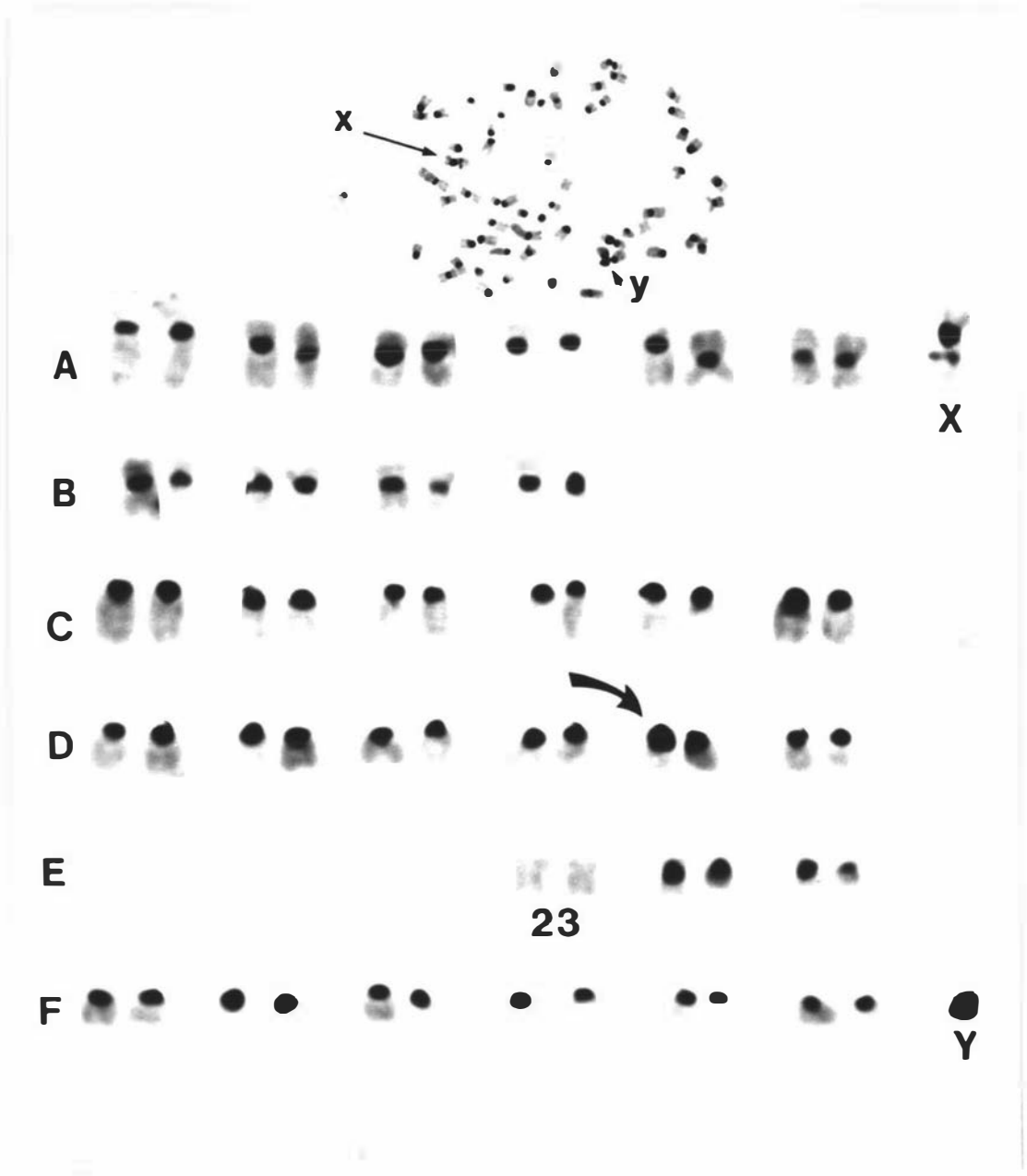


Figure IV.8. Karyotype from a normal stallion after centromere staining. The arrow indicates dimorphism shown by one pair of acrocentric chromosomes. (Magnification 5500x).

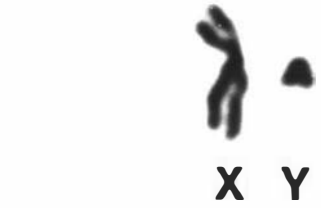


Figure IV.9. Characteristic appearance of the sex chromosomes of the horse after centromere staining. Giemsa-stained sex chromosomes are shown, as well as male and female sex chromosomes showing typical C-bands. (Magnification 5500x).

2. CYTOGENETIC STUDIES OF INFERTILE HORSES

i) Literature review

a) Sterility of equine hybrids

Little is known of the mechanisms responsible for hybrid sterility in mammals (White, 1954). Makino (1955) and Trujillo *et al.* (1962) compared chromosome differences between the horse, donkey and mule with those in other sterile hybrids. They found that the degree of infertility was related to the extent of chromosome disparity between the parent species. Hybrid sterility was attributed to structural dissimilarities between parental chromosomes.

The diploid chromosome number of the mule and the hinny has been conclusively established at 63 (Benirschke *et al.*, 1962; Trujillo *et al.*, 1962), intermediate between that of their parents; a male donkey (jack) and mare in the case of the mule, a stallion and female donkey (jenny) for the hinny. Similarly, the donkey/Grevy zebra hybrid (Benirschke, Low, Brownhill, Caday and Venecia-Fernandez, 1964) and Przewalski horse/domestic horse hybrid (Short, Chandley, Jones and Allen, 1974) possess a chromosome number intermediate between those of their parents.

Despite the well known sterility of the mule, numerous "fertile" mules have been described (Smith, 1939; Anderson, 1939). Reports of apparent fertility in mules relied on breeding history and phenotype to confirm that the animals were in fact hybrids. Craft (1938) disproved accounts of 11 allegedly pregnant mules and one pregnant hinny, and revealed the necessity for such claims to be supported by chromosome studies. More recently, Benirschke, Low, Sullivan

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and Carter (1964) found by chromosome analysis that a female mule described as fertile was an ordinary donkey. Discussing the role of the mule in research, Short (1975) wrote "in the absence of scientific justification and until there is conclusive proof to the contrary, it must be assumed that all mules and hinnies are completely sterile."

In the female, a germ cell must enter meiosis before it is capable of inducing the development of a normal Graafian follicle which is the endocrine apparatus of the ovary. Female mules have been known to demonstrate oestrous cycles and to produce normal follicles and corpora lutea (Benirschke and Sullivan, 1966), but follicles containing germ cells were virtually absent from the mature ovary. Taylor and Short (1973) found that although a few oocytes may survive until ovulation, oocytes in the ovaries of the female mule and hinny were severely depleted due to partial meiotic arrest.

No substantiated reports of fertile mules or hinnies occur in the literature but the ejaculate recovered from males may contain mature spermatozoa (Short, 1975), and spermatogenesis has been reported in a male hinny (Trujillo, Ohno, Jardine and Atkins, 1969). This hybrid was verified chromosomally as a male hinny but was considered to be probably sterile on the basis of mating trials and the absence of significant numbers of motile spermatozoa. Spermatozoa produced by such hybrids were found to be significantly smaller than those of a normal horse or donkey (Chandley, Short and Allen, 1975) and were probably incapable of fertilization.

From a study of spermatogenesis, Wodsedalek (1916) proposed that azoospermia of the mule was the result of

disorderly pairing of chromosomes in meiosis, accompanied by cell death. Histological examination of testes from adult mules revealed a lack of spermatozoa, atrophy of the seminiferous tubules, and the presence of very few germ cells (Anderson, 1939; Makino, 1955). Chandley et al. (1975) concluded that spermatogenesis in the mule and hinny was almost totally arrested due to difficulties of chromosome pairing, but a few mature spermatozoa were recovered from the ejaculate and epididymal flushings of a male hinny.

Unlike all other interspecies equine hybrids in which meiosis is arrested in prophase, male and female Przewalski horse/domestic horse hybrids are fertile (Short et al., 1974). Close homology between the karyotypes of the Przewalski horse (Equus przewalski, 2n = 66) and the domestic horse has been demonstrated by chromosome banding (Short et al., 1974). The only difference between the karyotypes of the two species is a single translocation of the centric fusion type, and the chromosomes are sufficiently alike to allow unimpaired pairing in meiosis.

b) The chromosomes of intersex horses

Disparities between the sex chromosomes and gonad morphology are known in several species of domestic animals (McFeely, Hare and Biggers, 1967; Bishop, 1972). Recent cytogenetic studies in the horse have revealed an association between intersex states and abnormalities of the sex chromosomes.

Bornstein (1967) found a normal female karyotype in two intersex horses; Gerneke and Coubrough (1970) described an intersex horse which was a genetic female; and Dunn, Vaughan and McEntee (1974) reported a bilaterally cryptorchid

stallion with a female karyotype. In the latter case the possibility of 64XX/64XY/65XXY was also suggested but the Y chromosome could not be positively identified. Male pseudohermaphrodites with an XX sex chromosome constitution in domestic animals are best known in the goat (Biggers and McFeely, 1966; Hamerton, Dickson, Pollard, Grieves and Short, 1969) and pig (Biggers and McFeely, 1966), but have also been found in the dog (Edols and Allan, 1968; Hare, McFeely and Kelly, 1974) and in man (Yunis, de De la Cruz, Nossa and Gutierrez, 1975).

Basrur, Kanagawa and Gilman (1969) reported a unilateral cryptorchid intersex horse with a mosaic karyotype, XX/XY/XXY/XO. With further study (Basrur, Kanagawa and Podliachouk, 1970) this was considered to be the result of either double fertilization or the fusion of two blastocysts.

A syndrome analogous to Klinefelter's syndrome in man (Klinefelter, Reifenstein and Albright, 1942) is well known in the mouse (Russell and Chu, 1961; Cattanach, 1961) and a similar chromosomal anomaly or its mosaic variants has been described in the cat (Thuline and Norby, 1961; Frota-Pessoa, 1962; Chu, Thuline and Norby, 1964; Loughman, Frye and Condon, 1970), dog (Clough, Pyle, Hare, Kelly and Patterson, 1970), sheep (Bruère, Marshall and Ward, 1969; Bruère and Kilgour, 1974), pig (Breeuwsma, 1968; Harvey, 1968) and ox (Rieck, Höhn and Herzog, 1969).

The equine equivalent of Klinefelter's syndrome was discovered in a sterile intersex horse with chromosome constitution 66XXXY.(Gluhovschii, Bistriceanu, Suiciu and Bratu, 1970). A similar condition was described in an intersex horse exhibiting strong male behaviour but with a female

appearance and ambiguous external genitalia (Bouters, Vandeplassche and de Moor, 1972). No uterus was present and undifferentiated seminiferous tubules contained only supporting cells. Although chromosome analysis detected a predominantly female karyotype, the animal was considered to be a 64XX/65XXY mosaic.

c) Sex chromosome anomalies in infertile mares

Cytogenetic studies of infertile horses or those with abnormalities of the reproductive system have been very limited in number. Payne et al. (1968) reported aneuploidy in an infertile mare with normal phenotype. In a complement of 63 chromosomes, no normal X chromosomes could be identified. The fact that sex chromatin was absent from vaginal cells suggested the lack of a sex chromosome but a normal level of erythrocyte glucose-6-phosphate dehydrogenase (G-6-P D), which is sex-linked in the horse (Trujillo, Walden, O'Neil and Anstall, 1965; Mathai, Ohno and Beutler, 1966), indicated that at least part of one X chromosome was present. Although the possibility of mosaicism could not be excluded, the author postulated structural rearrangement of the X during meiosis in the dam and fertilization of the abnormal egg produced by a sperm lacking a sex chromosome.

Three contemporary studies (Chandley, Fletcher, Rossdale, Peace, Ricketts, McEnery, Thorne, Short and Allen, 1975; Hughes, Kennedy and Benirschke, 1975a; Hughes, Benirschke, Kennedy and Trommershausen-Smith, 1975b) have demonstrated an association between sex chromosome aneuploidy and infertility in mares. The clinical details of these mares are recorded in Table IV.6. In each case the phenotypically normal mares were referred because of poor reproductive

performance, either irregularity or the absence of oestrous cycles and small gonads lacking follicular development. Hughes et al. (1975b) considered the XO mares to be "small but with none of the somatic anomalies frequently associated with gonadal dysgenesis in man."

From successful cultures of nine infertile mares, Chandley et al. (1975) discovered seven (76%) with chromosome anomalies. Sex chromosomes were positively identified with G-banding, and karyotypes included two XX's, two XO/XX mosaics, one 65 triple-X, one XY sex-reversed female and one normal karyotype with an additional autosomal fragment present in some cells. The last case exhibited regular oestrous cycles although follicles were absent, and the author doubted that the infertility was attributable to the observed chromosome aberration. Hughes et al. (1975a, 1975b) reported four cases of XO gonadal dysgenesis and an XO/XX mosaic, and Hughes and Trommershausen-Smith (1976) found a further five mares lacking one X chromosome, one XO/XY mosaic and one XY sex-reversed female (Table IV.6).

Reaction to the male varied. Oestrous behaviour was absent in most cases, yet four of the mares had been bred on several occasions (Hughes et al., 1975b) and their behaviour was considered similar to that recognized in normal anoestrous mares (Hughes, Stabenfeldt and Evans, 1972). The triple-X mare showed oestrus with an ovarian follicle on one occasion and an XO/XX mosaic in the same study (Chandley et al., 1975) also demonstrated oestrus but no follicle was produced.

In general, the uteri of these 12 infertile mares were smaller than normal but the distinction was not conclusive. In two cases, the XXX and one XO mare (Chandley et al., 1975),

the endometrium was hypoplastic. All the mares were characterized by small smooth ovaries devoid of follicles and germ cells and consisting primarily of ovarian stroma.

Further studies on XO mares (Hughes et al., 1975b) revealed normal levels of G-6-P D but reduced plasma concentrations of oestrogens. There were significant elevations of plasma LH* in four XO mares but not in the XO/XX mosaic. Presumably, increased gonadotrophin levels were due to absence of the normal feedback inhibition by oestrogens on the hypothalamus. Drumsticks were missing from polymorph neutrophils in XO mares but were detected at subnormal levels (1.75%) in the XO/XX mosaic.

* Luteinizing hormone.

TABLE IV. 6. CLINICAL DETAILS OF INFERTILE MARES WITH SEX CHROMOSOME DISORDERS.

Author	Mare		External appearance	Oestrous behaviour	Vagina cervix and vulva	Uterus	Gonads	Karyotype
	Breed	Age (years)						
Payne *et al.*, 1968	TB	12	Normal	Irregular cycles	Normal	Normal	Normal Follicles present Ovulated	2n=63 rearranged X?
Chandley *et al.*, 1975	Pony	2	Normal	No cycles	Normal	Slightly small	Very small No follicles	63X0
	TB x Arab	4	Normal	No cycles	Normal	Small Endometrium immature	Not palpable per rectum	63X0
	Arab	5	Normal	No cycles	Normal	Normal	Very small (2x1cm)	63X0/64XX
	TB	7	Slightly gelding-like	Some after irrigation	Pale, flaccid cervix	Small	Very small (2cm)	63X0/64XX
	TB	5	Normal	One recorded cycle with follicle	Normal	Small Endometrium hypoplastic	Small No follicles	65XXX
	TB	8	Normal	No cycles	Normal	Small	Very small	64XY
	TB	15	Normal	Regular but no follicles	Normal	Normal	Very small No oocytes	Extra fragment
Hughes *et al.*, 1975b	Qh	6	Normal or slightly smaller than average size	No cycles	Normal external genitalia, cervic flaccid	Small	Small. No palpable follicles	63X0
	Ap	6		Irregular		Small	Very small No follicles	63X0/64XX
	Arab			Oestrous cycles irregular or absent		Small	Small	63X0
	Arab					Small Endometrium hypoplastic.	Small No follicles	63X0
	Arab					Small Endometrium hypoplastic.	Small No follicles	63X0
Hughes and Tromershausen-Smith, 1976	Arab					Small	Small	63X0
	Arab					Small	Small	63X0
	Arab					Small	Very small. Not palpable per rectum	63X0
	Arab					Small	Very small	63X0
	Qh					Small	Small	63X0
	Qh					Small	Small	63X0/64XY
	Pony					Small	Very small	64XY

TB = Thoroughbred Qh = Quarterhorse Ap = Appaloosa

ii) Results

Chromosome analysis was undertaken for ten thoroughbred mares characterized by poor breeding performance and the failure to produce a live foal. The methods employed were described in Part II.3, and the clinical details of these infertile mares are shown in Table IV.7. Mitotic figures obtained by leucocyte cultures from each mare revealed a normal female karyotype (64XX) in nine of the ten infertile mares. Cell counts from the mares shown to have normal karyotypes are given in Table IV.8. Inconclusive results were obtained for three mares due to the small numbers of cells analyzed. However, one of these mares had previously conceived, and another ("Astrella") had had her left ovary surgically removed due to the presence of a granulosa cell tumour.

Metaphase spreads from the remaining mare disclosed a modal chromosome count of 63, and the consistent lack of one X chromosome (Table IV.9).

TABLE IV. 7. CLINICAL DETAILS OF INFERTILE MARES REFERRED FOR CHROMOSOME ANALYSIS.

Case number	Name	External appearance	Conceived?	Oestrous cycles	Vulva, vagina and cervix	Uterus	Ovaries	Karyotype
69/74	Astrella	Normal	Never	Irregular	Normal	Normal	Very small *	
4/75	First Fancy	Normal	Never	Normal	Normal	Normal	Normal	64XX
5/75	Sea Lark	Normal	Once	Normal	Normal	Normal	Normal	64XX
13/75	Geedee	Normal	Never	Irregular	Normal	Normal	Normal	64XX
35/75	Emily	Small	Never	Normal	Normal	Normal	Normal	64XX
36/75	Anne Gable	Normal	Twice	Normal	Normal	Normal	Normal	64XX
38/75		Small, 146cm.	Never	Absent	Normal	Small	Very small *	64XX
44/75	Banker's Delight	Normal	Never	Absent	Small	Small	Small	64XX
4/76	Fiola	Normal	Never	Irregular	Normal	Small	Small	64XX
PB	Excitation	Small, 146cm.	Never	Absent	Normal	Small	Very small *	63X0

* Smaller than 4cm x 2cm.

TABLE IV. 8.

CHROMOSOME COUNTS FROM INFERTILE MARES SHOWN TO HAVE NORMAL KARYOTYPES

Case Number	NAME	DISTRIBUTION OF CELL COUNTS						Cells counted
		< 63	63	64	65	>65	Polyploid	
69/74	Astrella	0	0	1	5	0	0	6
4/75	First Fancy	0	1	7	1	0	0	9
5/75	Sea Lark	0	0	12	1	0	0	13
13/75	Geedee	3	2	17	0	0	0	22
35/75	Emily	2	2	20	1	0	0	25
36/75	Anne Gable	0	4	16	3	0	0	23
38/75		9	14	105	5	0	0	133
44/75	Banker's Delight	3	3	21	2	1	0	30
4/76	Fiola	3	1	18	0	0	0	22

Studies on 63XO mare

a) History

Considered a "well-bred" thoroughbred ("Pretendre"-"Bikini"), this mare (named "Excitation") was born during the 1971-72 breeding season. (In New Zealand, the imposed breeding season for thoroughbreds commences on August 1st). Her growth was slow and as a four-year-old she measured 146cm in height (14.2 hands) at the wither (Figure IV.10). The average height for racing thoroughbreds in New Zealand is 155 to 160cm (15.2 to 16 hands).

"Excitation" never raced and was sent to a breeding stud as a three-year-old for the 1974-75 breeding season, where she was noted for her intractable behaviour. Apart from one doubtful report of mild oestrus (described as "semicycled"), this mare was never observed to demonstrate oestrous behaviour at stud, and was never mated. Her vulva and vagina appeared normal but rectal palpation detected a small uterus and smooth ovaries lacking palpable follicles (Table IV.7).

b) Chromosome studies

In addition to leucocyte cultures, mitotic figures were obtained after culturing tissue explants taken from sub-cutaneous fascia, mesentery and the right ovary. Details of all cell counts appear in Table IV.9. Among the 123 cells counted, 82% contained 63 chromosomes, and in each karyotype the missing element was an X chromosome (Figure IV.11).

The proportion of non-modal cells is comparable to that obtained from leucocyte and fibroblast cultures from normal horses and no normal cells were found. Karyotypes prepared from hypodiploid metaphase spreads were lacking in different chromosomes but in each cell one of the missing

elements was an X chromosome.

No evidence of mosaicism was detected. Several cultures of leucocytes and mitoses obtained from other tissues produced consistent results and the analysis of non-modal cells showed them as broken cells with the loss or gain of different elements. Although mosaicism is difficult to exclude, the karyotype of this infertile mare was concluded to be a presumptive 63XO.

Centromere staining

Centromere staining of the chromosomes of the horse permits positive identification of the X chromosome. Mitotic cells prepared from leucocytes and from cells from the right ovary were C-banded and karyotyped (Figure IV.12). Analyzed cells consistently contained only one X chromosome.

Figure IV.10. Phenotype of thoroughbred mare shown to have XO anomaly.

