A study of the bovine 1/29 Robertsonian translocation chromosome

by

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DECLARATION

The contents of this thesis are the work of the author. The thesis has not been previously submitted for the award of a degree to any university.

T. Douglas Wilson

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ABBREVIATIONS

BSS	balanced salt solution
C	centigrade
cm	centimetre
DNA	deoxyribonucleic acid
FSH	follicle stimulating hormone
g	gram
GUTS	ground up tick supernate
iu	international unit
IU	international unit
LH	luteinising hormone
mg	milligram
ml	millilitre
mM	millimole
MHC	major histocompatibility complex
N	normal
NOR	nucleolar organiser region
PBS	phosphate buffered saline
PG	prostaglandin
PHA	phytohaemagglutinin
PMSG	pregnant mare serum gonadotrophin
PSP	phenolsulphonphthalein
r	correlation coefficient
RNA	ribonucleic acid
SSC	saline sodium citrate
t	translocation

ABBREVIATIONS (Cont'd)

T	T statistic (two-sided t-test)
x g	relative centrifugal force
μ	micron
μg	microgram
μl	microlitre

Abbreviations used for journals correspond to those recommended by the Code for Abbreviation of Titles of Periodicals

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SUMMARY

Three hundred microscope slides containing fixed chromosome spreads from cattle with the normal karyotype were treated by either of two methods used to produce G-bands. Unsatisfactory results were obtained using one of these methods but distinct bands suitable for the identification of individual chromosomes were produced using the other. Karyotypes were prepared from 25 of the most clearly stained chromosome sets and the banding pattern of each individual chromosome was described in detail. Karyotypes were also prepared from 20 sets of C-banded chromosomes.

Conventionally stained chromosome preparations were examined from 59 British Friesian animals belonging to two pedigree herds situated in the South West of Scotland and North West of England. Of these, 21 were heterozygous for a centric fusion involving the largest and one of the shortest autosomes. Ten G-banded and ten C-banded karyotypes were prepared from four of these animals and the aberration was identified as the 1/29 Robertsonian translocation which is the first time this type of translocation has been recognised in the British Friesian breed.

The pedigree of each animal was examined and illustrated in a diagram which demonstrated that all the carriers were closely related to each other and that the translocation probably formed de novo in a recent predecessor. The age at first calving and the intervals between successive calvings of 13 cows heterozygous for the translocation were compared with figures obtained from normal cows. However, the number of animals available for investigation was insufficient to detect any statistically

significant difference.

Uterine tube patency was assessed in 37 cull-cows using the PSP dye test. Either one or both tubes were blocked in nine animals. Both tubes were patent in the remaining 28 animals. These and another nine cows were each stimulated to superovulate on up to four separate occasions by treating with PMSG between days 7 and 14 of the oestrus cycle. They were then inseminated with semen from either a bull heterozygous for the 1/29 Robertsonian translocation or a normal bull.

The number of corpora lutea counted by rectal palpation of each animal ranged between 0 and 20 with a mean of 5.0. The number of corpora lutea was counted by laparoscopic examination of the ovaries on 24 occasions and ranged from 0 to 24 with a mean of 7.0. Statistically significant differences in the superovulatory response were produced by animals of different breeds ($P < 0.03$) and when PMSG was administered on days 10 and 13 ($P < 0.02$). The age of the animal, batch of drug used and whether the animal had been previously treated had no significant effect.

Overall, 214 eggs were recovered from these animals 6-8 days after oestrus using a non-surgical technique giving a recovery rate of 60.8%. Of these, 163 were fertilised. They were at various stages of development and classified as being either normal (105) in the process of degeneration (39) or degenerate (19) although there was no significant difference between the condition of embryos sired by normal bulls and bulls heterozygous for the translocation.

Cytogenetic spreads suitable for chromosome analysis were obtained from 67 of the embryos after culture with colchicine. Mixoploidy was recognised in five of these which each contained one polyploid cell. Three embryos, which were all sired by an animal heterozygous for the 1/29 Robertsonian translocation, were aneuploid. Of these, two were monosomic and one was trisomic. However, due to the degree of contraction, it was not possible to identify the unbalanced chromosomes.

Lymphocytes belonging to six cows which were either heterozygous for the 1/29 Robertsonian translocation or had the normal karyotype were stimulated to divide for 15 weeks by infecting them with Theileria. Cytogenetic analysis of these cells at regular intervals showed that a range of karyotypic abnormalities developed in varying proportions of cells from each animal. G-banded karyotypes prepared from 24 of these cells contained chromosome aberrations in 16 of them. These included structural rearrangements, centric fusions and aneuploidy. The chromosomes most frequently involved in these aberrations were numbers 2, 15 and members of the 22-23 group, respectively. None of the aberrations corresponded to those, such as the 1/29 Robertsonian translocation, which are recognised in vivo and the possible reasons for this are discussed.

CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Historical Background

In 1859, Charles Darwin discussed the concept of heredity in "The Origin of Species". He observed that certain characteristics are common between successive generations of animals and plants and he proposed that such characteristics were inherited. In 1865, Mendel scientifically demonstrated the transfer of traits between generations of peas. However, he offered no explanation as to the mechanisms involved.

At this time, the understanding of biology was greatly advanced by use of the light microscope. In 1833, Brown demonstrated microscopically that each cell consists of two basic parts which are now called the nucleus and cytoplasm. When studying cells under the microscope, Virchow, in 1858, observed that they divided to form identical new cells. During the process of cell division, the nucleus was seen to change in appearance and form fibre-like strands. The term mitosis, derived from the Greek word meaning fibre, was used by Flemming in 1882 to describe this activity in dividing cells of the human cornea. The fibre-like strands were later named chromosomes, from the Greek word meaning coloured body, by Waldeyer in 1888.

In 1875, Strasburger observed, in plants, that fertilisation involved fusion of the nuclei of egg and sperm and this was also demonstrated in animals by Hertwig in the following year. Further microscopic studies by Strasburger in 1888, led to the discovery that there is a reduction in chromosome number during the production of egg and sperm, and that the full complement is restored in the new plant. The term meiosis, derived from the

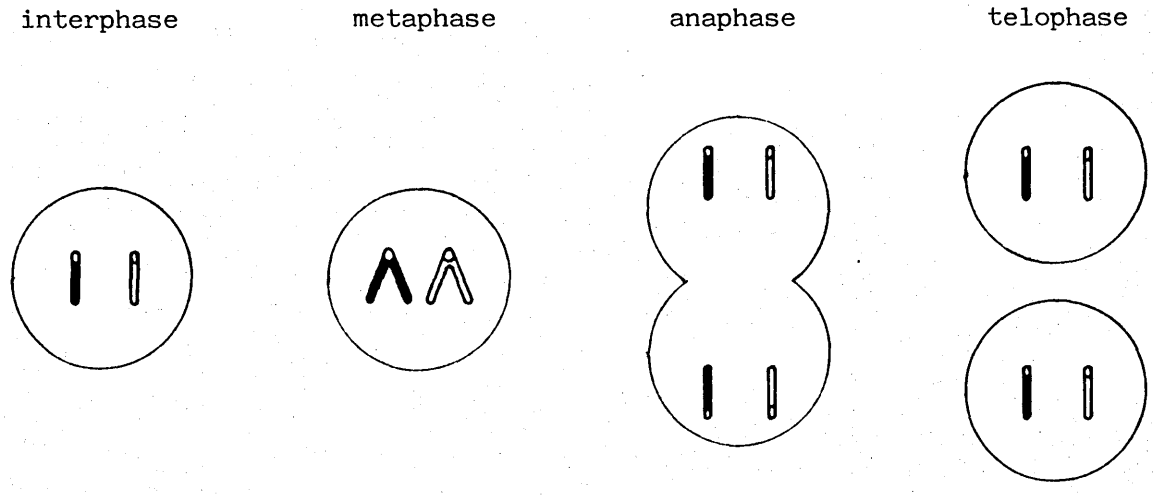

Figure 1. A diagrammatic representation of mitosis involving a homologous pair of chromosomes