TABLE IV. 9. CHROMOSOME COUNTS OF METAPHASE SPREADS FROM X0 MARE

Tissue cultured	COUNT DISTRIBUTION						Cells counted	Karyotype
	< 62	62	63	64	> 64	Polyploid		
Leucocytes	1	12	50	0	0	1	64	63X0
Muscle	0	2	26	0	0	0	28	63X0
Fascia	0	0	6	0	0	0	6	63X0
Mesentery	0	0	4	0	0	0	4	63X0
Right ovary	1	4	15	0	0	1	21	63X0
TOTAL	2 (1.5%)	18 (15%)	101 (82%)	0	0	2 (1.5%)	123	

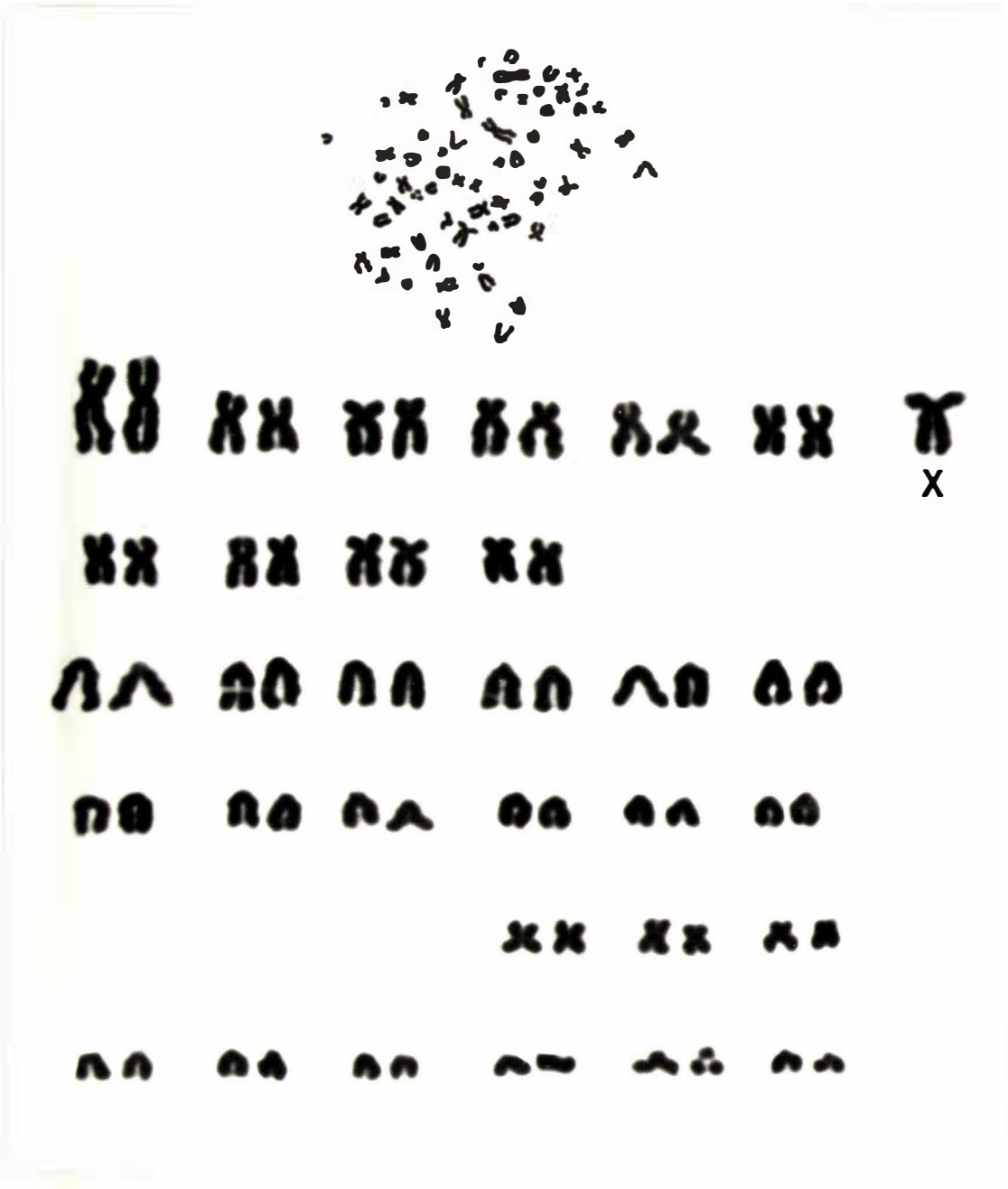


Figure IV.11. Karyotype of mare shown to have XO anomaly, 2n = 63. (Magnification 4500x).

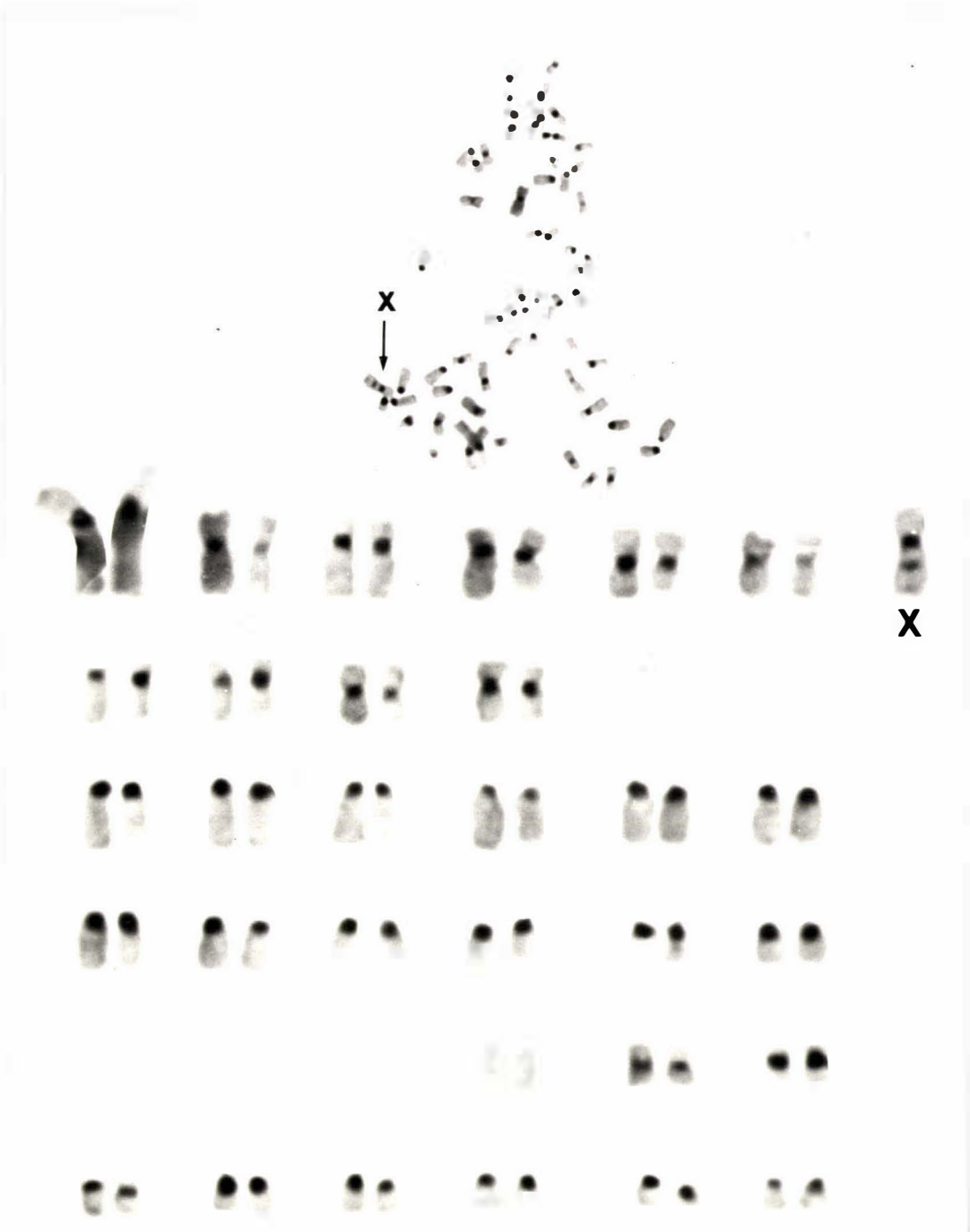


Figure IV.12. Karyotype of 63XO mare after centromere staining. (Magnification 4500x).

c) Examination of the reproductive tract

Following successful cell cultures of blood leucocytes, subcutaneous fascia and mesentery, the mare was destroyed with pentobarbitone sodium ("Euthatal", May and Baker) and autopsied. The reproductive organs are displayed in Figures IV.13, 14 and 15.

The vulva and vagina were normal in size and appearance except for the presence of an incomplete band of fibrous tissue cranial to the urethral orifice connecting the lateral walls of the vagina (persistent hymen). The cervix and uterus were of the normal size expected for a young maiden mare.

The ovaries were almost identical in appearance with a smooth firm texture (Figure IV.15). Both were markedly undersized, measuring only 3.5cm x 2cm, and follicles were absent from the ovarian surface. Sectioning of the ovaries during autopsy failed to reveal macroscopic follicles within the ovarian tissue.

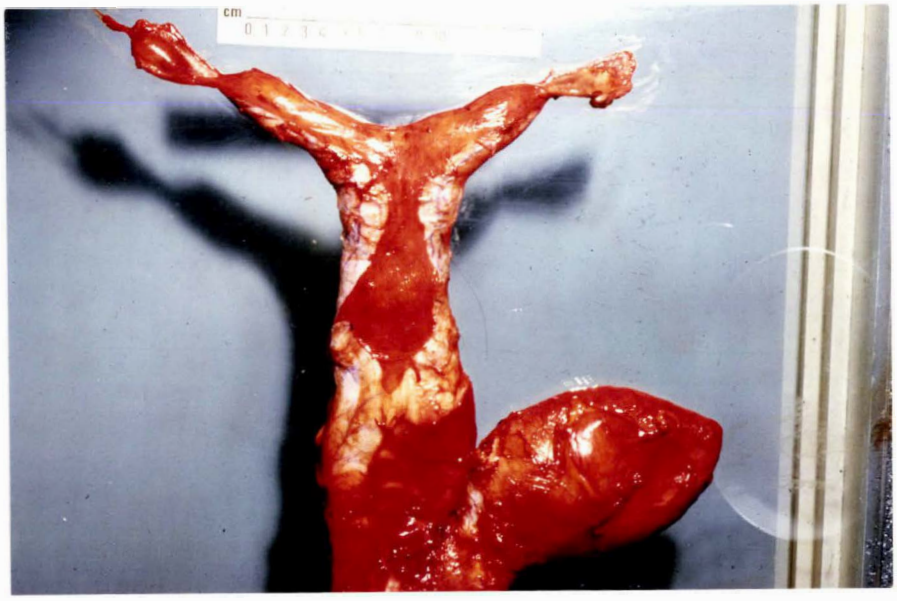


Figure IV.13. Internal genitalia of XO mare. The uterus is of normal size for a young virgin mare but the ovaries are lacking in overt follicular development.

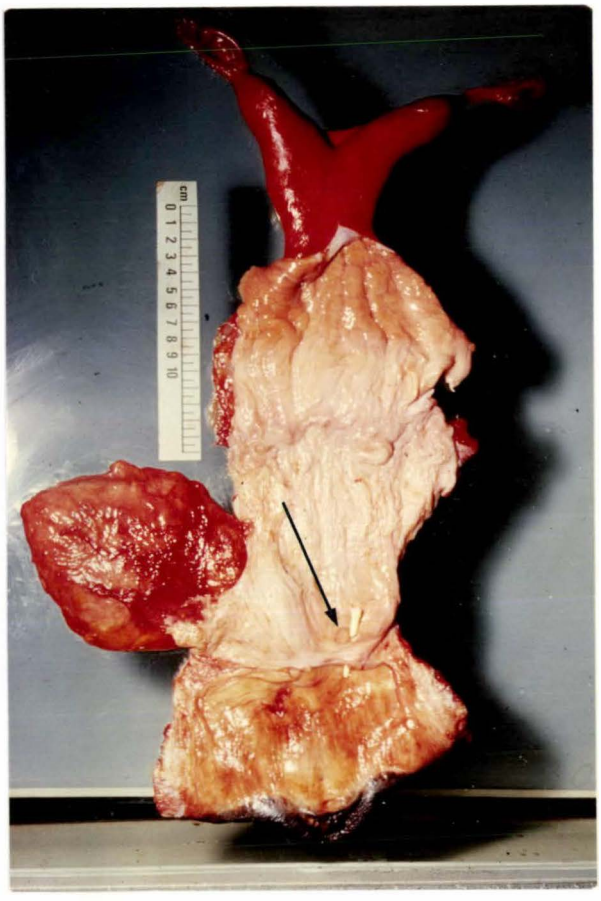


Figure IV.14. Internal genitalia of XO mare; vagina cervix and uterus opened. The persistent hymen is indicated by the arrow.

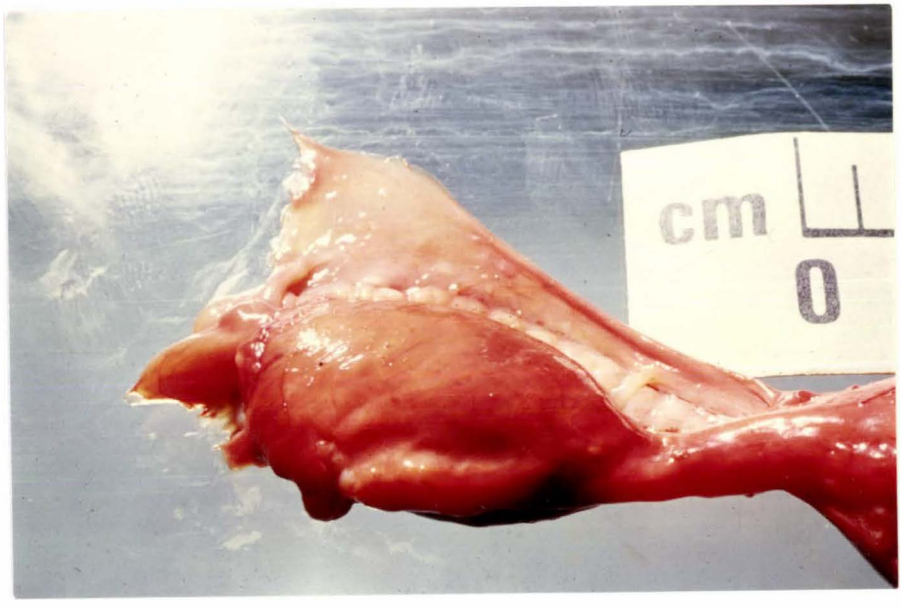


Figure IV.15. Left ovary of XO mare. Note the absence of Graafian follicles which, in a normal cycling mare, protrude from the ovarian surface.

Histology

Segments from both ovaries and the uterus were fixed in 10% formal saline and routinely prepared for sectioning. Slides containing histological sections 7μ thick were stained with haematoxylin and eosin, and each slide was mounted with a coverslip with DPX.

The hypoplastic appearance of the endometrium is illustrated in Figure IV.16. Little arborization is evident in the endometrial glands which are lined with cuboidal cells. Cuboidal cells indicative of low circulating levels of oestrogens compose the luminal epithelium of the uterus.

Most of the ovarian substance consisted of fibrous connective tissue or ovarian stroma. However, one section taken from the right ovary revealed the presence of a number of presumptive atretic Graafian follicles and a discrete area of functional luteal tissue (Figure IV.17). No primordial germ cells were observed and the structures presumed to be atretic follicles were few.

The wall of a normal Graafian follicle consists of a row of granulosa cells, initially columnar in shape, which proliferate to produce a stratified layer of granulosa cells at least five cells thick. The presumptive follicles in this infertile mare were lined with cuboidal granulosa cells in only one or two layers (Figure IV.18). The theca interna and theca externa were also poorly differentiated, completing the picture of an underdeveloped or atretic follicle.

The poorly developed wall of a Graafian follicle in the right ovary of this chromosomally abnormal mare is shown in Figure IV.19. The granulosa cell layer is only two cells thick, and the rich capillary network found in the theca

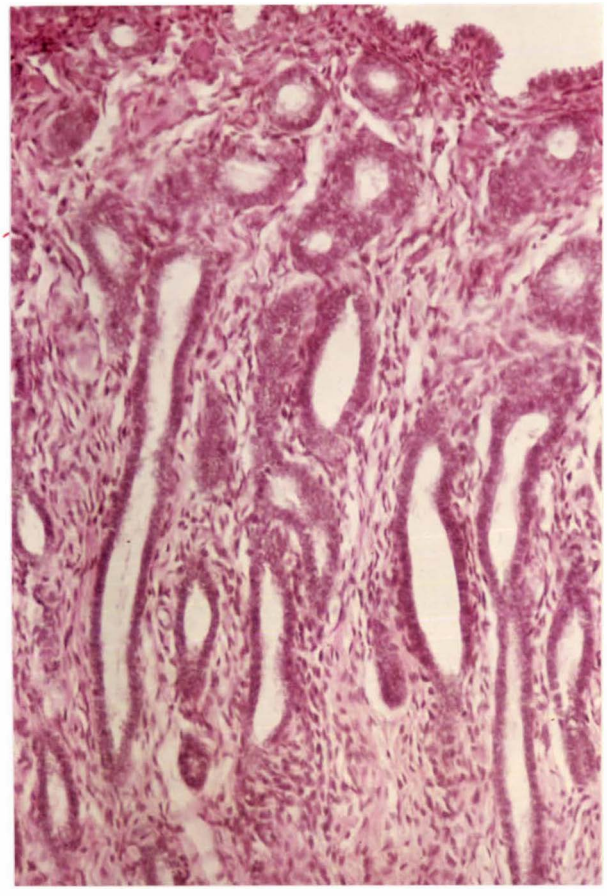


Figure IV.16. Histological section of endometrium from XO mare.
Note the hypoplastic appearance with little aborization of endometrial glands and cuboidal cells lining the luminal epithelium and endometrial glands. (Magnification 500x).

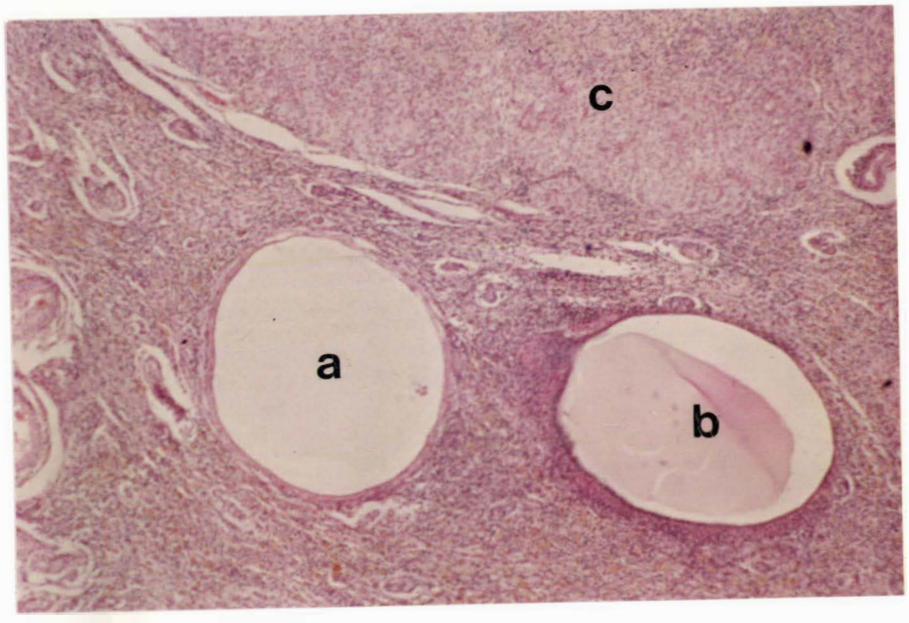


Figure IV.17. Presumptive atretic Graafian follicles (a and b) and luteal tissue (c) in the right ovary of the XO mare. (Magnification 50x).

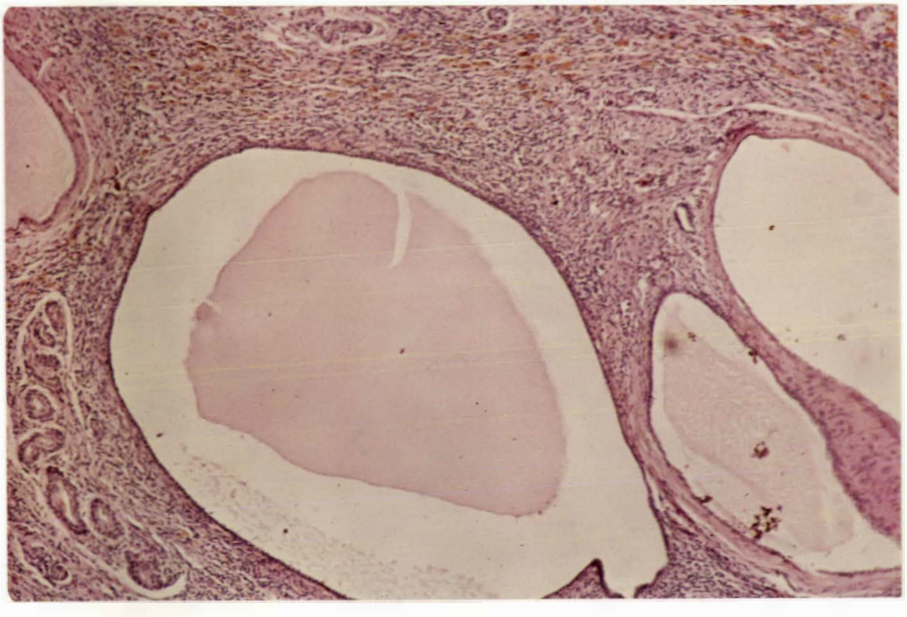


Figure IV.18. Presumptive Graafian follicles in the right ovary of the XO mare. Four follicles are evident, each lined with a narrow layer of cuboidal granulosa cells. (Magnification 80x).