Greek word meaning diminution, was used by Farmer and Moore in 1905 to describe the process of cell division involving a reduction in chromosome number. Thus, it was realised that each parent contributes half its chromosome complement to the new generation thereby maintaining the stability of chromosome number between successive generations.

By considering their observations of chromosome behaviour during mitosis, meiosis and fertilisation, Sutton and Boveri (Sutton, 1903) concluded that chromosomes are the structures on which hereditary information is stored and transferred between generations.

In 1910, Morgan, investigating the inheritance of different traits in Drosophila, concluded that the factors which determine heritable traits are arranged along the chromosomes and he called these factors genes.

Mitosis (see Figure 1)

The accepted principles of mitosis and meiosis were first outlined by Sutton in 1903. These define mitosis as a process involving a simple division of somatic cells which can be subdivided into five phases known as interphase, prophase, metaphase, anaphase and telophase.

Duplication of each chromosome occurs towards the end of interphase so that the duplicated chromosome consists of two identical sister strands called chromatids. The two chromatids are joined at a constriction called the centromere.

During prophase, the sister chromatids shorten in length and increase in diameter. This is caused by coiling of the

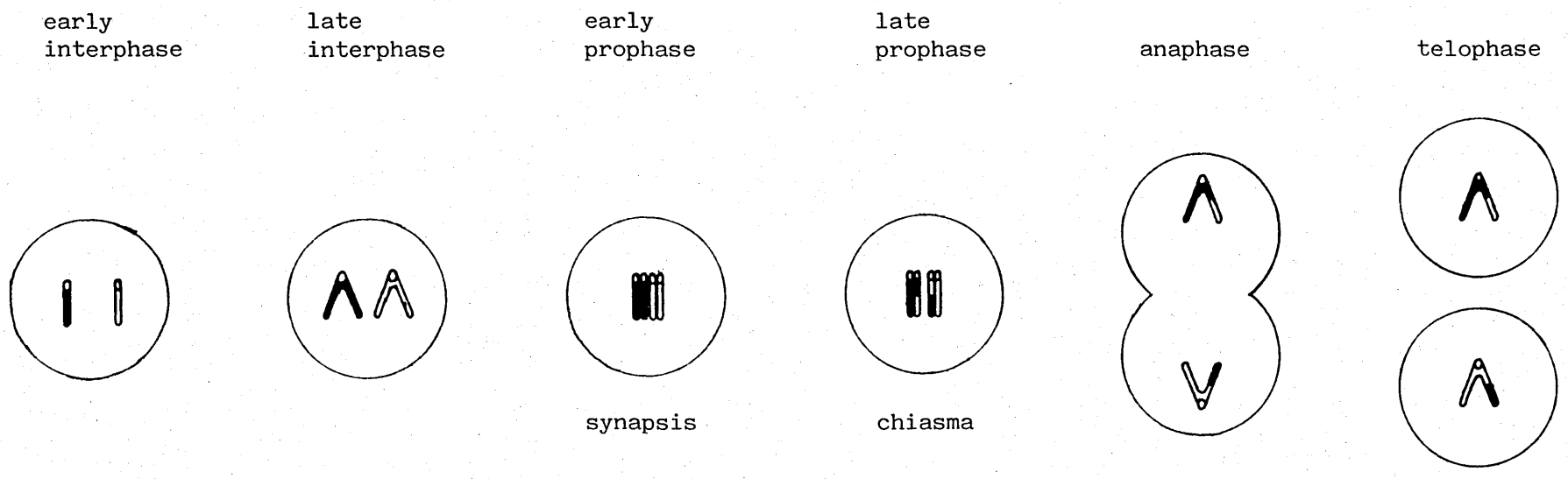

Figure 2. A diagrammatic representation of the first meiotic division involving a homologous pair of chromosomes

chromosome material (Du Praw, 1966) and at this point, two rod shaped structures called centrioles move towards opposite poles of the cell nucleus.

During metaphase, the chromosomes are arranged in a disc like area called the equatorial plate and a spindle of fibres connect the chromosomes by their centromere to the centrioles. The nuclear membrane disappears during late prophase or early metaphase.

During anaphase, the sister chromatids are pulled apart by the spindle fibres to produce two groups of chromosomes with identical hereditary information. These move towards opposite poles of the cell and form the nucleus of two daughter cells during telophase when cell division occurs.

Meiosis

The outcome of meiosis, which involves two successive divisions, is that the number of chromosomes is halved. This occurs during the first divison (Figure 2), and as with mitosis, each meiotic division is subdivided into five phases.

The periods of interphase and prophase are similar to those of mitosis with duplication, shortening and thickening of the chromosomes. However, in contrast to mitosis, each duplicated chromosome becomes attached to its homologous pair in a zipper-like fashion during prophase. This synapsis forms a bivalent structure containing two pairs of sister chromatids. The bivalent structure separates longitudinally but is held together at one or more points called chiasmata. These represent sites of exchange (or cross-over) of chromosome material between non-sister chromatids (Stern, 1931; Creighton and McClintock, 1931).