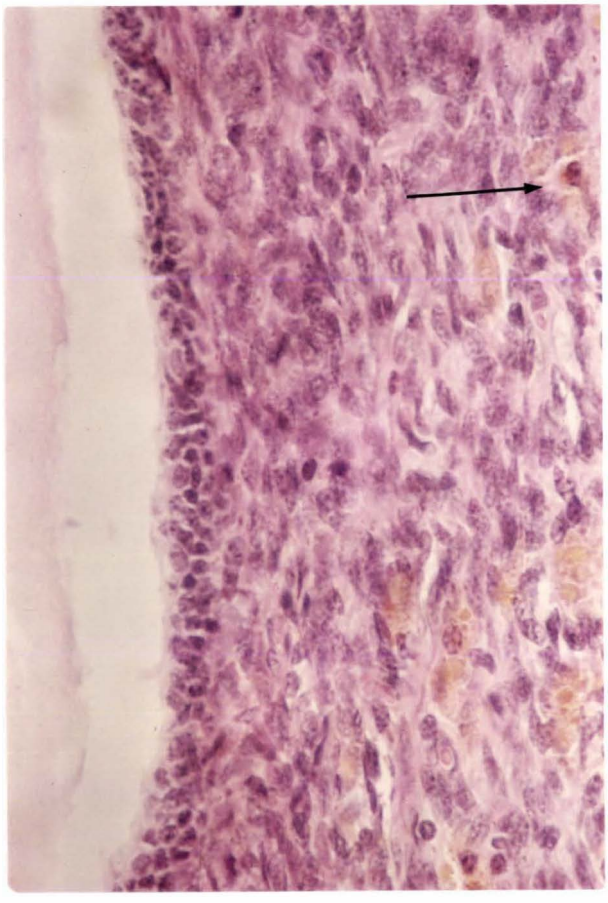


Figure IV.19. Wall of presumptive Graafian follicle. The granulosa cell layer is only two cells wide and the thecas are poorly developed. The arrow indicates a red blood cell in an early stage of phagocytosis within a macrophage. (Magnification 500x).

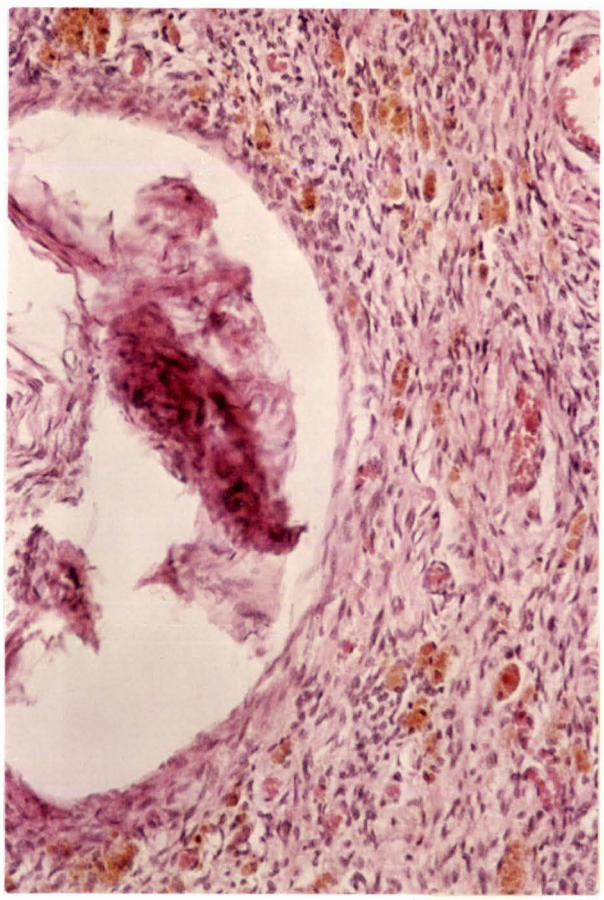


Figure IV.20. Atretic or degenerating Graafian follicle. The follicle has lost its granulosa cell layer and theca interna. Considerable recent haemorrhage and haemosiderin is present. (Magnification 200x).

interna of a normal follicle is not apparent. Many macrophages are present, having migrated into the tissues and engulfed and broken down erythrocytes, producing the pigment haemosiderin. In many macrophages the red blood cells can still be discerned, indicating that the haemorrhage and phagocytosis are recent occurrences. As their reparative function is completed, the phagocytes move out of an area, but in these sections they are still fully functional and present in large numbers.

A presumptive atretic Graafian follicle in a more advanced stage of degeneration is shown in Figure IV.20. This follicle has largely lost its finite structure and the granulosa cells and theca interna cannot be defined. The flimsy surrounding band of fibrous connective tissue is probably a remnant of the theca externa. Macrophages containing haemosiderin are particularly obvious, indicative of recent haemorrhage.

The discrete region of luteal tissue (Figure IV.21) has the characteristic appearance of a normal functional corpus luteum containing endocrine cells and a rich blood supply, and lacking a fibrous capsule. Often found in regions of cell activity, the pigment lipofuchsin ("wear and tear pigment") is probably represented by a yellow-tinged area among this luteal tissue (Figure IV.22). Fine lipid droplets are found in the lutein cells of a normal corpus luteum. These usually remain small in size and number while the corpus luteum is functional, but when involution begins the lipid droplets become larger and more numerous. As involution proceeds, lipids continue to accumulate in the lutein cells which finally undergo complete fatty degeneration. A yellow or brown pigment, lipofuchsin, is frequently formed during

this process, but is seen in only small amounts in Figure IV.22.

In normal horses, accessory corpora lutea may form from ruptured follicles or, more commonly, by the luteinization of unruptured follicles, in which case the corpus luteum would appear to be encapsulated due to the persistence of the theca externa. In Figure IV.21 there is no evidence of a fibrous capsule surrounding the area of luteal tissue.

Figure IV.23 reveals the typical appearance of active endocrine cells composing the region of luteal tissue. Almost all the endocrine cells contain normal nuclei with distinct nuclear membranes and prominent nucleoli. That these cells are functional is further supported by the presence of obvious cytoplasmic vacuolation, giving them a finely vacuolated appearance.

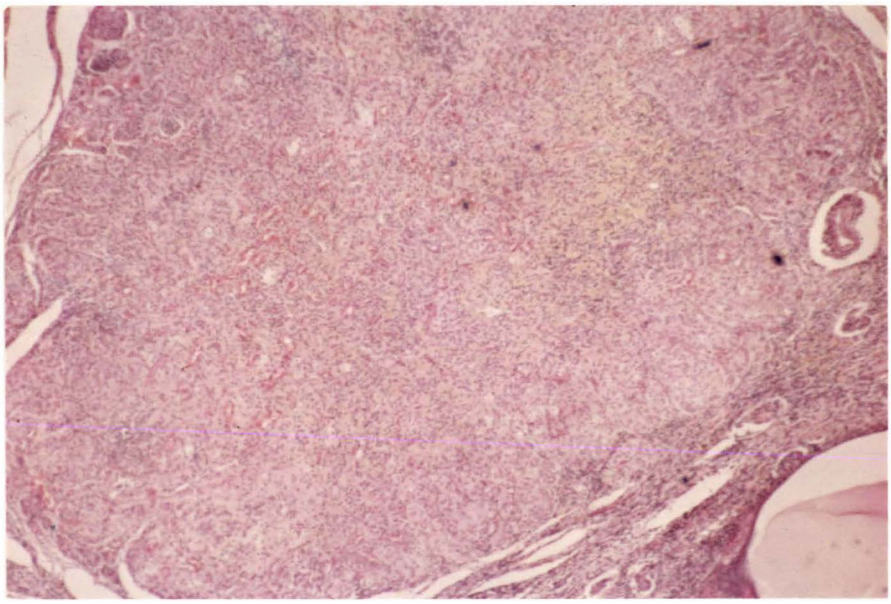


Figure IV.21. Discrete area of luteal tissue in the right ovary of the XO mare. (Magnification 50x).

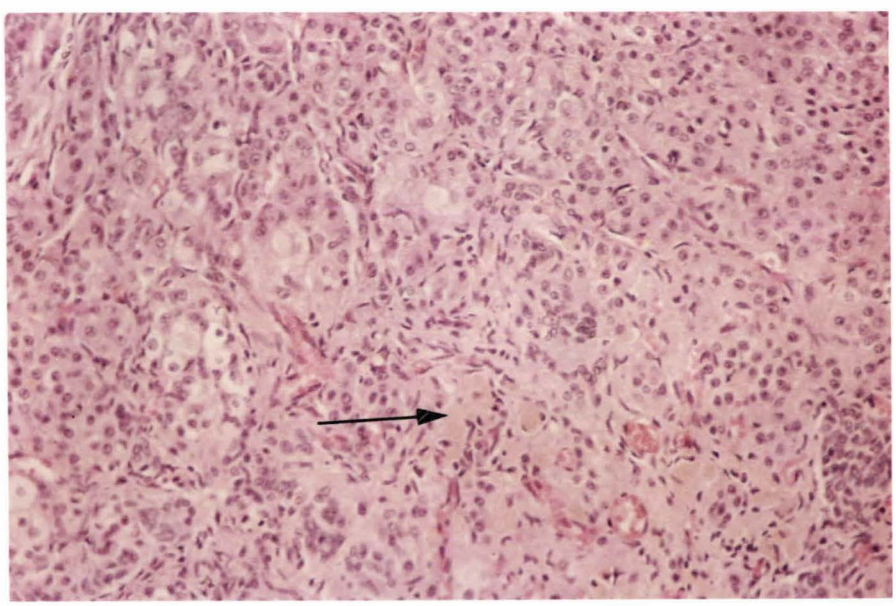
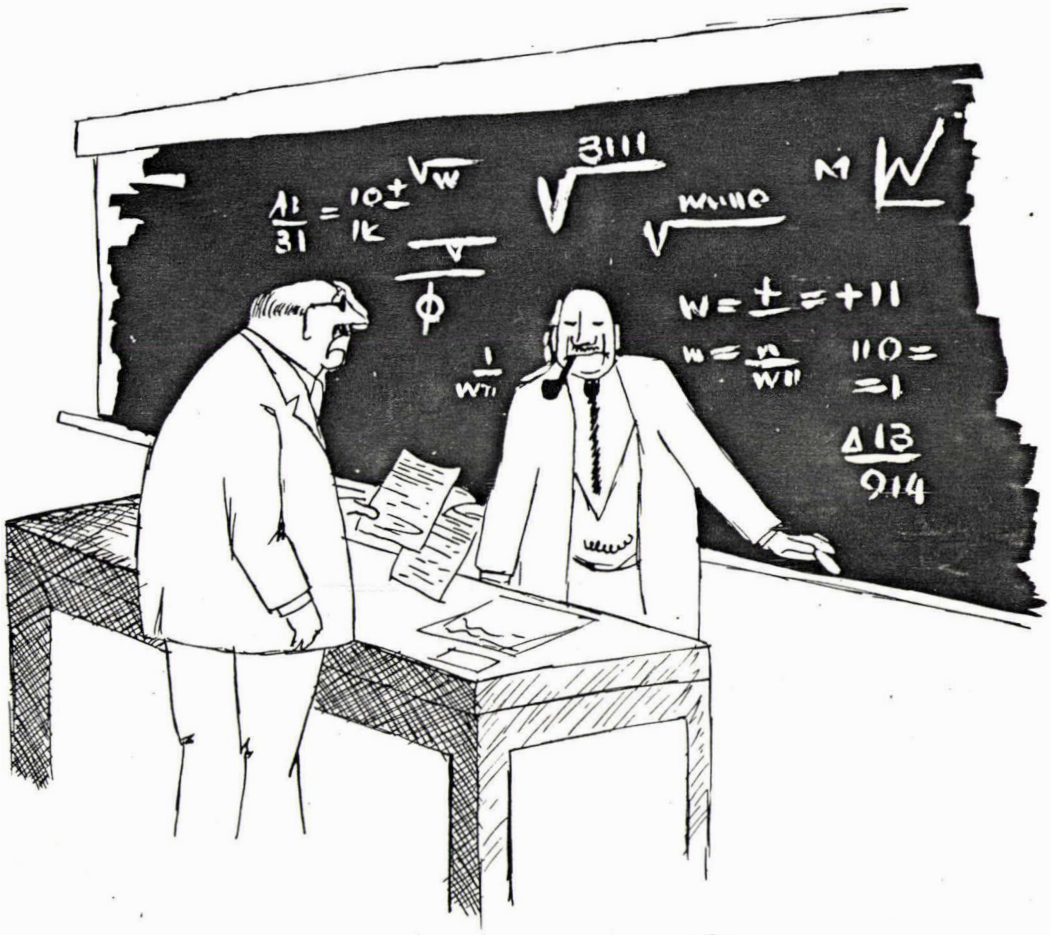


Figure IV.22. Typical histological appearance of functional luteal tissue from right ovary of XO mare. An area containing a faint yellow-brown pigment, probably lipofuchsin, is indicated by the arrow. (Magnification 200x).



"You Mean E <u>Doesn't</u> = MC^2?"

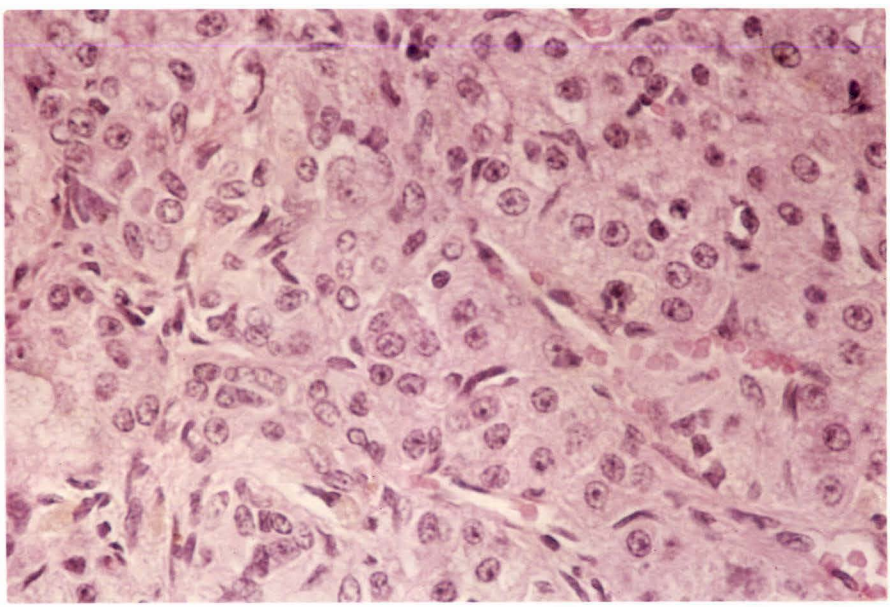


Figure IV.23. Characteristic active luteal endocrine cells with distinct nucleoli and nuclear membranes and fine cytoplasmic vacuolation. (Magnification 500x).

d) Sex chromatin studies

(1) Introduction

The discovery of sex chromatin, usually attributed to Barr and Bertram (1949) who first reported a sex difference in neurones of the cat, has proved invaluable as a screening procedure for the diagnosis of X chromosome abnormalities. At the same time, Barr and Bertram (1949) noted a similar feature in the nerve cells of women, and a smaller, less well-defined chromatin mass can be identified in males.

Staining properties with nuclear dyes indicated that the small discrete sex chromatin body was composed of DNA (Lindsay and Barr, 1955) but not until 1960 was the presence of sex chromatin related to the number of X chromosomes (Stewart, 1960). The formula relating the number of sex chromatin bodies per cell, B, with the number of X chromosomes and the number of autosomal sets, A, is

$$B = X - \frac{A}{2}$$ (Harnden, 1961).

From the formula, "halves" are considered an indication of the unstable effect of an abnormal autosomal complement, as with human XXY triploids.

Apart from the correlation between the sex chromatin and the number of X chromosomes, a size relationship has also been established. Abnormal size of the sex chromatin body is indicative of a structural abnormality of an X chromosome: a small sex chromatin body has been linked with partial deletion of an X chromosome (Jacobs, Harnden, Buckton, Court-Brown, King, McBride, MacGregor and Maclean, 1961); a large Barr body may represent an isochromosome (Fraccaro, Ikkos, Lindsten, Luft and Kaijser, 1960).

Since its initial discovery, sex chromatin has been

demonstrated in many cell types in man (Moore and Barr, 1954, 1955) and in domestic animals including the cow (Lang and Hansell, 1959), sheep (Bruère and MacNab, 1968), cat, pig, and horse (Hoshino and Toryu, 1959). In humans, the sex chromatin is readily distinguished from other particles of general chromatin by its larger size (0.7 to 1.2μ; Moore and Barr, 1954, 1955) and characteristic shape. In other species, problems of identification may arise and it may be difficult to differentiate sex chromatin from coarse autosomal chromatin, as in many cells of rodents (Moore, 1965).

Whereas chromosomes are visible as dense structures only during metaphase, sex chromatin can be identified only during interphase. As well as varying between tissues and with age, the frequency of sex chromatin bodies found in mammalian tissues may be greatly influenced by technical factors such as fixation and sectioning (Klinger, 1966). Of all body tissues, the most consistent for the identification of sex chromatin is nerve tissue (Moore and Barr, 1953).

The sex chromatin body may be found in three different positions in the cell: (1) adjacent to the nucleolus, (2) free in the nucleoplasm, and (3) adjacent to the nuclear membrane. The position of the sex chromatin body within the nucleus appears to vary with the type of neurone and with the species (Moore and Barr, 1953), and depending on its position in the nucleus, the sex chromatin may have several different shapes. It tends to conform to adjacent surfaces; when free in the nucleoplasm it assumes a spherical shape, when pressed against the nucleolus it is semilunar.

(2) Sex chromatin in the horse

In the domestic horse (Equus caballus), sex chromatin similar to that present in bovine cells has been described in nerve tissues and nuclei of epithelial and smooth muscle cells of the duodenum (Hoshino and Toryu, 1959). Benirschke et al. (1962) could not distinguish the sexes by examining buccal smears for sex chromatin due to the clumping of chromatin particles in male nuclei. They did, however, detect sex chromatin in the epidermal cells of an alleged fertile mare mule (Benirschke et al., 1964), and in skin cells cultured from a donkey-Grevy zebra hybrid (Benirschke et al., 1964).

Payne, et al. (1968) were unable to identify chromatin bodies in buccal cells due to poor staining quality of the cells and the presence within nuclei of chromocentres similar in appearance to chromatin bodies. Vaginal smears were more successful, and sex chromatin bodies could be identified in approximately 30% of cells from a normal fertile mare (Payne et al., 1968).

(3) Sex chromatin of polymorphonuclear leucocytes

Distinct from the Barr bodies present in interphase nuclei of tissue cells, sex chromatin is also represented by a small nuclear mass, the "drumstick", attached by a fine filament to the body of the nucleus of some polymorphonuclear leucocytes. Davidson and Robertson Smith (1954) first demonstrated "drumsticks" in human peripheral blood and drew attention to the presence of non-sex-specific structures liable to be confused with them. In the horse, sessile nodules resembling small "drumsticks" have been observed frequently in polymorphonuclear neutrophils of both males and females (Colby and Colhoun, 1963).

Even in normal subjects, the frequency of neutrophil drumsticks in humans and animals is extremely variable. Colby and Colhoun (1963), examining the polymorphonuclear neutrophils of domestic animals, considered that the presence of six accessory nuclear lobules per 500 cells was diagnostic of female sex.

Typical "drumsticks" have been described in 6% of polymorphonuclear leucocytes in a female mule and in 4% of similar cells in a female donkey (Benirschke et al., 1962). Dunn et al. (1974) found "drumsticks" in 6.25% of polymorphonuclear neutrophils from an intersex horse, in their view "a positive diagnosis of female sex". Hughes et al. (1975a, 1975b) reported a 4% incidence of "drumsticks" in a normal mare, a reduced frequency (1.75%) in an XO/XX mosaic, and no "drumsticks" present in a case of pure gonadal dysgenesis.

Although the frequency of "drumsticks" does not always bear a direct relation to the number of X chromosomes, drumstick analysis provides a means of "sexing" another tissue in the evaluation of mosaicism of X chromosome abnormalities. As with the sex chromatin in tissue cells, structural abnormalities of the sex chromosomes in humans are reflected in the leucocyte drumsticks (Maclean, 1962).

(4) Examination of neurones for sex chromatin

Nerve cells from the cerebellum and spinal cord of three horses, a normal male, normal female, and the XO mare, were scored for the presence of sex chromatin bodies. The results of these examinations are shown in Table IV.10.

A sex chromatin body was identified in 394 of 566 nerve cells obtained from the cerebellum and spinal cord of a normal mare. This frequency (70%) is slightly lower than the 80%

incidence found by Hoshino and Toryu (1959) in nerve cells taken from various regions of the nervous systems of cats, pigs, goats, cattle and horses.

The position of the sex chromatin body within the nucleus in the five regions of nerve tissue examined is shown in Table IV.11. Depending on the relative planes in which the sex chromatin and the nucleolus occurred, interpretation of the position of the sex chromatin was often difficult. In 75% of neurones containing sex chromatin, the semilunar sex chromatin body was situated adjacent to the nucleolus (Figure IV.24); in approximately 20% of cells it was located free in the nucleoplasm (Figure IV.25), and it was infrequently found adjacent to the nuclear membrane (Figure IV.26). The position of the sex chromatin appears to be partly species specific but also shifts its position within the nucleoplasm during altered states of metabolic activity of the cell (Bertram, 1966).

The absence of sex chromatin bodies among 539 neurones from a mare with sex chromosome constitution XO, is evidence for the lack of an X chromosome in cells of the nervous system. Since nerve tissue is epidermal in origin, it is probable that cells composing other body tissues derived from epiderm are also characterized by the absence of a sex chromosome.

(5) Examination of neutrophils for "drumsticks"

Following the examination of 100 polymorphonuclear neutrophils from a normal mare, "drumsticks" were identified in 4% of the cells examined (Figure IV.27). This finding is comparable to that reported from similar studies (Hughes et al., 1975b). No typical "drumsticks" were detected among 1000 polymorphonuclear neutrophils from a normal stallion,

nor among a further 1000 neutrophils from the infertile mare lacking one X chromosome. However, a small number (less than 5 per 1000) of nuclear appendages resembling small "drumsticks" were noticed in both the XO mare and the normal male (Figure IV.28).

When present, "drumsticks" occur in only a small proportion of neutrophils and may be confused with non-sex-specific structures. The size of the "drumstick" head is an important characteristic because of possible confusion with other nuclear appendages which may have a similar shape but are usually smaller. Further, the frequency of "drumsticks" alters with the degree of lobation of the neutrophil nucleus in different individuals.

Due to the very low frequency of accessory drumstick appendages in the polymorphonuclear neutrophils of domestic animals, Colby and Colhoun (1963) considered this test to be of doubtful value as a method of sex diagnosis. From this study too, the examination of blood smears for "drumsticks" was concluded to have limited diagnostic value in the horse.

TABLE IV. 10.

SEX CHROMATIN IN NERVE CELLS OF A NORMAL STALLION, NORMAL MARE AND XO MARE

ANIMAL		NERVOUS TISSUE EXAMINED					Total	%
		Cerebellum	REGION OF SPINAL CORD					
			Cervical	Thoracic	Lumbar	Sacral		
Normal stallion	Sex chromatin	0	0	0	0	0	0	0
	Cells examined	106	106	107	112	106	537	
Normal mare	Sex chromatin	87	98	69	65	75	394	69.6
	Cells examined	105	144	104	104	109	566	
XO mare	Sex chromatin	0	0	0	0	0	0	0
	Cells examined	105	104	108	113	109	539	

TABLE IV. II. POSITION OF THE SEX CHROMATIN BODY WITHIN THE NUCLEUS

Position of sex chromatin	REGION OF NERVOUS TISSUE					Number of sex chromatin bodies
	Cerebellum	Cervical	Thoracic	Lumbar	Sacral	
Adjacent to nucleolus	84 (96.5%)	81 (83%)	50 (72.5%)	45 (69%)	36 (48%)	296 (75%)
Free in nucleoplasm	3 (3.5%)	16 (16%)	16 (23%)	17 (26%)	29 (38.7%)	81 (20.5%)
Against nuclear membrane	0	1 (1%)	3 (4.5%)	3 (5%)	10 (13.3%)	17 (4.5%)
TOTAL	87	98	69	65	75	394

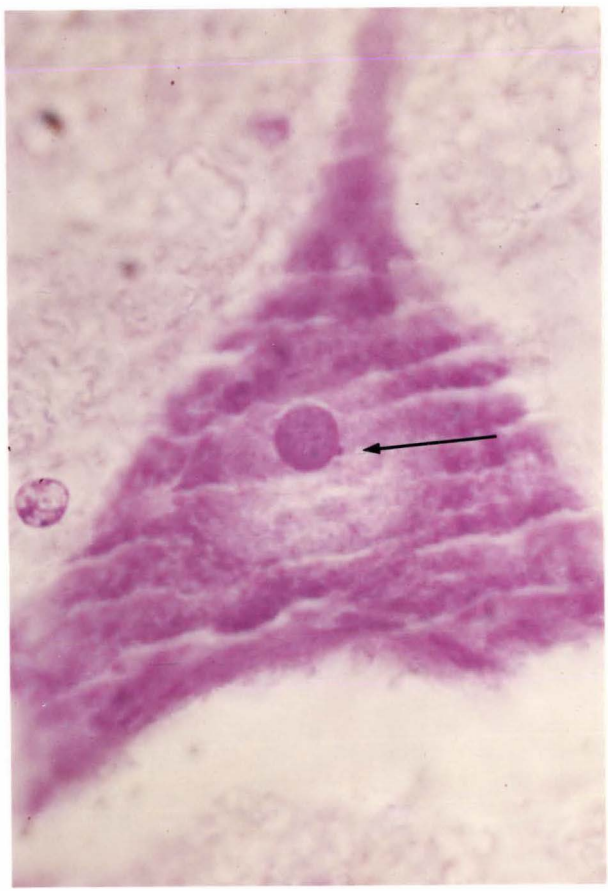


Figure IV.24. Sex chromatin body adjacent to the nucleolus in a neurone from a normal mare. (Magnification 1250x).

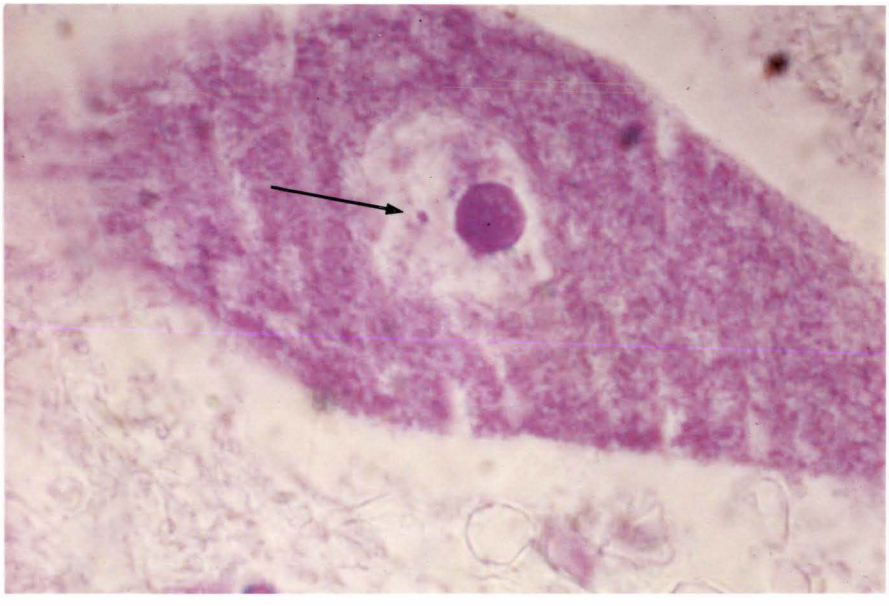


Figure IV.25. Sex chromatin body free in the nucleoplasm of a neurone from a normal mare. (Magnification 1250x).

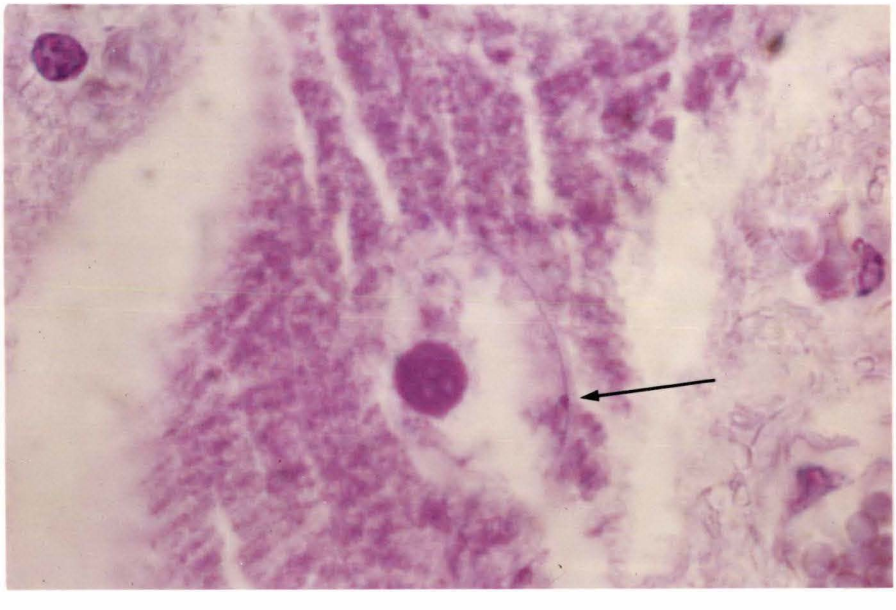


Figure IV.26. Sex chromatin body adjacent to the nuclear membrane in a neurone from a normal mare. (Magnification 1250x).

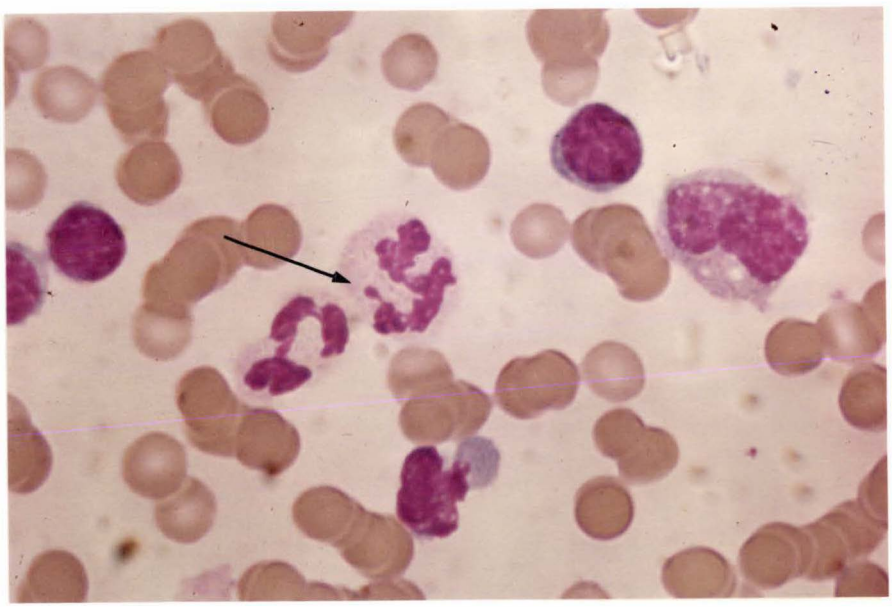


Figure IV.27. Characteristic nuclear appendage ("drumstick") on a polymorphonuclear neutrophil from a normal mare. (Magnification 1250x).

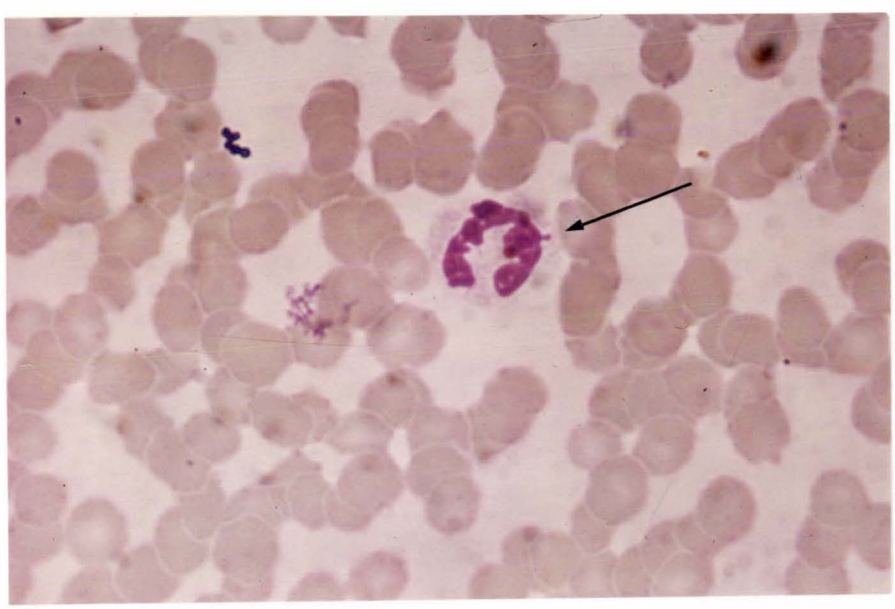


Figure IV.28. Nuclear appendage on a polymorphonuclear neutrophil from a normal stallion. (Magnification 1250x).

iii) Discussion

Disorders of the sex chromosomes associated with phenotypic abnormalities are well known in man, and comparable syndromes have been found among the domestic animals (Bishop, 1972). The best known of the sex chromosome disorders in humans are the sterile male with Klinefelter's syndrome (Klinefelter, Reifenstein and Albright, 1942) and the female with Turner's syndrome (Turner, 1938) characterized by sterility and infantile sexual development.

The animal counterpart of Klinefelter's syndrome with XXY sex chromosomes in either pure or mosaic form has been described in each of the domestic animals. The XO karyotype, as found in women with Turner's syndrome, is recorded in mice (Welshons and Russell, 1959; Russell, 1961) and has now been reported in three of the domestic animals: the pig (Nes, 1968), cat (Norby, Hegreberg, Thuline and Findley, 1974) and horse.

Origin of the XO anomaly

In the mare, as in other species, an XO sex chromosome constitution may arise by nondisjunction in a parental germ cell or in mitosis after fertilization, or by the loss of an X chromosome during gametogenesis or early cleavage of the zygote.

The XO anomaly in women is unassociated with maternal age (Tennes, Puck, Bryant, Frankenberg and Robinson, 1975) and is often found in mosaic form. This was considered indirect evidence that the loss of a sex chromosome most commonly resulted from a mitotic error between fertilization and first cleavage (Morishima and Grumbach, 1968). The XO karyotype in mice was also thought to result from sex chromo-

some loss, more frequently of the paternally-derived chromosome (Russell, 1962), and family studies of X-linked traits in man (colour blindness and Xg blood group) have further indicated the preferential loss of the paternally-derived X chromosome. Similar information concerning the XO anomaly in the horse is lacking.

In the only report of an XO cat, Norby et al. (1974) believed that the retention of the paternal X was due to nondisjunction in the dam's germ cell line or during very early embryonic division. Noting the family history of unusual coat colour patterns he suggested the possibility of an hereditary influence.

Frequency of the XO anomaly

In newborn children, the XXY anomaly is about five times more common than XO (Moore, 1959; Maclean, Harnden and Court-Brown, 1961). The incidence of the XO anomaly in live-born female children has been estimated at 0.4/1000 (Jacobs, 1966). No similar surveys have been undertaken in animals, but from this and other concurrent reports, the XO anomaly seems remarkably common among infertile mares.

Prior to this report, disorders of the sex chromosomes have been found in 19 of 28 infertile mares, an incidence of 68%. (An earlier but inconclusive report of a chromosome anomaly in a sterile mare was recorded by Payne et al. (1968)). These results are comparable to cytogenetic studies on women with a history of primary amenorrhoea (Jacobs et al., 1961), but anomalies of the sex chromosomes in infertile mares are apparently more frequent than the 50% incidence recorded in amenorrhoeic women (Table IV.12).

TABLE IV. 12. COMPARISON OF SEX CHROMOSOME DISORDERS AMONG INFERTILE MARES AND WOMEN.

Author	Patients examined	Number with chromosome anomaly	Number with abnormal sex chromosomes	SEX CHROMOSOME CONSTITUTION						Autosomal abnormality
				X0	X0/XX	X0/XY	XXX	XY female	Abnormal X	
Jacobs *et al.*, 1961	32 women having primary amenorrhoea	17	16	6	1	3 (1 X0/XYY)	1 (X0/XXX)	2	3	1
Chandley *et al.*, 1975 Hughes *et al.*, 1975b Hughes *et al.*, 1976	29 infertile mares	20	19	11	3	1	1	2	1(?) Payne *et al.*, 1968	1

Most placental mammals including the mouse, dog, cow, horse, donkey and man have X chromosomes similar in size and genetic constitution (Ohno, Stenius, Christian, Bezak and Bezak, 1964; Mathai et al., 1966) but the function of the X chromosome remains largely equivocal. The occurrence of a relatively high incidence of chromosomally abnormal mares may indicate that the X chromosome plays a less significant role in the embryonic development of this species.

The finding of a sex chromosome disorder in only one of ten infertile mares examined chromosomally in this study appears to represent a lower frequency of chromosome anomalies than is evident from other reports (Chandley et al., 1975; Hughes, 1976). There are several possible reasons for this discrepancy.

In this survey it was not possible for the author to apply rigid clinical standards of selection to the mares referred for chromosome analysis, and selection was based only on the failure to produce a live foal. Whereas the infertile mares in previous studies were characterized by the failure of follicular development and conception, and the presence of small rudimentary gonads, two of the ten mares reported here had previously conceived and seven had shown oestrous behaviour. In addition, the ovaries in five mares were considered normal by rectal palpation, and in one case ("Astrella") an ovary had been removed after it was found to contain a granulosa cell tumour. Had the author been able to apply more strict criteria of selection, only four mares would have been referred for chromosome analysis. Also in many instances, blood samples sent for screening were processed before the full history was available. There were considerable difficulties in liaison with field veterinarians

and in access to infertile broodmares. The mare shown to have a 63XO karyotype was a patient at Massey University during the period that blood samples were obtained from mares at studs. This mare was able to be adequately studied chromosomally while many of the others were not (Table IV.8).

The possibility of cell selection in culture cannot be ignored. Cell lines apparently grow at different rates, and one line may outgrow another (Fraccaro, Gemzell and Lindsten, 1960). Other factors which may influence the success of detecting chromosome errors include the range of tissues sampled and the number of cells analyzed for each mare.

Mosaicism is common in humans and horses with sex chromosome abnormalities, and it is often necessary to make chromosome preparations from several tissues to determine a patient's chromosomal constitution. In this study, cells other than blood leucocytes were analyzed in only one case, the XO mare. Due to difficulties of access to the individual mares, the multiple sampling of a range of tissues was usually not possible and chromosome analysis relied on the culture of a single sample of blood.

In any given case the presence or absence of mosaicism cannot be determined with absolute certainty. Court-Brown et al. (1964) commented that "in most cases the presence or absence of mosaicism may reasonably be determined after counting 30 metaphases". For only three of the ten infertile mares were 30 or more metaphases counted, and 22 cells were analyzed in a fourth mare having a similar phenotype and reproductive history to the XO mare.

That the presence of mosaicism can be easily overlooked was illustrated from the bovine true hermaphrodite described by Dunn, McEntee and Hansel (1970). Only diploid

cells were detected among the first 300 leucocytes examined, but a further 400 metaphases revealed one 88XXY cell. The author stated that "the triploid cell line would have gone undetected if only 200 metaphases per culture from blood, skin and bone marrow had been examined".

Mosaicism is probably much more common than has been recognized, and is difficult to exclude because chromosome constitution may vary in different tissues and even between areas of the same tissue (Morishima and Grumbach, 1968). A survey of Xg (a sex-linked blood group system) in Turner's syndrome disclosed a much greater incidence of mosaicism than found in other aneuploid states (Race and Sanger, 1969). This apparently high frequency of mosaicism in women with Turner's syndrome is due to their preferential survival in uteri in comparison with XO zygotes (Hecht and MacFarlane, 1969). Similarly in the horse, leucocyte cultures have shown that approximately one third of the chromosomally infertile mares reported have been mosaics. Although in these cases mosaicism was detected in leucocyte cultures, the possibility cannot be ignored that undetected mosaicism may account for the relatively low incidence of sex chromosome disorders found in this survey, particularly in view of the small numbers of cells examined.

Bishun, Rashad, Morton, Mannion, Neely and Burke (1964) suggested that the presence of gonosome mosaicism might lead to the production of some abnormal gametes and embryonic death. They reported XO/XX mosaicism in a women with a reproductive history of four pregnancies, of which three had resulted in stillbirths. In view of the relatively high incidence of mosaicism with sex chromosome anomalies in mares,

this may have considerable importance in the occurrence of embryonic death in the horse.

Effect of XO anomaly on phenotype

Infertile mares exhibit a range of phenotypes and not all infertile mares with small inactive ovaries contain detectable chromosome anomalies (Table IV.7). There were marked similarities in appearance and breeding histories between the XO mare and infertile mares shown to have normal karyotypes, and it was not possible to distinguish between them on the basis of phenotypic characteristics. In particular, one unnamed mare (Case 38/75, Table IV.7) was almost identical to the XO mare in physical appearance and reproductive behaviour, and from rectal palpation the internal genitalia of these two mares seemed very similar. However, leucocyte cultures showed one to have a normal female karyotype; she was returned to the stud and subsequently conceived.

The production of a live foal does not, however, preclude the presence of chromosome anomalies responsible for reduced fertility. In the woman reported by Bishun et al. (1964), XO/XX mosaicism was associated with reduced fertility rather than sterility, and the presence of parental chromosome anomalies in man has been causally associated with subfertility due to the embryonic death of chromosomally unbalanced gametes (Chandley, Christie, Fletcher, Frackiewicz and Jacobs, 1972). All of the reported mares having the XO anomaly in pure or mosaic form have been sterile. However, there may be a yet undisclosed pool of chromosomally abnormal mares characterized by reduced fertility or apparent embryonic

loss. This hypothesis is supported by the histological findings from the XO mare.