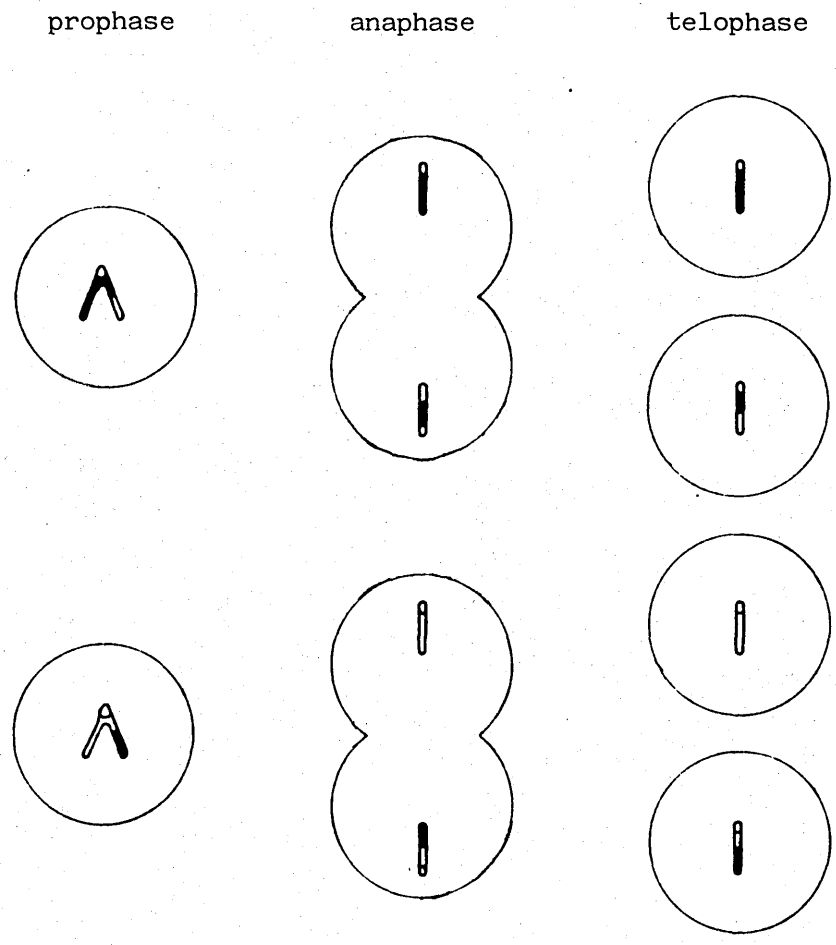

Figure 3. A diagrammatic representation of the second meiotic division involving a homologous pair of chromosomes

Consequently, when several chiasmata occur, each member of the two pairs of chromatids has a different genetic composition.

During metaphase, as in mitosis, the bivalent structures assemble at the equatorial plate. They are then connected by spindle fibres to centrioles at opposite poles of the cell and the nuclear membrane disappears.

During anaphase, the two pairs of sister chromatids which form the bivalent structure are pulled apart by the spindle fibres. The chromosomes separate with one member of each homologous pair moving towards opposite poles of the cell.

The cell divides during telophase to produce secondary spermatocytes or oocytes. These cells therefore contain half the parent number of chromosomes, although each chromosome contains two chromatids.

During the second meiotic division (Figure 3), the chromatids separate and move to opposite poles of the cell. When the cell divides to form gametes, each has one set of chromosomes. The gametes are called haploid to differentiate them from somatic cells which have a pair of each chromosome and are called diploid (Strasburger, 1905).

Chromosome Number and Morphology

Scientists became particularly interested in the number and morphology of chromosomes after Sutton demonstrated in 1903 that hereditary factors are stored on chromosomes. Initially, chromosomes were studied by examining histological specimens embedded in paraffin wax (e.g. Flemming, 1882). One disadvantage of this method, however, is that chromosomes are cut through when

preparing the section and this caused difficulty when attempts were made to examine the chromosomes. Nevertheless, by using this method it was possible to demonstrate that chromosomes vary in shape and number among different species of plant and animal.

In 1921, improved chromosome preparations were produced by Belling working with plant cells. This was achieved by squashing the tissue between a slide and coverslip and, in this way, chromosomes were obtained in one plane of focus, although they still overlapped each other.

In 1931, Lewitzki described metaphase chromosomes from several animals according to the position of the centromere. He classified them as metacentric, submetacentric, acrocentric or telocentric and arranged the chromosomes in pairs in order of decreasing size with the centromere at the same level and the short arms orientated upwards. It was agreed to classify human chromosomes in this manner at the Denver Conference held in 1960. To achieve suitable preparations for examination, chromosomes photographed during mitotic metaphase were cut out and arranged in pairs according to shape and size. Chromosomes presented in this way are called a karyotype.

Colchicine

A significant advance in chromosome studies was achieved by Blakesley and Avery in 1937, who discovered that an alkaloid called colchicine, from the bulb of the Mediterranean plant Colchicum, stops the process of mitosis during metaphase when the chromosomes are in their most contracted state. At this stage they can be recognised under the light microscope. In 1952, Inoue demonstrated that colchicine produces its effect by

disrupting the spindle fibres.

Later work by Taylor in 1965 demonstrated that the effect of colchinine and the related compound colcemid, varies with concentration and duration of exposure although very low concentrations of either compound do not stop the dividing cells at metaphase, even when exposure is continued for 20 hours. In 1966, Herreros and his co-workers demonstrated that high concentrations of colcemid produce some cells with more than two complete sets of chromosomes which is called polyploidy. The concentration of colcemid currently recommended for use with mammalian cells is 0.5 to 0.05 µg/ml for 1 to 6 hours (Sharma and Sharma, 1980).

Hypotonic

Unfortunately, accurate counts of chromosomes in a cell are difficult to make using the colchicine technique alone since even contracted chromosomes in metaphase are intertwined with one another. Neverthless, this problem was overcome in 1952 when Hsu observed that treating cell cultures with hypotonic solution allowed sufficient separation of the chromosomes such that they could be individually counted. Since then, various hypotonic solutions have been used including sodium chloride, sodium citrate and potassium chloride (Genest and Auger, 1963). However, most workers now use 0.075 Molar potassium chloride which was recommended by Hungerford in 1965 for use in human leucocyte cultures.

Phytohaemagglutinin

A further improvement in chromosome investigations was made

by Nowell, in 1960, who noticed that the addition of the plant derivative phytohaemagglutinin to blood cultures stimulated the leucocytes to divide. Phytohaemagglutinin is a mucoprotein extract from the Red Kidney Bean, Phaseolus vulgaris and is commonly referred to as PHA.

Systematic analysis of cell types found in leucocyte cultures demonstrated that only lymphocytes respond to PHA (McKinney, Stohlman and Brecher, 1962) and later work by Keast and Bartholomaeus in 1972, showed that PHA stimulates a population of lymphocytes which are dependant on or influenced by the thymus.

The process by which PHA stimulates lymphocytes to divide is still not fully understood. However, in 1962, Beckman discovered that PHA acts via the serum surrounding the cells and, in 1972, Younkin used specific PHA-antiserum to show that PHA exerts its stimulatory effect while on the cell surface. This was investigated further by Forsdyke in 1973 who found that an optimum response required an optimum ratio of PHA to non-diffusable serum macromolecules and suggested that a possible role of serum in the activation of lymphocytes is that the serum macromolecules facilitate either the reaction of the cells to PHA or their subsequent response after reaction to PHA has occurred.

Fixation

Examination of chromosomes under the light microscope is improved by staining the chromosomes. In order to do this, it is necessary to fix the chromosomes onto a microscope slide. Fixation also removes extraneous cytoplasmic debris which interferes with examination of the chromosomes and various

different fixatives have been used. In 1882, Flemming treated chromosomes with a mixture of osmic acid, chromic acid and acetic acid. However, this frequently produced unequal fixation of tissue. La Cour (1941) recommended a combination of acetic acid, methanol and formalin to fix blood smears used in chromosome studies and a modification of this fixative, using 3 : 1 methanol/acetic acid, was recommended by Moorhead and co-workers in 1960 for use in human leucocyte cultures. This is now probably the most commonly used fixative in cytogenetic studies.

There is a divergence of opinion as to the value of chilling the fixative before use. Ford and Hamerton (1956) and Hungerford (1965) recommend chilling, whereas Rothfels and Siminovitch (1958) found that chilling had no advantage over treatment at room temperature.

Staining

At the end of the last century, chromosomes were stained with non-specific basic dyes like haematoxylin (e.g. Strasburger, 1888). However, these dyes also stain the cytoplasm which interferes with examination of the chromosomes. An improved staining method was developed by Feulgen and Rossenbeck in 1924. This method is based on the Schiff's reaction of aldehydes which stains the nucleic acid of the chromosomes specifically and although well stained chromosomes were obtained using this technique, it was not simple to use. In 1927, Newton used crystal violet but this also stained the cytoplasm and La Cour used orcein in 1941 to stain chromosomes. This is effective as a 1 per cent solution in 45 per cent acetic acid but heating is

sometimes required which damages chromosome structure.

Several other basic dyes have been tried and now giemsa is routinely used to stain mammalian chromosomes (Moorhead et al, 1960). This stain is easy to use and produces an even stain on the chromosome with minimal staining of cytoplasm.

Chromosome Molecular Structure

In order to understand the functions of a chromosome, one must be familiar with its chemical components. In 1871, Miescher discovered that deoxyribonucleic acid (DNA) is a component of the cell nucleus and by using a technique which specifically stains DNA, Feulgen demonstrated in 1924 that DNA is localised in the chromosomes. However it was not until 1944 that Avery, McLeod and McCarty postulated that DNA is the molecular basis of heredity. One major difficulty in the concept that DNA is reponsible for coding hereditary factors is that DNA was understood to be a molecule constructed of a sequence of amino acids and this seemed a relatively simple structure to produce such a wide range of hereditary variations.

In 1953, Watson and Crick, who were investigating the physical chemistry of DNA by X-ray diffraction crystallography, demonstrated the structure of DNA. They showed it to have a double helix structure consisting of four types of molecules called nucleotides which differ from one another in one portion of the molecule called the base. The bases found in the nucleotides were adenine, thymine, cytosine and guanine. Nucleotides on one strand of the helix bonded with those on the opposite strand such that adenine always paired with thymine, and cytosine with guanine. Three successive bases on the DNA

structure was called a codon and genetic information is determined by the sequence of codons. These are found in an almost infinite array of permutations and combinations which explains how DNA is responsible for such a wide variation in hereditary factors.

In addition to DNA, the nucleus contains protein (Kossel, 1884). These proteins are of two main types: histones which have a low molecular weight and non-histones with a high molecular weight. The proportion of these proteins in rat nuclei was demonstrated by Strenran in 1957 who found 15% DNA, 15% histones, 50% non-histones and 20% nuclear membrane and miscellaneous components. Earlier work by Painter in 1933 demonstrated that only certain genes on a chromosome function at one time and that other genes are inactive and do not express themselves. In 1951, Stedman and Stedman suggested that nucleoproteins may be involved in gene expression. In 1962, Cole demonstrated that proteins are involved in maintaining the structure of DNA in the chromosome, and Du Praw showed in 1966 that histones are associated with chromosome construction during cell division.

The involvement of nucleoproteins in chromosome structure was confirmed in 1975 by Lewin for histones and by Douvas and co-workers for non-histones. However research is still being undertaken to clarify the role of nucleoproteins in gene expression.

Banding Techniques

Research workers used their knowledge of chromosome molecular structure to develop methods of differentially staining chromosomes and in this manner, they hoped to be able to identify individual chromosomes.

Q-Banding

In 1969, Caspersson and his co-workers were the first to recognise individual chromosomes by staining them with a fluorochrome which produced differential staining along the length of each chromosome. This technique is called Q-banding since the fluorochrome used was Quinacrine Mustard.

The reasons behind the pattern produced were not known at the time and are still unclear. Caspersson used Quinacrine Mustard because, in addition to binding non-specifically to DNA, it contains an alkylating agent which binds specifically to guanine - one of the nucleotide bases on DNA (Caspersson et al, 1969). He therefore assumed that areas of the chromosome with a high concentration of guanine would fluoresce intensely.

However, he subsequently repeated the technique using Quinacrine Hydrochloride which does not bind specifically to guanine, and produced the same banding pattern (Caspersson et al, 1970). Later work by Weisblum and de Haseth in 1972, using purified DNA solutions, demonstrated that the degree of fluorescence is correlated with the composition of bases on DNA such that DNA with high adenine-thymine ratios give brighter fluorescence than low adenine-thymine ratios and they suggested that Q-banding is specific for adenine-thymine rich regions of the chromosome.

In 1973, Sumner and Evans demonstrated that quinacrine binds to DNA and not to chromosome protein, however the binding was not related to specific ratios of base pairs. Sumner and his co-workers investigated fluorescent staining in cells of the edible crab, Cancer pagurus, in 1975. In this species, different cell types have different amounts of nuclear protein although the DNA content is identical. They found that fluorescence varied with the protein content and concluded that Q-banding results from some effect of chromosome protein on the interaction of quinacrine with DNA.

One disadvantage of using Quinacrine Mustard is that the banding pattern fades rapidly. However, in 1972, Hilwig and Gropp produced Q-bands, which do not fade quickly using a benzimidazole derivative called 33258 Hoechst.

Other fluorochromes have been found which preferentially attach to guanine-cytosine rich DNA. These include chloromycin A_3, mithramycin (Schweizer, 1977) and oligomycin (van de Sande et al, 1977) and the banding patterns are the opposite of those produced by Quinacrine Mustard.

Q-banded chromosome preparations can only be examined using an ultraviolet microscope to detect fluorescence, and since this equipment is expensive, other methods of producing banding patterns were investigated.

G-Banding

In 1971, Drets and Shaw produced a pattern of bands similar to Q-bands by treating fixed chromosome preparations with sodium hydroxide and saline sodium citrate (SSC) then staining with

Giemsa. This was also reported by other groups of research workers in the same year using similar techniques (Schnedl; Sumner, Evans and Buckland; and Patil, Merrick and Lubs). In 1972, Shiraishi and Yosida produced the pattern by pretreating chromosome preparations with phosphate buffered urea. Whilst other research workers (Dutrillaux and Lejeune; and Seabright, 1971) used pronase and trypsin respectively prior to staining with Giemsa and produced the same banding pattern. This pattern is called G-Banding (Paris Conference, 1972) since Giemsa stain is used.

Since the pattern produced by G-banding is the same as Q-banding, it might be thought that the underlying mechanisms which produce the bands are the same. However, there is no pretreatment of chromosome preparations prior to staining with fluorochrome in Q-banding (Caspersson et al, 1969) whilst G-banding depends on pretreatment with a denaturing agent (Sumner and Evans, 1973).