Reports of XO mares have drawn attention to their frequently small size and absence of oestrous cycles (Hughes et al., 1975a , 1975b; Chandley et al., 1975). These findings were confirmed in the present case. However, although the XO mare was never confirmed as being in oestrus, this finding is not uncommon among young maiden mares in their first season at stud, and Hughes et al. (1975b) noted that infertile mares having chromosome anomalies may exhibit similar reproductive behaviour to that of normal anoestrous mares, except that their behaviour is never cyclical.

The reasons for the irascible nature of the XO mare studied here are unknown. The general behaviour of mares with sex chromosome anomalies has not differed appreciably from that of normal mares (Hughes, 1976) and Tennes et al. (1975) reported that women with Turner's syndrome were also usually of normal intelligence. In the only available animal comparison Bruère and Kilgour (1974) found no detectable differences in behaviour between normal and Klinefelter sheep.

Women with an XO sex chromosome constitution tend to have a number of phenotypic abnormalities in common with XO mares, including shortness of stature and the absence of cyclical ovarian activity or menstruation. Most of the phenotypic characteristics of classical Turner's syndrome in women seem to be due to monosomy of the short arms of the X chromosome (Ferguson-Smith, 1965), but studies of patients with structural abnormalities of the X chromosomes have suggested that loci on both the long and short arms are involved in ovarian differentiation (Ferguson-Smith, 1965; Forabosco and Dallapiccola, 1974; Nankin and Loiudice, 1974). The

short arm of the X chromosome and long arm of the Y may contain homologous loci since the presence of either element prevents the short stature and many of the somatic abnormalities of Turner's syndrome (Ferguson-Smith, 1966).

Effect of XO anomaly on the reproductive organs

Except for the ovaries, which were small, firm and lacking in palpable follicles, the genitalia of the XO mare reported here were relatively normal. However, structures presumed to be Graafian follicles in various stages of degeneration, and a distinctive area of apparently functional luteal tissue were found in the right ovary. These findings conflict with other reports of XO mares (Chandley et al., 1975; Hughes et al., 1975a , 1975b) in which the ovaries were invariably devoid of germ cells. That the atretic follicles and luteal tissue were detected in only one section suggests that only few were present at the time of autopsy. The cutting of serial sections from a number of ovarian segments obviously enhanced the detection of the few germinal structures present.

The characteristic appearance of the rudimentary "streak gonads" in many women with Turner's syndrome was first described by Wilkins and Fleischmann (1944). Recognizable ovaries have occurred in all the XO mares reported, and the extreme situation where the gonad exists as a rudimentary streak has not been reported in mares. A kitten with 37XO karyotype also had normal ovaries with follicular development (Norby et al., 1974), while Nes (1968) discerned a 37XO karyotype in each of four Norwegian Landrace pigs characterized by ambiguous or hypoplastic genitalia, leg deformities and anoestrus.

The sex chromosome anomalies are associated with a range of phenotypes depending on the chromosome disorder and the species involved (Table IV.13). The XO anomaly in the horse has not yet been associated with increased prenatal mortality, further evidence that the X chromosome in this species, as in the mouse (Cattanach, 1962), has a more trivial function than in man. However, as in man, normal ovarian differentiation and fertility seem to require the presence of two normal X chromosomes.

Inactivation of X chromosomes

The relatively mild phenotypic changes seen with abnormalities of the sex chromosomes when compared to autosomal errors may be attributed at least partly to the phenomenon of X inactivation (Lyon, 1962) in which one X chromosome is genetically inactive in all female cells. Whether the paternal or maternally derived X is inactivated appears to depend on chance but once established, the inactive state of that particular X is transmitted to all its daughter cells. The female, therefore, has the same amount of active genetic material as the male, although in man, unlike the mouse, the X chromosome is not completely inactivated and some loci may express a dosage effect (Race and Sanger, 1969).

In hybrids of the horse and donkey, the individual X chromosomes can be identified from morphological differences and the presence of the species-specific enzyme, glucose-6-phosphate dehydrogenase, which is controlled by a locus on the X chromosome. Evidence from the mule and hinny has confirmed the Lyon hypothesis that only one X chromosome is functional in female cells and that this state is transmitted

TABLE IV. 13.

SPECIES CHARACTERISTICS ASSOCIATED WITH SEX CHROMOSOME DISORDERS

SPECIES	SEX CHROMOSOME CONSTITUTION				
	XY	XX	XXY	XO	XXX
Mouse	Normal male	Normal female	Phenotypically normal male. Sterile.	Phenotypically normal female. Fertile.	
Cat	Normal male	Normal female	Phenotypically normal male. Sterile.	Phenotypically normal female. Fertility unknown	
Dog	Normal male	Normal female	Phenotypically normal male. Sterile.		
Sheep	Normal male	Normal female	Phenotypically normal male. Sterile.		
Pig	Normal male	Normal female	Phenotypically normal male. Sterile.	Phenotypically abnormal female Sterile.	
Cattle	Normal male	Normal female	Phenotypically normal male. Fertility unknown		Phenotypically normal female. Infertile.
Horse	Normal male	Normal female	Phenotypically abnormal male. Sterile.	Phenotypically normal female. Sterile.	Phenotypically normal female Sterile.
Human	Normal male	Normal female	Phenotypically normal male Sterile.	Phenotypically abnormal female. Sterile.	Phenotypically normal female. Fertile.

to daughter cells. In addition, there seemed to be preferential inactivation of the donkey X chromosome (Hamerton, Richardson, Gee, Allen and Short, 1971).

Triple-X females

The inactivation of X chromosomes additional to one explains the relatively minor phenotypic changes in women with extra sex chromosomes when compared to the severe anomalies associated with trisomy of an autosome as small as number 21. However, it does not entirely prevent their expression in the phenotype. The abnormal features of Klinefelter's syndrome are more severe with extra X chromosomes and many women with additional X chromosomes are physically normal and fertile but have an increased tendency to mental retardation, the severity of which increases with the number of X chromosomes.

In cattle, two triple-X females have been reported. Rieck, Höhn and Herzog (1970) described a Simmental heifer with normal reproductive organs and normal sexual function, and Norberg, Refsdal, Garm and Nes (1976) recorded a triple-X heifer of normal appearance but considered to be probably sterile due to severe depletion of ovarian follicles. Human females with three X chromosomes exhibit a range of phenotypes (Tennes et al., 1975) but fertility is apparently unimpaired (Stewart and Sanderson, 1960), while a triple-X mare had irregular oestrous cycles and produced a presumptive follicle on only one occasion, and ovarian biopsy disclosed a lack of ovarian follicles (Chandley et al., 1975).

In contrast to the syndrome in cattle and humans where fertility is at least possible, a phenotypically normal mare with an extra sex chromosome was shown to be sterile. However,

a variable phenotype has been associated with triple-X females in other species and it may be that this sterile mare represents an extreme of a range of phenotypes yet to be discovered in the horse. Alternatively, these differences may be attributed to species differences in the function of the X chromosome.

Fertility and the lack of an X chromosome

The XO anomaly is not invariably associated with sterility. In fact it is the normal female karyotype in certain species of rodents (Bishop, 1972).

While XO mice (Cattanach, 1962) and rats (Yong, 1971) are normal fertile females, albeit with fertility lower than normal (Lyon and Hawker, 1973), all the XO mares reported have been sterile and XO women are almost invariably sterile and have Turner's syndrome.

The origin of XO species differences may have at least two explanations (Lyon, 1962). In some mammals a short pairing segment of the heterochromatic X may remain active (Hamerton, 1958) and the abnormalities in XO women may result from deficiency of this region. A second proposal related the variable phenotypes observed to the time of X inactivation in different species. Inactivation may occur at a later developmental stage in the horse and man, and anomalies may result from abnormal sex chromosome action before the stage when one X is normally inactivated. Sex chromatin is first detectable in the human embryo after 16 days gestation (Park, 1957); in the rat which has an embryology similar to the mouse it is first evident after seven days (Zybina, 1960), which probably corresponds to an earlier stage. The stage when sex chromaton is first detectable in the horse embryo

is unknown.

After migration into the primitive gonad, primordial XO cells degenerate more rapidly than cells with XX or XY sex chromosomes, and if an appropriate number of germ cells fail to survive, ovarian development does not occur. Similarly, testicular development will not proceed in the absence of XY germ cells in the gonad. Hamerton (1968) suggested that the migration and development of germ cells requires only one X chromosome but that the second X is necessary to control their rate of atresia and perhaps also to control oestrogen production.

Unlike the mouse, normal female development with complete ovarian differentiation in mares and women requires the presence of two normal X chromosomes. However, some breast development and even menstruation has occurred in a few 45XO women, and there is one report of an XO woman who gave birth to a normal son (Bahner, Schwarz, Harnden, Jacobs, Heinz, and Walter, 1960). In the latter case, sex chromosome mosaicism was not detected in any tissue.

A recent hypothesis by Lyon and Hawker (1973) related the degree of infertility associated with an XO sex chromosome complex to the longevity of the species. They suggested that in all XO individuals, oocyte development is similar, but in larger mammals with a longer delay before puberty, the degeneration of germ cells results in the exhaustion of viable oocytes before sexual maturity. Indeed, germ cells are present in the gonad of the human XO embryo (Singh and Carr, 1966) but have disappeared or are severely depleted at birth (Carr, Haggar and Hart, 1968), and when puberty is reached the gonad is usually devoid of gametes. Gametes persist, however, in the gonads of the post-pubertal XO mouse which has a much

shorter reproductive life. In older XO mice, however, reproduction stopped due to a shortage of oocytes (Lyon and Hawker, 1973). Conceivably, gametes might sometimes persist in the ovary of an XO woman so exceptional cases of fertility are not really surprising.

The presence of normal follicles in the ovaries of a newborn XO kitten (Norby et al., 1974) appeared to conflict with the findings in infertile XO women, but is not necessarily an indication of fertility. In human XO embryos the gonads develop normally up to the third intrauterine month and in this kitten too, the germ cells may gradually have been lost from the ovary before puberty. The domestic cat is relatively short-lived in comparison with the horse and man and may attain sexual maturity before degeneration of oocytes is complete. On the basis of the above hypothesis, the XO cat is more likely to be fertile than its equine or human counterpart.

In the sow and mare the XO anomaly has been invariably associated with sterility. These mammals have a much longer reproductive life than rodents so more severe depletion of oocytes might be expected, yet a number of degenerating Graafian follicles were discovered in the ovary of an XO mare reported in this study. While the occurrence of atretic follicles and organised luteal tissue does not affirm that ovulation has occurred, it does indicate that the ovary was capable of at least some cyclic activity. If fertility is related to the rate of oocyte degeneration, the finding of an occasional fertile mare would not be unexpected.

The full relationship of chromosome disorders with development and prenatal loss in the mare is unknown, but the

presence of chromosomal errors may help to explain the low fertility of the thoroughbred mare. From preliminary reports, the high incidence of chromosome anomalies among infertile mares is emphasized, and chromosome studies can obviously be of considerable value in the investigation of infertility problems in mares at breeding studs. Chromosome studies have added importance in this regard due to the difficulties in detecting chromosomally abnormal mares clinically when compared with similar chromosome disorders in women. The clinical effects of the XO anomaly are mainly manifested in the abnormal development of the primary and secondary sex characteristics, which are more difficult to recognize in mares. Further, in view of the high prices currently realized at thoroughbred and standardbred yearling sales in New Zealand, and other breeds of horses throughout the world, the chromosome analysis of animals offered for sale is an important practical application.

PART V

CHROMOSOME STUDIES IN ABORTION AND EMBRYONIC LOSS

PART V

CHROMOSOME STUDIES IN ABORTION AND EMBRYONIC LOSS

1. LITERATURE REVIEW

i) Chromosomes and human abortion

a) Incidence of abortion

Javert (1957) defined human abortion as the termination of pregnancy before 22 weeks of gestation or before foetal weight exceeds 500g. Although the actual incidence of spontaneous abortion is in doubt it has been widely believed that 10-20% of human gestations terminate in this way. This is probably a conservative estimate as in cases of early foetal loss the patient is often unaware that she is pregnant, and Creasy and Crolla (1974) considered that 25% of recognizable pregnancies end in abortion.

An early study by Hertig et al. (1956) indicated that as far as early conceptus loss was concerned, these estimates were low. In a series of human ova obtained by hysterectomy from women of known fertility, they discovered abnormal conceptuses, many of which would probably never have implanted. Accounting for early conceptual loss by the addition of about 30% failure of grossly abnormal conceptuses to the estimate of clinical abortion, Carr (1970a) proposed a total incidence of 45% for the spontaneous loss of conceptuses in women.

b) Chromosome studies in spontaneous abortions

Chromosome disorders were first reported almost simultaneously from three sources. Trisomy of chromosome 21 was found in association with mongolism or Down's syndrome (Lejeune, Turpin and Gautier, 1959) and abnormal sex chromo-

some constitutions were described in patients with Turner's syndrome (Ford, Jones, Polani, De Almeida and Briggs, 1959) and Klinefelter's syndrome (Jacobs and Strong, 1959; Ford, Jones, Miller, Mittwoch, Penrose, Ridler and Shapiro, 1959).

Since these original discoveries, an overwhelming amount of information has accumulated in this field. A wide variety of chromosome disorders have been found in clinical patients (Makino, 1964; Carr, 1969) and also occasionally in phenotypically normal persons (Warkany, Weinstein, Soukup, Rubinstein and Curless, 1964).

Initially it appeared that specific chromosomal abnormalities might be correlated with certain clinical syndromes. This association has gradually broken down although notable examples of such correlations do exist. The best known example is the association of mongolism or Down's syndrome with at least partial trisomy of chromosome 21.

The Geneva Conference (1966) concluded that chromosomal abnormalities were a significant factor in spontaneous abortions, since 19% of nearly 800 spontaneous abortions reported up to that time were found to have a chromosome anomaly. In order to standardize procedures for chromosome studies of abortion, definitions and nomenclature were defined by the Geneva Conference (1966). This terminology was adhered to in the present study.

Considerable variation was evident from separate cytogenetic studies of human abortion. From 18 studies believed to be unselected, the mean incidence of heteroploid abortuses was 21.5% (Carr, 1970a) and even in three studies involving the chromosome analysis of 100 or more abortions, the incidences of chromosome errors were 38% (Boué, Boué and

Lazar, 1957), 22% (Carr, 1967a) and 8% (Stenchever, Hempel and Macintyre, 1967).

Many factors are known to influence the incidence of chromosome anomalies in populations. Problems have arisen when comparing different populations, and to assess geographical variation, guidelines for standardization were drawn up at the Geneva Conference (1966).

The prevalence of unsuspected induced abortions in the population may influence the frequency of chromosome disorders. Hospital admission practices may be another source of bias; while women in some areas are admitted to hospital for early abortion, similar patients in other areas may be treated at home.

A more important factor is the possible selection of the abortus material itself. Unknown to the investigator, the abortus may have been selected on anatomical grounds by the physician. Intact sacs, either empty or containing only a stunted remnant of embryonic material are much more likely to yield a chromosome anomaly than those containing a normal embryo or foetus. Anomalous embryos apparently have only about a 50% chance of containing normal chromosomes (Singh and Carr, 1967) and by selecting for so-called "blighted ova", Thiede and Metcalfe (1966) found an extremely high incidence (88%) of chromosome anomalies.

Abortus material may also have been selected for age of the conceptus. Boué and Boué (1969) reported that 70% of embryos with a developmental age of six weeks or less are chromosomally abnormal. From collected studies, Carr (1970a) found that the incidence of chromosome anomalies remained at about 40% for the first 90 days of gestation, fell to approximately 25% between the 13th and 17th weeks, and at

later stages chromosome anomalies were found in only 3.5% of abortuses.

After correcting for the various sources of bias, Carr (1970a) calculated that approximately 36% of all spontaneous abortions in humans are chromosomally abnormal.

c) Types of chromosome anomalies in abortions

The reaction of the organism to chromosome disorders varies between the plant and animal kingdoms as well as among different animal classes. Not all chromosome aberrations of a given type are prenatally lethal, while some chromosome defects may never be found because of their lethality at the gametic stage.

In general, the chromosomal abnormalities described in spontaneous abortions are the same as those reported from livebirths. Most of the chromosome anomalies found in abortuses have been numerical, involving one of three types: autosomal trisomy, monosomy X and polyploidy, although a variety of miscellaneous anomalies have been reported. The frequencies of the chromosome disorders among spontaneous abortions in women are shown in Table V.1; the results of cytogenetic studies in spontaneous abortion are summarized in Table V.2.

Table V.1. Chromosome anomalies among human spontaneous abortions (Carr, 1970a)

	Chromosome anomaly					
	Trisomy	XO	Polyploidy			Other
			3X	4X	Other	
Frequency	45%	20%	19%	5%	1%	10%

The commonest chromosome anomaly among abortions and liveborns is autosomal trisomy, characterized by the presence of one extra chromosome. Autosomal trisomy has been found in about 45% of chromosomally abnormal spontaneous abortions in women.

Polyploidy is the term used to define multiples of the haploid number of chromosomes, including triploidy and tetraploidy. Approximately one quarter of the chromosome anomalies in spontaneous abortions have involved whole extra sets of chromosomes.

About 20% of human spontaneous abortions are due to the absence of one sex chromosome (45XO), the only monosomy common in man, and the remaining 10% of chromosome disorders include autosomal monosomies, double trisomies, mosaics and structural anomalies.

TABLE V. 2 SUMMARY OF CHROMOSOME STUDIES IN HUMAN SPONTANEOUS ABORTIONS

AUTHOR	Number of specimens	Age (weeks)	Analyzed		Abnormal Karotype		CHROMOSOMAL ANOMALY								
			Number	%	Number	%	Trisomy	XO	Triploidy	Tetraploidy	Mosaics	Structural abnormality	Double trisomy	Autosomal monosomy	Other
Arakaki and Waxman, 1970 Waxman, Arakaki and Smith, 1967	358	<20	127	35.5	63	49.5	19	18	7	1	14	1		1	2
Boué, Boué and Lazar, 1967 Boué and Boué, 1970	187	<20	132	70	50 267	37 51	27	8	10	1	2		2		
Bowen and Lee, 1969	163	<15	41	25	12	29	3	4	3		1	1			
Carr, 1963, 1965, 1967a	395	<16	227	57.5	50	22	26	12	9				1		
Clendenin and Benirschke 1963	18	9–17	10	59	3	30	1	1				1			
Hall and Kallén, 1964		<13	8		2	25	2								
Inhorn, Therman and Patau, 1964	135		?88	65	8		1		2	2				3	
Jacobson and Barter, 1967	52	<13	35	67	8	23	3	2						1	2
Kajii, Ohama, Niikawa, Ferrier Avirachan, 1973	216	<13	152	70	82	54	50	12	10	5		3	1	1	
Kerr and Rashad, 1966	62	<term	35	56	6	17	3				1		2		
Larson, 1969	69		40	58	16	40	8	5	3						
Lauritsen, Jonasson, Therkelson, Lass, Lindsten and Peterson, 1972	71	<16	68	96	34	50	14	12	3	4			1		
Makino, Ikeuchi, Sasaki, Muramoto Shimba, Fujimoto and Matsuda, 1967	73	6–24	37	51	4	11	1		1			1			1
Mikamo, 1970	150	<17	67	45	17	25.4	7	2	3	2					3
Smith, MacNab and Ferguson-Smith, 1969	22	<24	16	72	3	19		1	1		1				
Stenchever, Hempel and Macintyre, 1967		<20	101	65	8	8	1	2	3		2				
Szulman, 1965	75		25	33	16	64	6	5	5						
Thiede and Metcalfe, 1966	179		54	30	24	44	15	5	1	3					
Thiede and Salm, 1964	51	<20	12	24	7	58	4	4	1	1					

d) Trisomy and monosomy in spontaneous abortions

The processes of chromosome splitting and polar migration are referred to as disjunction. Nondisjunction is the most frequent functional error of both meiosis and mitosis and accounts for many of the abnormalities of chromosome number (aneuploidy). A less common cause of aneuploidy involves premature disjunction with the loss of a chromosome through anaphase lagging.

Within a group of chromosomes, different trisomies are eliminated at different stages of development. Trisomy of a large autosome has such a severe effect on the embryo that it usually results in abortion. In general, the larger the chromosome involved, the less commonly it has been encountered in trisomic form. If all trisomies occurred with equal frequency, their occurrence should be related to the size of the group. Such has not been the cause so the chance of occurrence of a particular trisomy cannot be random. As well as double trisomies (Carr, 1967a), members of all the chromosome groups have been represented in trisomic abortuses, with Groups D, E and G being the most frequent (Kajii et al., 1973).

Theoretically, monosomy should be just as common as trisomy, but trisomies have outnumbered monosomies by about 20 to one. Autosomal monosomy has a more severe effect on the conceptus than trisomy and the majority are presumably lost before developing to the stage of clinically recognizable abortion. While monosomy X is common, autosomal monosomy is rare, but monosomies of some autosomes (Pawlowitzki, Cenami and Frischbier, 1973) and even double monosomy have been described.

Monosomy of the X chromosome is the only monosomy common in man, accounting for 20% of all heteroploid abortuses. The XO disorder is apparently one of the commonest chromosome anomalies in man but owing to a large foetal loss it is relatively uncommon at term.

e) Polyploidy

Polyploidy is apparently common in man and other mammals but is lethal before birth. As long as the chromosome complement is an exact multiple of the haploid number, polyploidy may be considered an anomaly of fertilization or post-fertilization rather than an abnormality of the chromosomes themselves. Triploidy, involving three haploid chromosome sets has been more common in abortuses, with an incidence three times greater than that of tetraploidy.