In 1972, Kato and Moriwaki suggested that the G-banding pattern was produced because certain regions of the chromosome are more susceptible than others to the effect of denaturing agents used during pretreatment and this was supported by the results of research by Ridler and O'hara (1972) which demonstrated that banded regions of the chromosome contain proteins which are resistant to proteolytic enzymes. In 1973, Sumner, Evans and Buckland demonstrated that almost all histone proteins are removed during the fixation of chromosomes with methanol : acetic acid and suggested that the remaining non-histones may interact with dye and contribute to the banding

pattern. In the same year, Sumner and Evans showed that chemical bands are broken during the pretreatment process and that the degree of staining varies with the concentration of DNA. Consequently, these authors concluded that the process of G-banding is dependant on the action of several different factors including the denaturing of certain proteins and variability in the uptake of dye according to the amount and type of exposed DNA.

The influence of chemical bonds on G-banding was reinforced by Sumner in 1974 when he demonstrated that G positive bands are rich in protein disulphides whereas sulphydryls predominate in G negative bands.

The identification of individual human chromosomes from their banding pattern was described in 1971 (Paris Conference, 1972). In 1976, the G-banding pattern of chromosomes from domestic animals was described, although this was not published until 1980 (Ford et al.1980). During the intervening period, Gustavsson (1980) compared the use of different banding techniques in domestic animals and concluded that G-banding is an accurate technique for identifying individual chromosomes since differential staining is produced along the chromatid arms of each chromosome.

C-Banding

A different banding pattern was discovered by Pardue and Gall in 1970, when attempting to hybridise RNA into mouse chromosomes. Their technique involved separation of DNA into single strands (denaturation) followed by reconstruction of DNA

into double strands (annealing). In 1968, Britten and Kohne had discovered that separate DNA strands reassociate at different rates. In particular, one faction of mouse DNA, called satellite DNA, reassociates very quickly. Furthermore, Britten and Kohne demonstrated that satellite DNA is located in the centromeric region of the chromosome and consists of highly repetitive nucleotide sequences. When Pardue and Gall stained the treated chromosomes with Giemsa, they produced a banding pattern which stained the centromere dark and the chromosome arms pale and they concluded that they had found a staining method to identify areas of the chromosome with highly repetitive nucleotide sequences on the DNA. The pattern was called C-banding since the centromeric region stains darkly. Furthermore, they assumed that the repetitive DNA in the stained areas was more condensed than non-stained areas and, therefore, more resistant to denaturation.

Work by Comings and co-workers in 1973 suggested that interaction between DNA and chromosome protein may influence the pattern of C-banding. However, it is not clear whether these interactions are between DNA and histones or non-histones (Matsukuma and Utakoji, 1977).

In 1978, Comings pointed out that C-bands are produced wherever highly repetitive nucleotide sequences occur on the chromosome, regardless of the base pair which predominates. However, the precise chemical mechanisms of C-banding are still not fully understood.

By using Q-, G- and C- banding techniques, it was possible to identify most chromosomes accurately. However, difficulty was experienced in studying the terminal parts of certain chromosomes

which did not stain with these techniques. Consequently further attempts were made to develop techniques which would stain these regions.

R- and T- Banding

In 1971, Dutrillaux and Lejeune incubated chromosome preparations in phosphate buffer of pH 6.5 at 87°C for 10-12 minutes and found that staining with Giemsa produced a banding pattern which was the reverse of Q- and G-banding. This method is called R-banding.

A variation of this technique was reported by Dutrillaux in 1973 whereby the pH was reduced to pH 5.1 and acridine orange was used instead of Giemsa. Using this method, only the terminal part of human chromosomes were stained and this method is called T-banding.

In 1973, Daniel and Lam-Po-Tang demonstrated that adenine-thymine nucleotide base pairs are denatured at 87°C and they suggested that this interferes with the uptake of stain and, therefore, areas of the chromosome which are rich in these nucleotides stain less intensely.

The techniques of R- and T-banding are slightly more complex than G-banding. However, they are frequently used to study structural changes involving the terminal regions of chromosomes which are lightly stained by G-banding and darkly stained by R- and T-banding.

Changes in Chromosome Morphology and Number

Now that techniques were available to identify individual chromosomes by differential staining, these techniques were used

to demonstrate rearrangements of chromosome structure. Early investigations, in 1923 by Bridges using Drosophila and in 1931 by McClintock using Maize, demonstrated that, in certain circumstances, changes in chromosome structure occur which interfere with mitosis and meiosis. However, many of these changes are not visible in conventionally stained chromosome preparations and, consequently, it was not until the development of banding techniques that these structural aberrations were seen. Nevertheless, earlier research workers were able to identify several different types of chromosome aberrations and, as early as 1927, Muller demonstrated that certain chromosome aberrations were a consequence of breaks in the chromosome. which joined with one another to produce a different chromosome structure.

Sister chromatid reunion

If a break is present in adjacent chromatids of a duplicated chromosome, then the broken ends lying side by side may unite to form a sister chromatid reunion (Muller, 1927). This results in the formation of two different chromosome structures. One structure does not have a centromere and, therefore, is lost during cell division. However, the other structure contains a centromere and two conjoined chromatids.

In 1942, McClintock investigated sister chromatid reunion in maize and demonstrated that the conjoined chromatids form a bridge between the two daughter cells during anaphase. She also observed that the chromatid bridge breaks before cell division is complete and that the break does not occur precisely in the middle of the chromatid bridge and, therefore, the chromosome

material is not equally distributed between the two daughter cells. Furthermore, when chromosome duplication occurs in the daughter cells, the broken ends of the two chromatids unite, as in the parent cell, and a chromatid bridge forms again during mitosis. This process is repeated for several generations, resulting in the chromosome content being distributed less evenly between daughter cells at each cell division until the cells are no longer viable.

Isochromosomes

When a transverse break occurs through the centromere of a duplicated chromosome, then two new structures are formed, each with a metacentric centromere. These structures are called isochromosomes since each contains a pair of identical chromatid arms (Rhoades, 1940) which can be identified by G-banding and Rhoades demonstrated that cells with isochromosomes are capable of meiosis.

Deletions

Chromosome rearrangements resulting from two breaks in the same chromosome were investigated in 1931 by McClintock who demonstrated that three chromosome sections are produced and providing the centromere is situated on one of the outer sections, then these sometimes join together and exclude the middle section to form a chromosome with a deletion and an acentric fragment. This fragment is lost during cell division and, although the chromosome with the deletion undergoes mitosis as usual, meiosis is only possible if a loop, corresponding to the deleted section, forms on the homologous chromosome to

facilitate synapsis between the two homologues.

Duplications

If two breaks occur on each chromatid of a duplicate chromosome then Bridges (1923) demonstrated that the section between the two breaks of one chromatid is sometimes inserted into the sister chromatid at one of its break points to produce one chromosome with a deletion and another with a duplication after completion of cell division. However, as with deletions, meiosis is only possible if the duplicate section forms a loop to facilitate synapsis between the homologous pair of chromosomes.

Ring Chromosomes

If a break occurs on both ends of a chromosome and these subsequently unite, then this produces a ring chromosome. These abnormal structures are capable of mitosis and are frequently seen in somatic cells after X-irradiation (Evans, 1967). However, meiosis is impossible since complicated structures which are formed by crossing over prevent separation of the duplicate chromosomes into daughter cells.

Inversions

Another structural rearrangement, called an inversion, results when a section of chromosome between two breaks rotates through 180^{o} and rejoins. If both breaks occur on the same side of the centromere, the inversion is paracentric and does not change the shape of the chromosome. Consequently, banding techniques are required to identify paracentric inversions In contrast, inversion of a chromosome section with one break on either side of the centromere is called pericentric inversion and often results in a change in the length of each chromosome arm

which is visible in conventionally stained preparations.

In 1939, McClintock showed that inversions often form a loop to facilitate synapsis during meiosis and viable gametes are produced providing crossing-over does not involve the section of chromosome within the loop. However, in 1955, Rhoades demonstrated that meiosis is impossible if crossing-over occurs within the loop since this results in the formation of complicated structures which prevent separation of the chromosomes into daughter cells.

Translocations

As early as 1916, Bridges used the term translocation to describe the transfer of a chromosome segment from its original position.

(a) Shifts

Relocation of the segment within the same chromosome requires three breaks in the chromosome and is now called a shift. A shift between different arms of a chromosome results in a change in its arm to length ratio which, providing the section is sufficiently large, is detectable in the cell karyotype. However, a shift within the same chromosome does not affect the shape of the chromosome and therefore banding techniques are required to demonstrate the shift. Since there is no loss of genetic material in the chromosome with the shift, the cell is viable and mitosis occurs normally. However, the rearrangement in chromosome structure prevents pairing of homologous chromosomes during meiosis and, therefore, chromosomes containing a shift are not inherited by subsequent generations.

(b) Insertions

Transfer of a chromosome segment from one chromosome to another also requires three separate breaks and is called an insertion. Mitosis is unaffected in cells containing chromosome insertions. However, changes in the length of the chromosomes involved prevents synapsis of the homologous chromosomes during meiosis and, in 1965, Lejeune and Berger demonstrated that this results in unbalanced gametes which are non-viable.

(c) Reciprocal translocations

A reciprocal translocation results when an exchange of genetic material takes place between two chromosomes following a break in each chromosome. The exchange of acentric segments produces two new chromosomes, of altered morphology, which contain the same total genetic material as their precursors and, therefore, cell viability and mitosis are unaffected. However, if the centric segments of the two chromosomes fuse with each other and the acentric segments fuse, then two new chromosomes are produced, one of which is dicentric and the other acentric. The acentric fragment is lost during cell division since it lacks a centromere for attachment to the spindle. However, the two chromatids of the dicentric chromosome may interlock during mitosis which results in either a break occurring in the chromatids at cell division, or both chromatids remaining interlocked and passing into the same daughter cell (Buckton et al, 1962).

The behaviour of chromosomes with a reciprocal translocation during meiosis is intricate since a complex quadrivalent structure forms to enable synapsis between homologous segments on

different chromosomes (McClintock, 1945). This structure is further complicated by crossing-over and, in 1969, Ford and Clegg demonstrated that genetically unbalanced gametes are produced.

(d) Robertsonian translocations

As early as 1916, Robertson described the fusion of two telocentric chromosomes at the centromeres to form one metacentric chromosome. This type of structural rearrangement is now called a centric fusion or Robertsonian translocation. These terms are also used to describe the centromeric fusion of two acrocentric chromosomes which is produced when a break occurs in the centromere of both chromosomes and the long arm segments fuse together to form a metacentric chromosome containing virtually all the genetic material, whereas the short arm fragments fuse to form a minute acentric chromosome which is lost during subsequent cell division (White, 1957).

In 1970, using the tobacco mouse (Mus poschiavinus), Tettenborn and Gropp demonstrated that the synapsis produced by the pairing of a Robertsonian translocation with its homologous chromosomes during meiosis forms a trivalent structure and that the chromosomes separate from each other in either of two alternative ways during cell division. If the homologous chromosomes separate into one daughter cell and the translocation into the other then genetically balanced gametes are produced and this is now called alternate segregation. However, if one of the homologous chromosomes segregate with the translocation, then genetically unbalanced gametes are produced and this is now called adjacent segregation or non-disjunction.

Changes in Chromosome Number

Differences in the normal diploid complement of chromosomes are described under two categories. If a cell has three or more times the normal haploid number then it is polyploid whereas cells with more or less than an integral multiple of the haploid number of chromosomes are aneuploid (Tackholm, 1922).

Polyploidy

In 1939, Geitler showed that the normal complement of chromosomes is doubled by the process of endomitosis whereby each chromosome is duplicated without the disappearance of the nuclear membrane, formation of a spindle, or division of the cytoplasm. Polyploidy also occurs as a result of endoreduplication whereby the chromosomes duplicate two or three times instead of once as is in normal mitosis (Levan and Hauschka, 1953).

In 1948, Wilson and Leduc demonstrated that fusion of two cells causes polyploidy and polyploid cells are occasionally produced in cells treated with colchicine or its related compounds due to interference in the spindle formation during mitosis (Herreros et al, 1966).

Under normal circumstances, a diploid zygote is produced by the fertilisation of a haploid ovum with a haploid spermatozoon (Strasburger, 1888). However, in 1949, Beatty and Fischberg demonstrated that suppression of the second meiotic cytoplasmic division prevents the formation of the second polar body and creates a diploid ovum which, when fertilised, produces a triploid zygote. In 1957, Braden demonstrated that suppression of the first polar body formation also results in a diploid ovum. Furthermore, he showed that a polyploid zygote is produced when a