A high proportion (20%) of triploid embryos have shown focal disorders, but the most consistent phenotypic feature in abortions with triploidy has involved the placenta rather than the embryo itself. Cystic degeneration of the chorionic villi produces the condition known as hydatidiform degeneration, first noticed in association with triploidy by Makino, Sasaki and Fukushima (1964).

Viable triploid individuals have been found only in certain amphibia and birds (Ohno, Kittrell, Christian, Stenius and Witt, 1963). Triploid embryos in the mouse survive only until mid-gestation (Fischberg and Beatty, 1952) and probably as in man, where less than 1% of triploid embryos survive (Edwards, Yuncken, Rushton, Richard and Mittwoch, 1967), the majority of triploid conceptuses in domestic animals and birds die during early pregnancy. Whereas ten children with diploid/triploid mosaicism have survived beyond the neonatal period (Niebuhr, 1974; Dewald,

Alvarez, Cloutier, Kelalis and Gordon, 1975), human foetuses with complete triploidy have died either *in utero* or in the early neonatal period (Niebuhr, 1974; Henrikson, Häkansson and Sandahl, 1974). Mammalian triploids are apparently not viable in post-natal life unless they contain a sufficient number of diploid cells, and of domestic animals the only two triploid individuals surviving to adulthood, a tortoiseshell cat (Chu *et al.*, 1964) and a bovine true hermaphrodite (Dunn *et al.*, 1970) proved to be diploid/triploid mosaics.

Tetraploid abortuses are eliminated earlier in gestation than triploids and have been characterized by extremely poor development (Carr, 1971). Tetraploidy apparently has a more severe effect on embryonic development than triploidy and it is not surprising that there are no reports of live-born individuals with this chromosome constitution.

Origin of Polyploidy

Evidence for the development of triploidy has been summarized by Austin (1960) and Niebuhr (1974). The condition results from the presence of three haploid nuclei, two from one parent and one from the other. The extra haploid set of chromosomes may be of maternal (digyny) or paternal (diandry) origin.

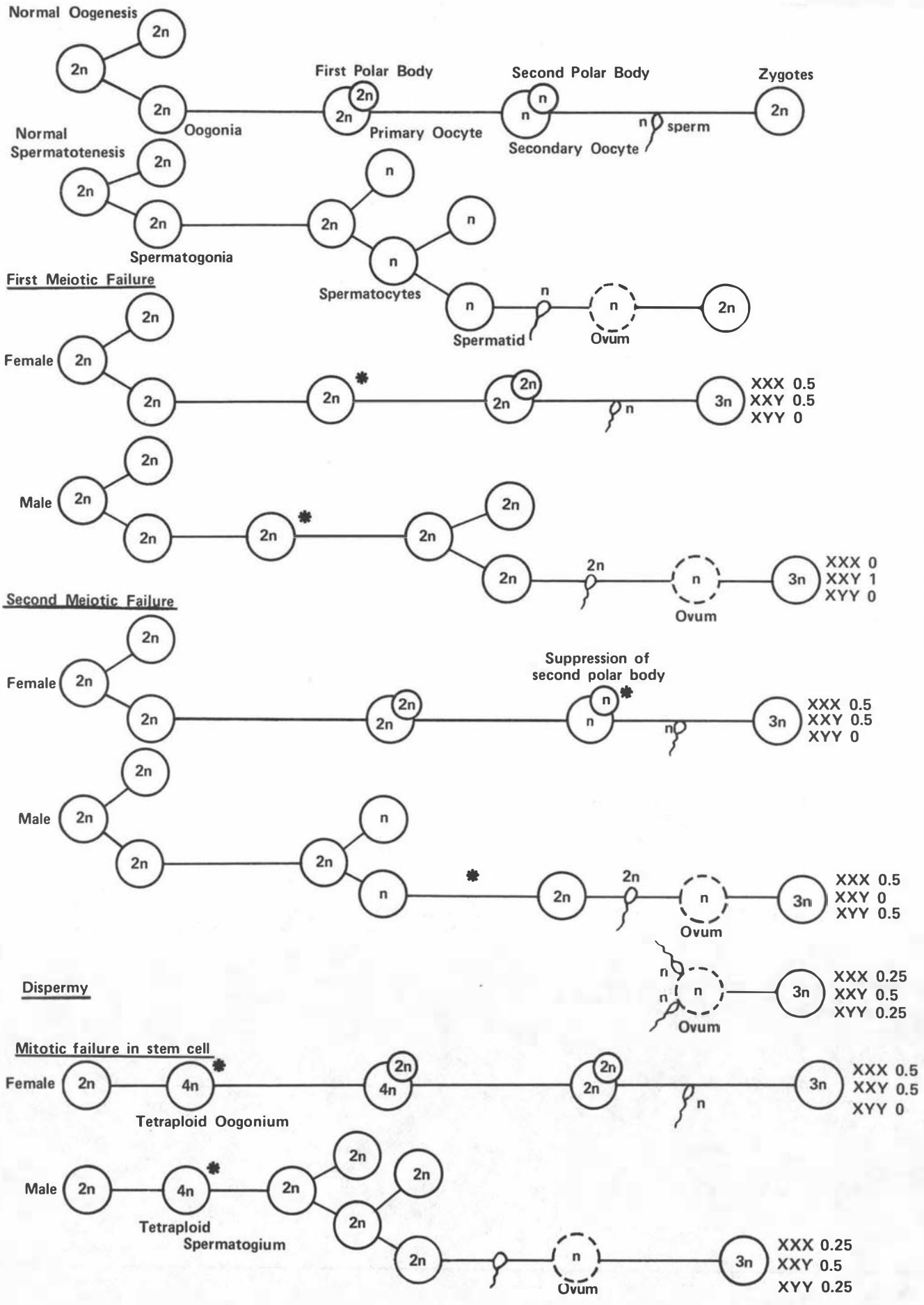
In theory, triploid zygotes may arise from failure in the first or second meiotic division of oocytes or spermatocytes, from dispermy, or from mitotic disturbances in the germ cell precursors. The possible mechanisms for the origin of triploidy are shown in Figure V.1. Complete triploidy may also result from other mechanisms (Edwards *et al.*, 1967) including the malsegregation of one set of haploid chromosomes from the zygote and tripolar division of a triploid

or tetraploid zygote.

Dispermy is one of the major causes of triploidy in the pig (Hancock, 1959) and has been increased by aging of the ovum with delayed mating (Austin, 1960). It has also been observed in the cat, sheep and cow at a probable incidence of 1-2% (Austin, 1960).

Tetraploidy probably arises by normal division of the chromosomes at the first mitosis and suppression of the first cleavage of the zygote.

FIGURE V.I. POSSIBLE MECHANISMS FOR ORIGIN OF TRIPLOIDY



f) Miscellaneous chromosome anomalies in abortions

A small proportion (10%) of chromosomally abnormal abortions have contained a variety of miscellaneous chromosome errors. These have comprised mosaicism (Arakaki and Waxman, 1969), and a range of structural aberrations including translocations (Bass, Crandall and Marcy, 1973; Kajii, Meylan and Mikamo, 1974), ring chromosomes (Arakaki, Waxman and Nonomura, 1969; Moore, Heller and Thomas, 1973), a dicentric chromosome (Aula and Hjett, 1962), deletions and unexplained chromosome breaks (Makino et al., 1967).

g) Parental chromosomes and spontaneous abortion

From the accumulated results of 23 surveys, Papp, Sandor and Dolhay (1974) noted that chromosome anomalies responsible for multiple aberrations had occurred in 70 (6.5%) of 1066 married partners and that translocations were more frequent than in the general population. Many different types of chromosomal aberration including reciprocal translocations (Makino, Aya and Sasaki, 1965; McKay, Hodgkin and White, 1967; Kadotani, Ohama, Sofuni and Hamilton, 1970; Lindenbaum and Bobrow, 1975), centric fusions (Lucas, 1969) and mosaicism (Bishun and Morton, 1968) have been incriminated in recurrent abortion, but unless the abortus can be examined chromosomally it is not possible to directly relate the parental chromosome aberration to the abortion. As well as a higher risk of producing chromosomally abnormal gametes, carriers of balanced translocations are more prone to meiotic nondisjunction of other chromosomes (Hamerton, Giannelli and Carter, 1963).

Although the occurrence of translocations or mosaicism predisposes the carrier to abortion (Jacobsen, Dupont and Mikkelsen, 1963; Bishun et al., 1964; Bishun and Morton,

1968), parental chromosome anomalies are probably not a common cause of recurrent abortion. Surprisingly few translocations have been found among spontaneous abortions but many aberrations may be undetectable by direct staining techniques, and Kim, Hsu, Paciuc, Cristian, Quintana and Hirschhorn (1975) have demonstrated structural rearrangements by chromosome banding.

h) Polymorphisms

Several early reports (Schmid, 1962; Carr, 1963) suggested that observed chromosomal defects such as the extra chromatin piece translocated to the short arm of one chromosome in both the propositus and its father described by Schmid (1962) may have caused recurrent abortion. Many such "defects" are now known to be normal variants.

Court-Brown, Jacobs and Brunton (1965) found a structural chromosome rearrangement in about 0.5% of normal human adults. These changes seemed to be "without effect on their carriers not only in terms of development but also in regard to the risk of the conception of abnormal children".

i) Chromosome anomalies in induced abortions

The occurrence of chromosome errors in spontaneous abortions is of major significance when compared with the low frequency of chromosome anomalies among induced abortions. The findings from chromosome studies in induced abortions are summarized in Table V.3. From a total of 891 therapeutic abortions in which chromosome analysis was undertaken, chromosome anomalies were detected in 29 specimens, an incidence of 3.25%. These figures emphasize the marked contrast between the low frequency of chromosome errors in induced abortions and the frequency among spontaneous abortions which has been conservatively estimated at 36% (Carr, 1970a).

TABLE V. 3. CHROMOSOME STUDIES IN INDUCED ABORTIONS

AUTHOR	NUMBER OF ABORTIONS	CHROMOSOMES		
		NORMAL	ABNORMAL	
			NUMBER	%
Thiede and Salm, 1964	37	34	3	8.1
Szulman, 1965	15	15	0	
Kerr and Rashad, 1966	15	14	1	6.7
Carr, 1967a	22	22	0	
Jacobson and Barter, 1967	53	50	3	5.7
Sasaki, Makino, Muramoto, Ikeuchi and Shimba, 1967	358	348	10	2.9
Yasuda, Matsuda and Nonomura, 1967	149	146	3	2
Suzoromi, 1968	10	10	0	
Valenti, 1968	18	17	1	5.5
Boué *et al.*, 1967	35	34	1	2.9
Bowen and Lee, 1969	7	6	1	14.3
Hahnemann, 1973	172	166	6	3.5
TOTAL	891	862	29	3.25

ii) Chromosomes and embryonic death in animals

More than one third of the spontaneous abortions in women can be attributed to chromosome disorders. Cytogenetic studies of embryonic loss in animals have been few, but preliminary reports have indicated the potential for further investigation in this area.

The chicken

Among 344 early chick embryos, Miller, Fechheimer and Jaap (1971) found a high frequency with abnormal karyotypes, including many cases of triploidy and its mosaic variants. Other studies have revealed a variety of chromosome anomalies, many in phenotypically abnormal embryos (Fechheimer, Zartman and Jaap, 1968; Bloom, 1969).

The rabbit

From a chromosome study of 463 rabbit blastocysts, Fechheimer and Beatty (1974) recorded a 5% incidence of spontaneous heteroploidy, including triploidy, trisomy and five mosaic karyotypes. One haploid and two diploid/triploid mosaics were described in embryos from normally-mated rabbits (Hansen-Melander and Melander, 1971).

Shaver and Carr (1967, 1969) demonstrated an increase in the incidence of chromosome anomalies, particularly triploidy, in blastocysts from rabbits in which fertilization had been delayed. Austin (1967) also found that aging of ova by delaying fertilization led to an increase in chromosomal aberrations in the embryos. Between six and ten days after fertilization, however, the difference in chromosome counts between embryos from normally-mated rabbits and those in which insemination had been delayed had disappeared, and Shaver and Carr (1967) further noted that an ovulation-

mating interval of more than ten hours resulted in a marked reduction in the number of blastocysts recovered. Thus it appeared that most embryos with abnormal chromosome constitutions were eliminated early in gestation.

Domestic animals

McFeely (1967) demonstrated a variety of chromosome defects, particularly polyploidy, in 10% of blastocysts from normal pigs. A further 2.3% of blastocysts were already degenerating. Among 15 blastocysts, Moon, Rashad and Mi (1975) found one tetraploid karyotype and three diploid/triploid mosaics. Since these anomalies have not been discovered in living pigs, at least some of them are presumably associated with embryonic death. Smith and Marlowe (1971) reported only one abnormal karyotype, a monosomy, among 76 25-day pig embryos, although a further eight embryos were already dead and in various stages of degeneration. They concluded that chromosomally abnormal pig embryos rarely survive implantation.

Following delayed insemination in sows, Bomsel-Helmreich (1965) found 6% of triploid embryos after 18 days gestation, but no chromosomal abnormalities after 26 days. As in rabbits, chromosomally aberrant pig embryos seem to be eliminated during early pregnancy.

The only other domestic animal in which a cytogenetic study of embryonic loss has been reported is the cow. From a study of 12 bovine blastocysts aged between 12 and 16 days of gestation, McFeely and Rajakoski (1968) reported a presumptive diploid/tetraploid mosaic. A case of embryonic death due to autosomal trisomy in one of four foetuses from a pregnant cat was described by Benirschke *et al.* (1974).

Pulos and Hutt (1969) attributed a genetic cause to one type of embryonic death in horses. From mating trials, they decided that when homozygous, the autosomal gene for white coat colour was lethal early in development.

A chromosomal translocation in a Swedish Landrace boar was shown to be associated with reduced fertility due to embryonic loss (Henricson and Bäckström, 1964). Five types of unbalanced karyotypes were detected in foetuses sired by the translocation heterozygote, and 41% of the progeny had the same translocation as their sire (Äkesson and Henricson, 1972). Since none of the 111 surviving offspring possessed an unbalanced chromosome constitution, the defective karyotypes were assumed to be lethal at the embryonic stage. A similar mechanism whereby the rearrangement of chromosomes results in reduced fertility from embryonic loss may also exist in cattle (Hansen, 1969).

The significance of translocations on reproductive performance in animals is by no means fully understood. Despite traditional emphasis on their possible deleterious effects (Gustavsson, 1969), translocations of the centric fusion type may be advantageous (Bruère, 1975), and have been suggested as a basic evolutionary process (White, 1969). The first report of an autosomal translocation in a horse was described in a normal stallion (Quéinnec, Berland, Darré and Carlotti, 1975). The abnormality was present in all tissues examined but details of fertility were unavailable.

iii) Aetiology of chromosome disorders in abortions

Many factors of exogenous and endogenous origin can influence the frequency of chromosome disorders in human abortions (Fechheimer, 1972). These include certain virus groups (Stoller and Collman, 1966; Nichols, 1966), some drugs and other chemical compounds (Stevenson, Bedford, Hill and Hill, 1971), maternal exposure to ionizing radiation (Uchida, Holunga and Lawler, 1968; Rugh and Budd, 1975) and possibly autoimmune disease (Fialkow, 1966, 1967). The aging of gametes prior to fertilization is a popular explanation for the origin of chromosomally abnormal zygotes, and a maternal age effect has been noticed in some species.

Hormones

From women who conceived within six months of discontinuing the use of oral contraceptives, Carr (1969, 1970b) reported a dramatic increase in the incidence of triploid abortuses. Similar studies have revealed no increase in polyploidy among abortions (Boué et al., 1973; Lauritsen, 1975) and the role of steroid hormones in the production of chromosome aberrations has yet to be defined.

Abnormal oestrogen levels have resulted in anomalous embryonic development in rabbits (Widmeyer and Shaver, 1972) and mice. Following the treatment of pregnant mice with diethylstilboestrol diphosphate, there was a significant increase in the number of aneuploid cells from eight day mouse embryos (Chrisman, 1974) and from the bone marrows of adult male mice (Chrisman and Hinkle, 1974). The degree of aneuploidy depended on both the dose and the duration of exposure to the drug.

Maternal age

An association between maternal age and the occurrence of certain chromosome disorders has been noticed in some species. The frequency of trisomic embryos has been found to increase with maternal age in women (German, 1968), and mice (Gosden, 1973; Yamamoto, Endo and Watanabe, 1973), while the proportion of triploid embryos has not. The higher frequency of certain chromosome disorders from older dams has been related to hormonal disturbances (Carr, 1967b), delayed fertilization secondaryto decreased frequency of coitus (German, 1968), and reduced frequency of chiasmata formation in older oocytes (Henderson and Edwards, 1968), but the factors responsible for the maternal age effect have remained unknown.

Aging of gametes

The aging of gametes is apparently an important cause of chromosomal defects in the mammalian conceptus, and many types of chromosome aberrations have been produced.

Delayed ovulation has been shown to result in chromosome anomalies and embryonic death in rats (Fugo and Butcher, 1966; Butcher and Fugo, 1967), mice (Vickers, 1969) and rabbits (Shaver and Carr, 1969). In particular, there was a dramatic increase in the incidence of triploidy.

The timing of ovulation is an important factor in the development of mammalian triploidy. If fertilization is delayed, shedding of the second polar body or the normal block to polyspermy is defective, but there are marked species differences. Whereas delayed mating in rabbits and mice leads to an increased frequency of triploid blastocysts from digyny, in the rat and pig a longer interval between

ovulation and mating renders the ovum more susceptible to polyspermy.

2. RESULTS AND DISCUSSION

A cytogenetic study was undertaken on a series of equine embryos surgically removed from mares' uteri. The materials and techniques employed were previously detailed in Part II.4.

i) Clinical details of equine embryos

Tissue was obtained from 26 equine embryos following their surgical removal from uteri. The clinical details of these specimens are given in Table V.4.

Embryos of the same size are not necessarily the same age. Variations may relate to the low precision of measurement, differences in the initial size of the egg and its genetically controlled rate of development, arrival time of the egg into the uterus and speed of implantation. Douglas and Ginther (1975) reported a maximum variability in crown-rump length of the equine foetus between 50 and 80 days of pregnancy.

The embryos examined in this study varied in estimated gestational ages from 28 to 64 days. Figure V.2 shows a normal horse embryo of approximately 32 days gestation. The embryo is clearly visible within its intact allantochorion which is fully formed and slightly opaque due to the early development of allantochorionic villi over its entire surface. A picture of the embryonic pole of the same conceptus shows the remnant of the yolk sac which is surrounded by an indistinct pale avascular area representing the chorionic girdle (Figure V.3). The sinus terminalis is clearly visible.

After the removal of its surrounding membranes, the embryo appears well differentiated and the limbs and tail are recognizable (Figure V.4).

An embryo slightly more advanced in development (34 days gestation) is shown in Figure V.5. The umbilical arteries are well developed and the well formed embryo is surrounded by a distinct amnion.

TABLE V. 4. CLINICAL DETAILS OF EQUINE EMBRYOS.

Specimen number	"Crown-rump" length (cm)	Estimated duration of pregnancy (days)	First cells studied (days)
1B	4.0	42	2
2B	4.5	46	2
3B	4.0	42	2
4B	3.5	37	2
5B	1.5		2
6B	3.6	38	2
7B	3.0	32	3
10B	3.4	36	2
11B	3.4	36	3
12B	4.7	48	3
13B	3.0	32	3
14B	5.0	50	6
15B	7.5	64	6
16B	2.8	30	6
17B	7.1	62	8
18B	4.6	47	6
19B	3.2	34	5
20B	7.0	61	6
21B	3.2	34	8
22B	2.6	28	9
23B	2.8	30	5
24B	3.8	40	4
25B	6.5	59	20
26B	3.8	40	4

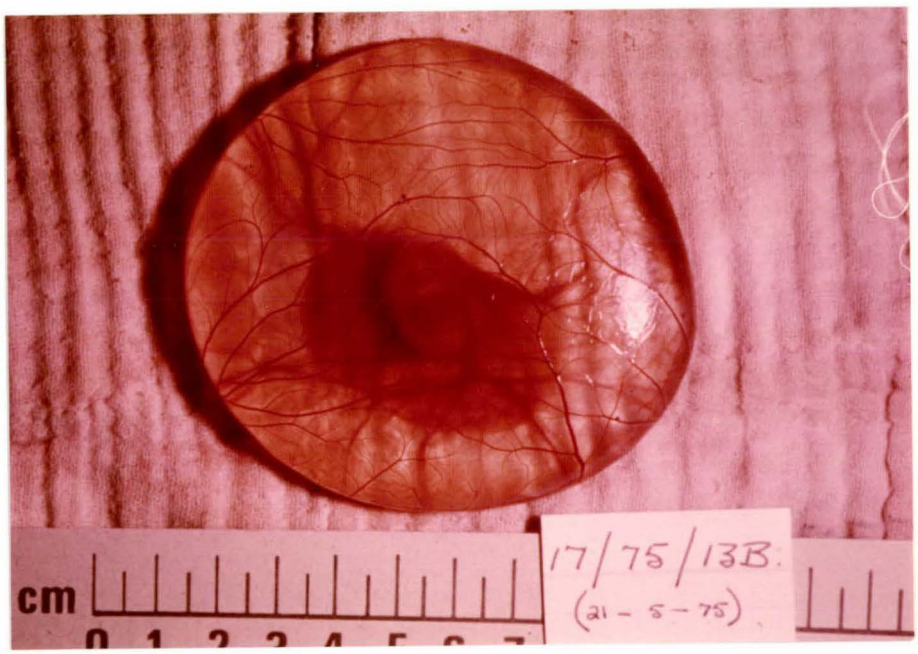


Figure V.2. Normal horse embryo (E. caballus) within intact allantochorion (32 days gestation).

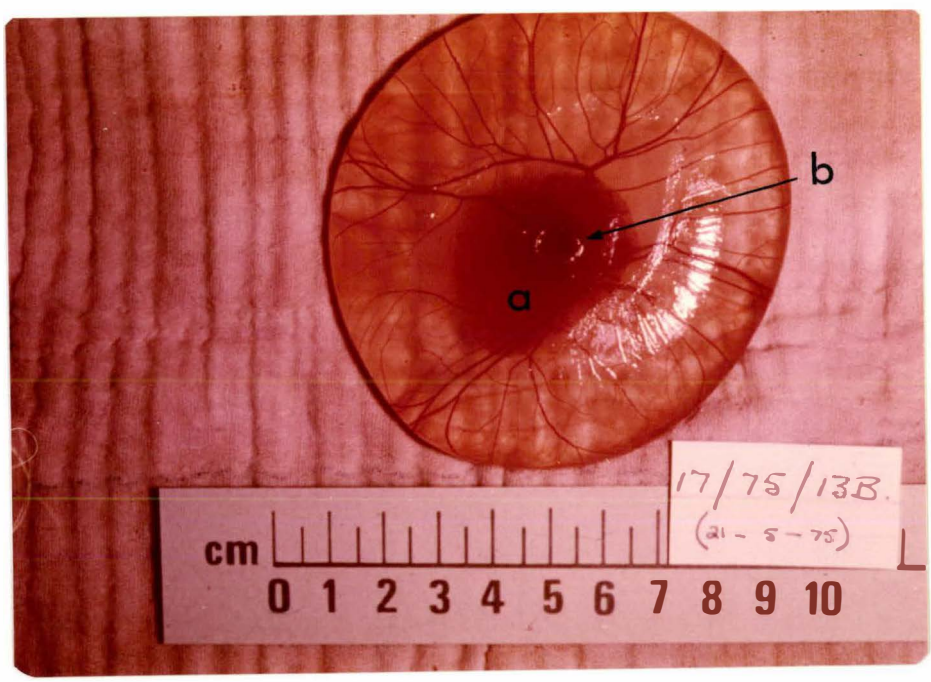


Figure V.3. Normal horse embryo (32 days gestation) showing remnant of yolk sac (a) and the sinus terminalis (b).

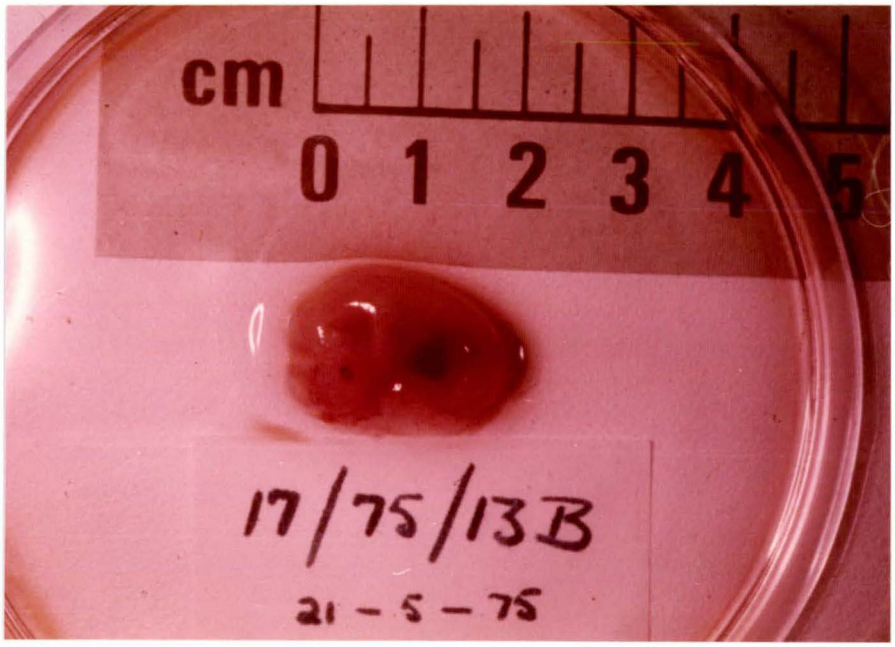


Figure V.4. Normal horse embryo (32 days gestation) with membranes removed.

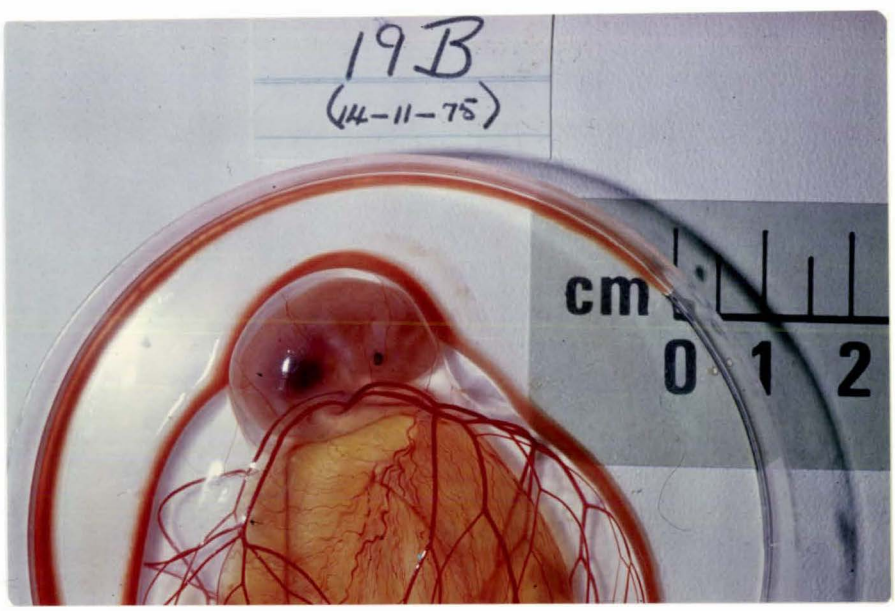


Figure V.5. Equine embryo (34 days gestation) with well developed amnion and umbilical arteries.

ii) Chromosome studies

Cell cultures from 22 horse embryos grew sufficiently well to provide adequate chromosome preparations. The results of these chromosome studies are shown in Table V.5.

Due to problems of bacterial contamination, insufficient cells were counted and no cells could be analyzed for cases 4B and 5B. The chromosome constitutions of these specimens are therefore unknown. Specimens 8B and 9B were obtained from a twin pregnancy of fewer than 20 days duration (Figure V.6). Cell cultures were initiated, but viable cells died out before a sufficient number were present for harvesting.

The 22 equine embryos which were successfully cultured and chromosomally analyzed were found to have normal karyotypes. A variable number of metaphase spreads, including the majority of hypodiploid cells, were karyotyped for each specimen. For each embryo, the karyotyped aneuploid cells were found to be broken cells with irregular losses or gains of elements. As mentioned previously, the quality of metaphase preparations obtained from equine embryos compared favourably with results from similar studies of human abortions and rabbit blastocysts (Table IV.5).

One metaphase among 207 cells examined from a 38 day embryo was found to contain a structural aberration (Figure IV.8). This anomaly was previously discussed in Part IV, 1, ii(b). It was considered to represent an association between two homologous chromosomes, with premature separation of the chromatids.

The normal horse sex ratio at birth is 52.5% (Short, 1975). Sex chromosomes from embryos in this study revealed an equal number of males and females.

TABLE V. 5. CHROMOSOME STUDIES OF EQUINE EMBRYOS

Specimen number	COUNT DISTRIBUTION						Cells counted	Number of karyotypes	Sex chromosomes
	<63	63	64	65	>65	Polyploid			
1B	16	12	148	7	1	1	185	24	XY
2B	17	17	123	2	0	2	161	19	XY
3B	5	10	90	6	0	0	111	15	XX
4B	0	1	1	4	0	0	6	0	
5B	0	1	7	1	0	0	9	0	
6B	16	23	159	6	3	0	207	47	XY
7B	1	3	20	0	0	0	24	5	XY
10B	0	7	46	4	0	0	57	4	XY
11B	10	8	135	2	3	9	167	21	XX
12B	9	15	111	8	0	0	143	9	XX
13B	19	15	111	4	1	0	150	10	XX
14B	7	10	144	5	1	0	167	9	XX
15B	7	10	131	5	0	0	153	8	XX
16B	9	6	59	6	0	0	80	5	XY
17B	4	2	36	2	0	0	44	3	XY
18B	5	4	49	2	0	2	62	4	XX
19B	4	5	21	0	1	0	31	4	XX
20B	10	6	101	1	0	2	120	4	XY
21B	3	5	62	1	1	1	73	3	XY
22B	7	3	51	0	0	10	71	3	XY
23B	2	7	57	0	0	3	69	6	XX
24B	6	13	70	1	0	0	90	5	XY
25B	12	6	84	1	0	9	112	4	XX
26B	14	3	79	2	0	2	100	3	XX

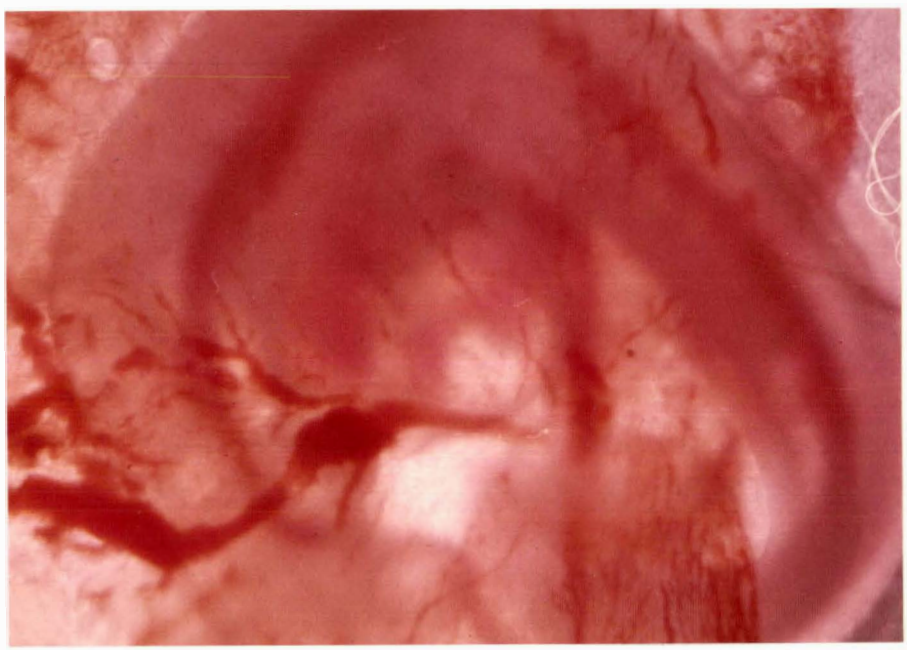


Figure V.6. Equine twin embryo of fewer than 20 days gestation. (Magnification 40x).

Problems with cell cultures

The efficiency of the cell culture technique is of vital importance to a study of this type. Success rates for the culture of aborted material from women have shown a wide variation, and from two large series of studies of human abortions, attention was drawn to the high failure rate of cell cultures (Carr, 1967a; Boué et al., 1967). In the current report, successful cultures were obtained from 84.6% of equine embryos. This is a considerably higher rate of success of chromosome analysis than the 56% achieved for 2276 spontaneous abortions in women (Table V.2).

Cell cultures initiated from spontaneous abortions in women have sometimes failed after only minimal growth in vitro. Waxman et al. (1967) suggested that the abnormal growth of these cultures was due to the presence of chromosomal and/or biochemical lesions incompatible with sustained cell division in vitro as well as in vivo. Cell cultures prepared from two early equine twin embryos failed before sufficient cells were present for harvesting. This was attributed to the small number of cells available and the relatively cell-wasteful method of monolayer cell culture by trypsin disaggregation.

The varying success rates of cell culture may have influenced the relative frequencies of chromosome disorders in human abortions. Indeed, the study with the highest success rate of cell culture (96%) (Lauritsen et al., 1972) revealed an unusually high frequency of the XO anomaly (35%), while the incidence of triploidy was relatively low (8.8%). Carr, Bateman and Murray (1966) attempted to judge the relationship between failed and successful cultures on the basis of

maternal age, gestational age of the specimens and the mothers' previous obstetrical histories, and Carr (1967a) concluded that "the 42.5% of spontaneous abortions which failed to grow in culture did not differ in any measurable way from the successfully cultured specimens". Although the success rate of cell cultures in this study was relatively high, in view of the small number of embryos examined the possibility that chromosome anomalies were present in the specimens which failed to grow cannot be excluded.

A common problem with cell cultures from human abortions is the possible contamination with maternal cells. In this study the foetus was recovered intact within its placental membranes and tissue for cell culture derived only from the foetus.

Chromosome analysis involved the cell culture of the entire foetus, and often entailed several subcultures. In theory, the results could have been influenced by the differential growth of various tissues, but evidence concerning the relative viability in cell culture of cells with different chromosome constitutions is conflicting. Dewald et al. (1975) noted the relative loss of triploid cells during the in vitro growth of human fibroblasts while others have found no disadvantage for triploid cells (Aspillaga, Schlegel, Neu and Gardner, 1964). Undetected mosaicism in some abortuses reported as complete triploids may explain the relative scarcity of 2n/3n karyotypes among abortions.

Mosaicism

Mention has previously been made of the difficulties in detecting mosaicism. Whether metaphase preparations from secondary cultures introduce a bias against mosaicism is

unknown, but the Geneva Conference (1966) recommended that some chromosome preparations be made from the primary growth phase and additional cultures continued. They further advised that for embryo studies, at least 50 metaphases should be counted, of which ten modal cells and all or a majority of non-modal cells should be analyzed or preferably karyotyped. These criteria were followed in the present study and for the majority of the embryos studied, many more than 50 metaphases were counted. As well as analyzing cells from both the primary and secondary growth phases, a proportion of cells produced from disaggregation of the embryo were stored at -70°C for subsequent study if necessary.

Mosaic karyotypes have been found at a relatively high frequency in sex chromosome anomalies among infertile mares. If mosaicism is more common in the horse, the presence of undetected abnormal cell lines may partly explain the absence of chromosome anomalies among the 22 embryos studied.

iii) Possibility of a sire effect

Attempting to account for unexplained fertility differences between sires, Bishop (1964) suggested that a considerable part of embryonic death is attributable to the male. The frequency of chromosome errors among the embryos analyzed may have been influenced by the use of only one stallion in the mating group. From mating trials there is some evidence of a sire effect on prenatal mortality in pigs (Crossman, Wijeratine, Imlah, Buckner and Gould, 1973), and it is possible that the stallion used in this study was characterized by a low incidence of embryonic loss. The examination of stud records for evidence of a sire effect on pregnancy wastage may have important practical applications.

iv) Chromosome anomalies and embryonic loss in the horse

Abortion and embryonic loss are a major source of reduced productivity in farm animals but the investigation of prenatal loss has been hindered by the lack of accurate data on its incidence. Stallion fertility figures released by the New Zealand Racing Conference (1976) revealed an annual abortion rate (foetal loss later than six weeks gestation) of 13%, similar to other reports (Platt, 1973b). These figures showed that only 54% of 7057 mares mated in the 1974-75 breeding season produced a live foal. The horse, like other animals studied including man, probably suffers a zygotic loss of at least 30%, about twice the figure for clinically recognizable abortion.

Not more than 12% of chromosomally abnormal human zygotes are carried to term, yet many live individuals with chromosome anomalies have been described. The small proportion of chromosomally abnormal embryos not rejected during pregnancy are mostly restricted to trisomies of the small autosomes and the XO anomaly. Many of the clinical syndromes associated with abnormalities of the sex chromosomes in man seem to have their equivalent in the domestic animals. In addition, the well documented autosomal trisomy syndromes in humans may have parallels in some of the recently described autosomal trisomies in cattle (Bruère, 1974). As with the early work in humans, chromosome anomalies were discovered after attention was focussed on individuals having phenotypic abnormalities. In the horse, chromosome studies have been directed towards phenotypically abnormal intersex horses and infertile mares, and the most frequent chromosome anomaly reported has been X monosomy. Doubtless, many further types of chromosome anomaly will be discovered in horses,

many of them subject to severe intrauterine selection.

There are major species differences with respect to the origins of chromosomal aberrations and their relative importance in embryonic loss. Consequently, extrapolation between species may be misleading. In chickens, the majority of chromosomally aberrant embryos found have been euploid mosaics, while abnormal embryos of the mouse, rabbit and pig have shown a preponderance of triploid karyotypes. Among spontaneous abortions in women the most common chromosome anomaly has been trisomy, followed by X monosomy and polyploidy. Due to a lack of data, the frequencies of the various chromosome errors in the horse are unknown, but the relatively high frequency of mosaicism associated with sex chromosome disorders suggests that post-fertilization errors may be common in this species.

The XO disorder is probably the most common chromosome anomaly occurring in man, but less than 3% of XO zygotes survive to term (Polani, 1970). The average incidence of 0.7% for XO in the mouse (Russell and Saylors, 1961) is very similar to its probable incidence in man (0.83%), but the XO mouse is a normal fertile female with no evidence of increased prenatal lethality (Cattanach, 1962). The absence of XO equine embryos may indicate that the X chromosome in the horse plays only a trivial part in embryonic development and XO embryos are not subjected to the same degree of intrauterine selection as in man. This hypothesis is supported by the finding of a relatively high incidence of the XO anomaly among infertile mares. Since the phenotype of the XO mare is relatively normal in comparison with XO women, it may be that XO embryos in the horse have a greater chance of survival,

and are extremely rare among abortions. In addition, mosaicism is apparently common with sex chromosome anomalies in infertile mares, and the presence of a normal cell line enhances the survival and subsequent development of XO embryos.

Alternatively, many XO embryos may be rejected during pregnancy in the mare but have not been detected. As in man, the occurrence of adult horses with an XO sex chromosome constitution may be indicative of a considerable embryonic loss.

The fact that no chromosome errors were found among the 22 equine embryos examined in this study does not preclude their occurrence. In contrast to the high frequencies found in spontaneous abortions, studies of induced abortions in women have yielded very low frequencies of chromosomal aberrations. In fact, there is no significant difference between the incidence of chromosome errors in induced abortions in women and the results of this study. The incidence of chromosomal abnormalities in induced abortions in women is approximately 3% (Table V.3). If a similar loss occurs in the horse, on average at least 30 embryos would need to be studied to detect one chromosome anomaly. Although these results of the chromosome analysis of 22 equine embryos are of limited value by themselves, they form an initial and important contribution towards cumulative studies of embryonic loss in the mare.

Another possible reason for the absence of chromosomal abnormalities among the embryos studied may be that chromosomally abnormal embryos in the horse are rejected at a very early stage of pregnancy and may be almost impossible to

detect without the aid of more refined techniques. The vast majority of chromosomally abnormal abortions in women occur during the first trimester and many are lost before pregnancy is clinically detectable. In other species too, early pregnancy is the critical period for embryo survival, in pigs the first 25 days (Dufour and Fahmy, 1975), and from studies of the effects of delayed fertilization in rabbits (Shaver and Carr, 1967) and pigs (Bomsel-Helmreich, 1965) the respective authors concluded that most chromosomally abnormal embryos were rejected during very early stages of pregnancy. In the sheep and cow the indications are that chromosomally abnormal embryos are lost very early in gestation, probably within the course of one heat cycle (Quinlivan et al., 1966).

From induced abortions in women it is evident that the chances of finding chromosome anomalies depends upon the stage at which the pregnancy was interrupted (Boué et al., 1967). The author encountered a major problem in procuring a sufficient number of equine embryos in early stages of pregnancy. Due to the occurrence of embryonic resorption (Van Niekirk, 1965b) it appears virtually impossible to obtain specimens of early abortions in mares. As in many small animals, early clinical abortion is often prevented and an abnormal foetus may be resorbed or, in polytoccous species, delivered as a mummified foetus incorporated in the placental membranes. In addition, the free grazing conditions typical of pasture management on thoroughbred studs in New Zealand are not conducive to the detection of abortuses. McFeely and Rajakoski (1968) reported a chromosome anomaly in an early bovine embryo. The small number of embryos in

their study emphasizes the difficulties in obtaining early embryos from the domestic animals. Although the anomaly reported was based on the examination of only a small number of cells (18) and is not necessarily conclusive, it certainly indicates the potential for further investigation in this direction.

v) The aetiology of chromosome disorders in relation to embryonic loss in the horse

Drugs

Hormonal disturbances have been implicated in chromosome disorders in human abortions and in abnormal embryonic development in rabbits and mice, and German (1968) suggested that the increasing frequency of some chromosome errors among the progeny of older women may be an hormonal effect. Steroid hormones have been used to prevent pregnancy by disturbing ovum transport and may result in chromosome errors in the embryo by causing aging of gametes. Hormones are commonly used for the treatment of infertility in the broodmare and may interfere with ovulation or the transport of the ovum or sperm, resulting in aging of gametes.