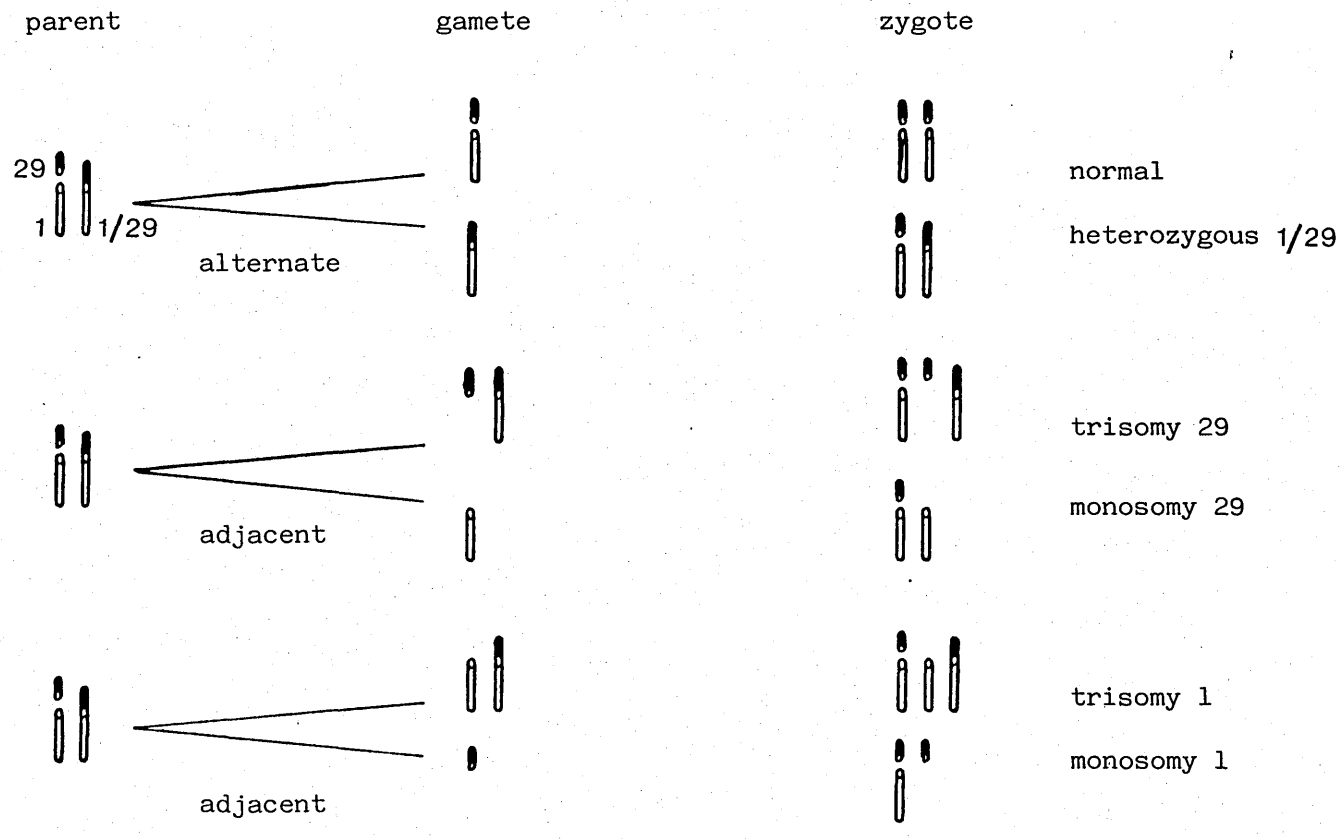

Figure 4. A diagram illustrating the types of segregation of chromsomes numbers 1 and 29 during meiosis in an animal heterozygous for the 1/29 Robertsonian translocation, and the zygotes produced by fertilisation with normal gametes

haploid ovum is fertilised by more than one spermatozoon and, in addition, his results supported earlier research (Beatty and Fischberg, 1949) which showed that polyploid zygotes subsequently die early in gestation.

Aneuploidy

In 1921, Mavor discovered that X-irradiation can produce cells which are deficient in one chromosome and such cells are now described as monosomic. These cells occur when one chromosome lags behind the others during anaphase and is excluded from the nucleus of the daughter cells, but they also occur as a consequence of non-disjunction whereby one daughter cell is monosomic and the other is correspondingly trisomic.

In 1970, Hamerton demonstrated that non-disjunction in the human is more likely to involve chromosomes belonging to a Robertsonian translocation. This is also the situation in other species including the mouse (Tettenborn and Gropp, 1970) and sheep (Chapman and Bruere, 1975), and in cattle Logue and Harvey (1978[a]) demonstrated that approximately 6% of secondary spermatocytes are aneuploid in bulls heterozygous for the 1/29 Robertsonian translocation. The various aneuploid gametes are shown in Figure 4.

Causes of Chromosome Aberrations

Irradiation

Muller was the first research worker to clearly demonstrate a cause of chromosome aberrations when, in 1927, he demonstrated that chromosome rearrangements and mutations are produced in Drosophila exposed to X-rays. In 1938, Sax demonstrated that

breaks occur in the chromosome structure of plants exposed to X-rays and suggested that changes in chromosome structure result when the broken ends join together in an altered sequence. Further investigation by Sax in 1941 demonstrated that the frequency of chromosome breaks is proportional to the dose of X-rays. He also demonstrated that X-rays applied to chromosomes immediately after replication, when the sister chromatids are closely apposed, result in an identical break in both chromatids. However, if the chromosomes are in metaphase at the time of exposure, the chromatids are no longer closely apposed and X-rays act on each chromatid independently.

In 1947, Thoday and Read showed that the frequency of chromosome abnormalities is not only related to the dose of X-rays by determining that chromosomes from the root tips of Vicia faba have three times as many breaks when exposed to X-rays in the presence of oxygen alone, than in the presence of nitrogen alone and they concluded that the susceptibility of chromosomes to the effect of X-rays is partly regulated by environmental factors. Further supportive evidence came from the results of investigations, in 1948, by D'Amato and Gustafsson which showed that different rates of chromosome abnormalities are produced by the same dose of X-rays when the environmental parameters of temperature, oxygen tension and hydration are varied.

Another cause of chromosome aberrations was discovered by Stadler in 1939 when he demonstrated that various structural rearrangements, including translocations, inversions and deletions, are produced in chromosomes of maize when exposed to ultraviolet irradiation. This was also observed in Drosophila

chromosomes by MacKenzie in 1941, although the incidence of abnormalities was less then that produced by X-rays.

Chemical agents

In 1941, Auerbach and Robson discovered that Mustard Gas [S ($CH_2CH_2Cl_2$] causes mutations in Drosophila chromosomes. However, their results were not published until 1947 for reasons of national security. The chromosome rearrangements which were identified included deletions, inversions, translocations and duplications. In 1949, Ford demonstrated that a delay may occur between the time of exposure to Mustard Gas and the onset of mutations in Vicia faba root tip cells and he also demonstrated that, as with X-rays, the frequency of chromosome aberrations is influenced by the chemical composition of the environment.

Many other chemicals are now known to cause changes in the chromosome structure and in 1960, Sharma and Sharma listed 50 unrelated compounds which interfere with the molecular composition of chromosomes. Furthermore, in 1961, Lerman demonstrated that acridine and related dyes intercalate between nucleotide pairs of DNA resulting in chromosome aberrations.

Infective agents

Certain infective agents are also responsible for chromosome aberrations since, in 1965, Fogh and Fogh demonstrated that chromosome breaks are produced in cell cultures infected with mycoplasma and, in 1969, Nichols reported on several viruses which cause chromosome defects both in vivo and in vitro. Nevertheless, the mechanism by which such infective agents cause chromosome aberrations is not yet fully understood.

The Incidence of Embryonic Death due to Chromosome Aberrations

Having recognised the existence of chromosome abnormalities, research workers began to study their effect on embryonic death by examining the chromosome complement of embryos. Nevertheless, to date, relatively few cytogenetic studies of this kind have been carried out and, therefore, the true incidence of embryonic death due to chromosome aberrations is unknown.

Human

In 1967, Carr demonstrated that certain cases of spontaneous abortion in the human are due to chromosome abnormalities of the foetus, and suggested that such abnormalities may also cause embryonic death. However, Boué and Boué pointed out, in 1973, that the true incidence of embryonic or foetal death is difficult to determine accurately since, in many cases, affected embryos are resorbed before pregnancy is diagnosed and, therefore, their presence is not recognised. Consequently, estimations of the frequency of human embryos with an abnormal karyotype ranges between 50 per cent (Boué, Boué and Lazar, 1975) and 20 per cent (Ford, 1975).