Many diverse chemicals are known to cause chromosomal abnormalities in other species and Stevenson, Hastie and Archer (1972) reported chromosome damage in horses treated with phenylbutazone. Evidence in this area is, however, lacking.

Heritable chromosome aberrations

The majority of chromosome disorders in human abortions arise as sporadic events during meiosis or early mitotic division, but some are the result of unbalanced gametes pro-

duced after the segregation of parental translocations. Most genetically unbalanced offspring are lost, so a history of pregnancies ending in abortion or the production of abnormal progeny may indicate a parental chromosomal abnormality. The birth of a Down's syndrome child has been frequently preceded by abortions (Zellweger and Mikamo, 1961), but clinical syndromes have varied depending on the chromosomes involved in the translocation (Jacobs, Frackiewicz, Law, Hilditch and Morton, 1975).

Approximately one in 150 newborn babies carries a structural aberration (Hamerton et al., 1975), sometimes associated with reduced fertility or sterility. Chromosome studies in recurrent abortion in women have revealed many parental chromosome abnormalities and Kim et al. (1975) attributed 10% of the foetal wastage of the general population to such anomalies. Whereas many phenotypically normal humans have been screened for the presence of structural chromosome anomalies, similar surveys in animals have yet to be undertaken. A chromosomal translocation in a boar was causally associated with embryonic loss (Äkesson and Henricson, 1972) and infertility of genetic origin has been reported in horses (Pulos and Hutt, 1969). However, infertility in the horse has not yet been associated with parental translocations.

Maternal age

Chromosome anomalies have been found more commonly in human abortions presenting one or more of three factors: short gestation, 'blighted' or structurally abnormal ova and advanced maternal age. The selection for similar criteria in the mare was not possible in this study. Because female germ cells complete their stem cell mitoses before birth,

their chromosomes are susceptible to damage from birth until the time of fertilization. However, the relationship between maternal age and chromosome errors in spontaneous abortion is unclear, and increasing age in women has been associated only with trisomies of specific chromosomes (Boué and Boué, 1970).

A maternal age effect may also occur in the horse. Since 1903, the fertility of thoroughbred mares in New Zealand has gradually declined and maiden mares at stud are now older and matings less successful than formerly (Dewes, 1973). Dewes (1973) further noted that there is a sharp decline in live foal production in mares more than 14 years old. Stallions and mares of proven performance are in greatest demand when past their period of optimum fertility and mating is often between older animals. Such breeding among the domestic animals is peculiar to the horse and in this regard the horse may be more similar to man than are the other domestic species.

Aging of gametes

The aging of gametes, particularly ova, may lead to chromosome errors in the embryo. The retention of spermatozoa in the female genital tract may also be detrimental to normal embryonic development (Austin, 1975) and reduced fertility due to embryonic loss was observed in cows inseminated with semen that had been stored at 4°C (Salisbury and Hart, 1970).

Polyploid embryos after delayed mating have been found in mice, rats and rabbits, and delayed insemination in pigs has resulted in polyploid embryos and smaller litters. A similar effect has not yet been demonstrated in monotoccus

species but there is evidence to suggest that the fertilization of aged ova increases the risk of chromosome anomalies in human abortions (Boué and Boué, 1970) and German (1968) suggested that the maternal age effect in women may relate to aging of ova. Following the study of pregnancies where the times of conception were known, Iffy (1963) postulated that some chromosomally aberrant embryos may have resulted from delayed fertilization. Indeed, polyploidy has been found at a relatively high frequency in human abortions.

In humans, many factors such as disease, drugs, nutrition and diverse psychological factors may influence the release of luteinizing hormone and lead to intrafollicular overripeness of ova. Similar mechanisms may operate in the horse. In domestic animals, delayed ovulation was considered to be more important as a cause of ovum aging than delayed fertilization. However, in amphibia, a similar effect was achieved whether the eggs became 'aged' before or after ovulation (Mikamo, 1962, 1968).

Aging of gametes in the oviduct predisposes towards errors of fertilization and early cleavage. Various types of chromosomal aberration may result depending on the species involved and the length of the aging process (Fechheimer, 1972). Whereas delayed fertilization in the sow predisposes to the formation of polyploid embryos from dispermy, delayed fertilization in the mare may be manifested by an increase in the frequency of mosaicism due to errors during cleavage. The relatively high incidence of mosaicism among sex chromosome anomalies in mares suggests that aging of the ovum before fertilization may be a common occurrence in this species.

In theory, post-ovulatory aging due to delayed fertilization should not occur in domestic animals having a defined oestrous period which ensures the synchronization of coitus and ovulation. However, especially in the thoroughbred mare under New Zealand stud conditions, ova have ample opportunity to become 'aged'. Breeders are well aware of the marked irregularity of the oestrous cycle in individual mares. Oestrus is often prolonged and the time of ovulation unpredictable, creating a situation resembling that in man where coitus and ovulation occur independently. The frequency of mating, particularly to popular sires, is limited, and human interference with normal ovulation and fertilization times may further predispose to the aging of ova and subsequent chromosome disorders in the embryo.

vi) Conclusion

The importance of chromosome disorders to fertility in farm animals is unknown, but infertility attributable to chromosome errors has been described in cattle, pigs, sheep and horses. Some of the infertility in cattle and pigs has been assigned to structural chromosome aberrations in phenotypically normal animals and in the horse there has been one report of a structural chromosome anomaly in a phenotypically normal stallion (Quéinnec et al., 1975). In man, chromosome anomalies have been established as an important cause of spontaneous abortion and approximately a third of the pregnancy wastage in pigs may be due to chromosome disorders (McFeely, 1967). It is suggested that chromosomal abnormalities may account for a significant proportion of embryonic loss in mares.

In human medicine, current emphasis is directed

towards defining those factors which predispose to chromosome anomalies in the conceptus. Whereas it is well advanced in humans, the era of defining the extent of the problem and the types of chromosome errors in abortions in other mammals has only just begun. This thesis is an initial study of possibly one of man's most interesting domestic species which, because of its size and singular reproductive cycle, is a perplexing subject for investigation.

APPENDICES

Appendix I. Parasite control of experimental animals

a) Equizole. Merck Sharp and Dohme (N.Z.) Ltd.
(Thiabendazole 33.3% w/w).

b) Neguvon. Bayer.
(O,O-dimethyl 1-hydroxy-2,2,2-trichloroethyl phosphonate).

c) Louse Powder. Cooper, N.Z. Ltd.
(Active ingredient: Rotenone).

Appendix II. Drugs used for anaesthesia and surgery

a) Acetylpromazine. The Boots Company Ltd., Nottingham, England.
(contains Acepromazine Maleate B.P.C. 1968 equivalent to 10 mg Acepromazine base per ml).

b) 'Intraval' Sodium. May & Baker Ltd., Dagenham.
(Thiopentone sodium 5%).

c) Tetanus antitoxin. Tasman Vaccine Laboratory Ltd., Upper Hutt, New Zealand.
(1500 international units).

Appendix III. Materials required for blood leucocyte culture

a) Preparation of culture.

10 ml Venoject tube with sodium heparin (Jintan Terumo Co., Ltd., Tokyo, Japan) + 20g x 1" needle.

Adapter for venoject tube.

Universal jar (25 ml) with lid.

Sterilized syringes: 1 x 10 ml syringe + 20g x 2" needle.

1 x 1 ml syringe + 26g x ½" needle.

Pasteur pipettes (9") + rubber bulbs.

Medium 199 (Wellcome Reagents Ltd., Wellcome Research Laboratories, Beckenham, England).

Phytohaemagglutinin (Wellcome Reagents Ltd., Wellcome Research Laboratories, Beckenham, Kent, England).

Sodium hydrogen carbonate, $NaHCO_3$, as 10% aqueous solution.

Incubator set at 37°C.

Laminar-flow Bio-Clean Work Station (Gelman Clemco Pty. Ltd., Australia.

General Laboratory centrifuge (Sorvall, Newtown, Connecticut, 06470, U.S.A.).

Rotor diameter 18 cm.

b) Harvesting of cultures.

Colchicine (Aqua Colchin, 0.5 mg colchicine in 1 ml per ampoule) Park, Davis and Company, Sydney, Australia.

To make stock colchicine solution: add 9 ml phosphate buffered saline (PBS) to one ampoule.

For use: add 1 ml stock solution to 49 ml PBS.

Concentration = 1µg/ml.

Add 0.1 ml colchicine solution to each leucocyte culture.

Graduated centrifuge tubes.

Pasteur pipettes (9") and rubber bulbs.

Hypotonic solution: 0.075M KCl (Hungerford, 1965).

Preparation: add 5.6 gm KCl to 1 litre distilled water.

Fixative: Methanol : glacial acetic acid (3 : 1).

Cleaned slides in beaker containing iced distilled water.

Refrigerator.

c) To mount slides.

Cover glasses (Matsunami 22 x 64 No. 2, Watson Victor Ltd., Wellington, New Zealand).

D.P.X. (DePex mounting medium, G.T. Gurr Ltd., London, England).

Glass rod, Xylol, Absolute alcohol.

Appendix IV. Preparation of Giemsa stain

Giemsa powder (Allied Chemical Corporation, New York) 0.5g dissolved in 33cc glycerine at 55°C to 60°C for 1.5 to 2 hours. Methyl alcohol - 33cc - added. This is STOCK solution.

For use: 1 ml stock dissolved in 50 ml buffer at pH 6.8 (Buffer tablets pH 6.8, Geo. W. Wilton and Co. Ltd., Lower Hutt, New Zealand: 1 tablet to 100 ml distilled water).

Appendix V. Procedure for centromere staining.

Slides prepared routinely for mitotic chromosomes.

Allowed to age for at least 4 days.

Stand slides 1 hour at room temp. in 0.2M HCl.

Rinse in de-ionized water.

Stand slides in 5% solution barium hydroxide ($Ba(OH)_2.8H_2O$) at 50°C for 10 minutes.

Rinse slide thoroughly in de-ionized water.

Incubate for 60 minutes at 60°C in 2 x SSC (below).

Rinse with de-ionized water.

Stain 60 minutes with Giemsa (above).

Rinse and air dry.

Formula for 2 x SSC (0.3M Sodium chloride containing 0.03M tri-sodium citrate).

NaCl	17.53g
$Na_3C_6H_5O_7.2H_2O$	8.82g
De-ionized H_2O	1 litre

Store at 4°C. Do not use hot 2 x SSC more than two days old.

Appendix VI. Preparation of glassware.

All debris, blood etc. removed.

Soaked 12 hours in Pyroneg. (Diversey Wallace Ltd., New Zealand).

Brush-washed and rinsed.

Machine-washed in dri-decon solution (Decon Laboratories Ltd., Brighton).

Rinsed in de-ionized distilled water.

Dried.

Autoclaved 10 lb. pressure for 25 minutes.

Appendix VII. Materials required for fibroblast culture: Plasma clot embedding technique.

a) To obtain tissue from live horses:

Local anaesthetic, Xylocaine 2%, Astra Chemicals Pty. Ltd., 10 Khartoum Rd., North Ryde, N.S.W.

10 ml sterilized syringe + 25g x 1" needle.

Scalpel, rat-toothed forceps.

Universal jar (25 ml) + lid.

HBSS. Hanks' Balanced Salt Solution (Hanks, 1948).

b) Growth medium:

Medium 199 (60%) Wellcome Reagents Ltd., Wellcome Research Laboratories, Beckenham, Kent, England.

Foetal bovine serum (20%). Laboratory Services Ltd., P.O. Box 6562, Auckland.

Equine serum (20%).

Streptomycin (0.2 mg/ml). Streptomycin sulphate, B.P. Glaxo Laboratories, Ltd., Greenford, England.

Benzylpenicillin (200 u/ml) 'Crystapen 600 mg'.
Benzylpenicillin (sodium) B.P. Glaxo Laboratories.
Tricine buffer.

c) To prepare tricine buffer:

Add 1.8g Tricine (N-tris-(hydroxymethyl) methyl glycine, Calbiochem., San Diego, California, 92112, U.S.A.) to 10 ml PBS.
Filter through millipore (0.22μ).
Use 1 ml per 100 ml medium.

d) To prepare plasma clot:

10 ml Venoject tube.
Bijou bottle and lid.
Refrigerator.
Centrifuge.
Alsever's solution.

De-ionized water	100 ml
Sodium citrate	0.8g
Sodium chloride (NaCl)	0.42g
Glucose	2.05g

Make up to near volume and adjust to pH 6 with citric acid. Filter and dispense.
Keep 3 days before use. Store at 4°C.
Calcium chloride $CaCl_2$ 2%.

e) A.T.V. (Antibiotic-trypsin-versene solution).

Trypsin 1 : 250 (Difco Laboratories, Detroit, Michigan, U.S.A.) 0.5g

E.D.T.A. (Sequestric acid, Tetrasodium salt), Hopkin and Williams Ltd., Chadwell Heath, Essex, England.	0.2g
Sodium Chloride NaCl	8.0g
Potassium chloride (KCl)	0.4g
Dextrose	1.0g
Sodium hydrogen carbonate ($NaHCO_3$)	0.58g
Penicillin	2×10^5 units
Streptomycin	100 mg
Phenol red	0.02g

Make up to 1 litre. Sterilize by filtration.
Store at -20°C.

f) PBS (Phosphate-buffered saline). pH 7.5.
(Dulbecco and Vogt, 1954).

Solution (1)	NaCl	8.0g
	KCl	0.2g
	Na_2HPO_4	1.15g
	KH_2PO_4	0.2g
	Ion-exchange H_2O	800 ml
Solution (2)	$CaCl_2$	0.1g
	Ion-exchange H_2O	100 ml
Solution (3)	$MgCl_2.6H_2O$	0.1g
	Ion-exchange H_2O	100 ml

Autoclave solutions (1), (2) and (3) separately.
Mix when cool and store at 4°C.

g) Sterilised scissors, forceps, petri dishes.
Laminar flow cabinet.
Incubator set at 37°C.
Pasteur pipettes (9") + rubber bulbs.

Tissue culture flasks. Falcon 3013, 25cm² growth area. Falcon: 1950 Williams Dr., Oxnard, CA 93030, U.S.A.

Appendix VIII. Materials required for fibroblast culture.

Trypsin disaggregation technique.

a) Transport medium. Composition:

Medium 199

Streptomycin sulphate	1.25g/litre
Crystalline benzylpenicillin B.P.	1,000,000 u/litre
Amphotericin B ('Fungilin', Squibb).	5 mg/litre

b) Trypsin solution 0.25% (Difco 1 : 250)

c) Antibiotic solution:

500 ml HBSS

5g Streptomycin sulphate

5,000,000 u. Crystalline benzylpenicillin B.P.

2 litres H_2O.

Stainless steel filter.

d) Colchicine (Appendix IIIb). Final dilution 1µg/ml.

0.1 ml added to each 5 ml culture.

e) Long term storage:

Deep freeze at -70°C.

Glass ampoules, 2 ml.

Dimethyl sulphoxide (DMSO). B.D.H. Laboratory Chemicals Division, Poole, England.

f) General equipment

Laminar flow cabinet, scalpel blades, petri dishes, Pasteur pipettes (9") + rubber bulbs, 125 ml conical flasks.

Tissue culture flasks (above).

Magnetic stirrer, Chiltern, Smith, Biolab Ltd., Auckland, New Zealand.

Inverted light microscope. Ernst Leitz Wetzlar, Germany.

Eyepiece 6.3x magnification.

Objective lens 6x magnification

Appendix IX. Analysis of results.

a) Examination of slides.

Wild MII Microscope. Wild Heerbrugg Ltd., Switzerland.

Hand operated Tally counter. English Numbering Machines Ltd., England.

b) Development of film.

Dektol print developer (Kodak, New Zealand Ltd.) 20°C for 3 minutes.

Universal fixer.

c) Development of prints.

Enlarger: Focomat IIc, Leitz, Germany.

Dektol print developer (diluted 1 : 3 with water)

Stop bath: water containing acetic acid.

Hypo bath: 2.5 lb. hypo crystals + 8 oz. meta-bisulphate + 1 teaspoon alum.

Water bath.

Glaze.

APPENDIX X.

CHROMOSOME COUNTS OF HORSES PREPARED FROM LEUCOCYTE CULTURES

Case number	Sex*	COUNT DISTRIBUTION						Cells counted
		<63	63	64	65	>65	Polyploid	
96/74	F	0	8	42	2	0	0	52
98/74	M	0	1	4	0	0	0	5
101/74	M	0	3	17	5	0	0	25
102/74	M	0	2	27	2	0	1	32
105/74	M	0	2	15	1	0	0	18
4/75	F	0	1	7	1	0	0	9
5/75	F	0	0	12	1	0	0	13
8/75	F	0	2	8	0	0	0	10
11/75	F	0	2	0	0	0	0	2
13/75	F	3	2	17	0	0	0	22
15/75	F	1	1	14	4	0	0	20
35/75	F	2	2	20	1	0	0	25
37/75	F	7	9	74	6	0	1	97
38/75	F	9	14	105	5	0	0	133
39/75	F	3	0	13	0	0	0	16
40/75	F	6	5	50	1	1	0	63
42/75	F	0	1	5	0	0	0	6
44/75	F	3	3	21	2	1	0	30
46/75	F	2	2	24	0	0	2	30
48/75	F	0	4	16	3	0	0	23
54/75	M	30	20	134	3	5	7	199
4/76	F	3	1	18	0	0	0	22
5/76	F	0	0	1	0	0	0	1
TOTALS		69 (8%)	85 (10%)	644 (76%)	37 (4%)	7 (1%)	11 (1%)	853

* M = male; F = female.

APPENDIX XI.

CHROMOSOME COUNTS OF HORSES PREPARED BY FIBROBLAST CULTURES OF TISSUE EXPLANTS

Case number	Sex*	COUNT DISTRIBUTION						Cells counted
		<63	63	64	65	>65	Polyploid	
2/73	M	1	3	10	0	0	0	14
3/73	F	2	0	3	0	0	0	5
6/74	M	7	2	8	0	0	0	17
10/74	F	17	4	23	1	0	1	46
11/74	F	0	2	14	1	0	1	18
13/74	M	1	2	16	0	0	0	19
14/74	M	0	0	2	0	0	0	2
15/74	M	0	0	4	0	0	0	4
18/74	F	0	0	6	1	0	0	7
24/74	F	0	0	1	0	0	0	1
25/74	F	0	0	1	0	0	0	1
26/74	F	5	2	16	5	0	0	28
27/74	F	0	0	1	0	0	0	1
29/74	F	0	0	3	0	0	0	3
45/74	F	0	2	7	1	2	1	13
48/74	M	1	0	3	0	0	0	4
69/74	F	0	0	1	5	0	0	6
85/74	F	0	5	2	0	0	0	7
92/74	M	1	0	3	2	0	0	6
97/74	M	0	1	4	3	1	0	9
107/74	M	6	8	73	3	0	0	90
TOTALS		41 (14%)	31 (10%)	201 (67%)	22 (7%)	3 (1%)	3 (1%)	301

* M = male; F = female.

APPENDIX XII. CHROMOSOME COUNTS PREPARED BY LEUCOCYTE CULTURE FROM HORSES WITH NORMAL KARYOTYPES. CASES WITH FEWER THAN 30 CELLS COUNTED ARE EXCLUDED.

Case number	COUNT DISTRIBUTION						Cells counted
	< 63	63	64	65	> 65	Polyploid	
96/74	0	8	42	2	0	0	52
102/74	0	2	27	2	0	1	32
37/75	7	9	74	6	0	0	97
38/75	9	14	105	5	0	0	133
40/75	6	5	50	1	1	0	63
44/75	3	3	21	2	1	0	30
46/75	2	2	24	0	0	2	30
54/75	30	20	134	3	5	7	199
TOTALS	57	63	477	21	7	11	636

APPENDIX XIII. CHROMOSOME COUNTS FROM FIBROBLAST CELL CULTURES EXCLUDING CASES WITH FEWER THAN 30 CELLS COUNTED.

(a) CHROMOSOME COUNTS FROM HORSES WITH NORMAL KARYOTYPES PREPARED BY CELL CULTURE OF TISSUE EXPLANTS.

Case number	COUNT DISTRIBUTION						Cells counted
	< 63	63	64	65	> 65	Polyploid	
10/74	17	4	23	1	0	1	46
107/74	6	8	73	3	0	0	90
TOTALS	23	12	96	4	0	1	136

(b) CHROMOSOME COUNTS FROM EQUINE EMBRYOS WITH NORMAL KARYOTYPES

Case number	COUNT DISTRIBUTION						Cells counted
	< 63	63	64	65	> 65	Polyploid	
1B	16	12	148	7	1	1	185
2B	17	17	123	2	0	2	161
3B	5	10	90	6	0	0	111
6B	16	23	159	6	3	0	207
10B	0	7	46	4	0	0	57
11B	10	8	135	2	3	9	167
12B	9	15	111	8	0	0	143
13B	19	15	111	4	1	0	150
14B	7	10	144	5	1	0	167
15B	7	10	131	5	0	0	153
16B	9	6	59	6	0	0	80
17B	4	2	36	2	0	0	44
18B	5	4	49	2	0	2	62
19B	4	5	21	0	1	0	31
20B	10	6	101	1	0	2	120
21B	3	5	62	1	1	1	73
22B	7	3	51	0	0	10	71
23B	2	7	57	0	0	3	69
24B	6	13	70	1	0	0	90
25B	12	6	84	1	0	9	112
26B	14	3	79	2	0	2	100
TOTALS	182	187	1867	65	11	41	2353

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