In 1978, Japanese research workers (Kajii and Mikamo, 1978) induced abortion in pregnant women during 3-4 weeks of gestation and found 9.3 per cent of the conceptuses with a chromosome abnormality. However, the true incidence may be even higher since this figure does not include chromosome aberrations in embryos which died earlier in gestation.

Several authors have published the frequency of different types of chromosome abnormalities recorded in cases of spontaneous human abortion and these are displayed in Table 1.

TABLE 1

Frequency of different types of chromosome abnormalities found in six spontaneous abortion studies in the human

Survey	Polyploidy	Monosomy	Trisomy	Structural Abnormalities	Others	Total
Lauritsen et al.(1972)	7	12	14	1	0	34
Therkelsen et al.(1973)	24	39	66	6	4	139
Kajii et al.(1973)	15	12	51	3	1	82
Boué et al.(1975)	240	140	495	35	11	921
Creasy et al.(1976)	50	68	143	10	16	287
Hassold et al.(1980[a])	103	112	212	20	16	463
Totals:	439	383	981	75	48	1926
Percentage of Abnormalities:	22.8	19.9	50.9	3.9	2.5	100.0

These results demonstrate that aneuploidy accounted for over 70 per cent of the chromosome abnormalities, although monosomic abortuses were only identified occasionally. Nevertheless, this observation concurs with the results of research by Gropp and his co-workers in 1974 which demonstrated that the majority of monosomic mouse embryos are eliminated before implantation in the uterus.

Pig

Early studies of embryos in the pig, by McFeely in 1967, revealed nine out of 88 (10.2 per cent) pig embryos with abnormal polyploid karyotypes which, he suggested, may result in death of the embryo and later work, in 1971, by Smith and Marlow demonstrated monosomy in 1.3 per cent of 25 day old embryos. However, the second group of research workers suggested that some embryos with an abnormal karyotype may already be dead by this stage.

Further investigations, by Moon, Rashad and Mi in 1975, recorded 26.7 per cent of 11 day old pig embryos with polyploid cells. However, subsequent research by Dolch and Chrisman in 1981 and Long and Williams in 1982, tended to suggest that the incidence is lower. Recent work by Hamlet in 1983, demonstrated 12 per cent of 4-7 day old pig embryos with a chromosome complement likely to cause embryo death and, in 1985, van der Hoeven, Cuijpers and de Boer recorded 7.3 per cent of 4 day old pig embryos with chromosome abnormalities including polyploidy and mixoploidy.

Several investigations have also been made into the incidence of embryo death in pigs heterozygous for a chromosome translocation. In 1972, Åkesson and Henricson compared the numbers of corpora lutea and live piglets and calculated that embryo mortality was 37.2 per cent when a boar heterozygous for the 11 p+; 15 q- reciprocal translocation serviced karyotypically normal females. Embryo mortality of 70 per cent was reported in 1981 by King and his co-workers by examining the chromosomes of embryos from pigs with the 13 q-; 14 q+ reciprocal translocation and these research workers were able to demonstrate that the non-viable embryos were due to deletions and duplications which formed during meiosis. In 1982, Popescu and Boscher studied the karyotypes of embryos and newborn piglets from pigs heterozygous for the 4 q+; 14 q- reciprocal translocation and reported that 40.7 per cent of the embryos had an unbalanced karyotype although the newborn piglets all had normal karyotypes. The authors concluded, therefore, that the unbalanced karyotypes were lethal and resulted in death of the embryo.

Sheep

Initial investigations in the sheep, by Long in 1977, demonstrated no abnormal karyotypes in 102 10-18 day old sheep embryos. However, she suggested that any abnormal embryos may already have died by this stage. This suggestion is supported by the results of further studies by Long and Williams in 1980, involving 2 day old sheep embryos, which revealed 13 out of 89 (14.6 per cent) embryos with chromosome abnormalities. The predominant abnormality was trisomy which accounted for 4.7 per cent of embryos karyotyped and although hypodiploid embryos were

found, Long and Williams suggested that some were possibly artifacts due to loss of individual chromosomes whilst preparing chromosome spreads. If these are excluded, 6.0 per cent of embryos had chromosome abnormalities including trisomy and mixoploidy.

In 1980, Williams and Long examined the chromosomes of embryos recovered from superovulated sheep at either 2, 3 or 20 days post-coitum and recorded abnormal chromosome complements in 21 of the 91 (23.1 per cent) 2-3 day old embryos which were karyotyped although they suggest that the process of superovulation may have resulted in a higher incidence of abnormal embryos since the rate was higher in this study than that of an earlier study using non-superovulated sheep (Long and Williams, 1980). Only one abnormal chromosome complement, a 54 XY/55 XY mosaic, was identified in embryos recovered 20 days post-coitum which suggested to the authors that most embryos with an abnormal karyotype die before this stage. This was further supported by finding a high proportion of embryos in the process of degeneration at day 20 post-coitum.

Recent studies by Hamlet in 1983, revealed 9.0 per cent of 4-5 day old sheep embryos with chromosome complements, mostly polyploidy, likely to cause early embryonic death.

Cattle

The first report of chromosome abnormalities in bovine embryos, by McFeely and Rajakoski in 1968, revealed one out of 11 10-16 day old embryos with a diploid-tetraploid mosaic. In 1978, Eldridge, Larsen, James and Cowan reported on one triploid embryo

(quoted in Eldridge, 1985)
from a group of twelve 3-13 day old embryos. However, it was not until 1980 that more extensive investigations into the cytogenetics of bovine embryos were reported.

During that year, Hare, Singh, Betteridge, Eaglesome, Randall, Mitchell, Bilton and Trounson (1980) studied the chromosomes of 198 12-18 day old embryos recovered from 46 superovulated cattle. Chromosome analysis was possible in 159 embryos including three polyploid embryos and 69 embryos with polyploid cells in addition to diploid cells. The authors also reported that the frequency of polyploid cells increased between days 12 and 18 and was higher in certain donor cows than others.

The Effect of the 1/29 Robertsonian Translocation on Embryonic Death in Cattle

In 1969 Gustavsson reported that the 56- and 273- day non-return to service rates of animals inseminated by semen from bulls heterozygous for 1/29 Robertsonian translocation was significantly reduced by 3 and 6%, respectively. He suggested that this reduction in fertility was due to the formation of trivalent structures during meiosis which result in non-disjunction and the production of genetically unbalanced gametes capable of fertilisation although the fertilised embryos subsequently die. This suggestion was supported by the results of Logue and Harvey (1978[a]) which showed aneuploidy in approximately 6% of secondary spermatocytes from bulls heterozygous for the 1/29 Robertsonian translocation.

Further support was provided in 1980 by the results of research by Popescu who studied the chromosomes from 123 13 day old embryos recovered from 17 superovulated cows inseminated with

semen from a bull heterozygous for the 1/29 Robertsonian translocation, and compared the results with karyotypes prepared from 24 embryos, of the same age, from four cows inseminated with normal semen. It was possible to karyotype 11 embryos from the control group, of which all were normal. However, two of the 52 (3.9 per cent) karyotypes prepared from embryos in the translocation group were considered to be monosomic for chromosome number 1.

A brief report into the effect of the 1/29 Robertsonian translocation on embryo mortality was published by King, Linares, Gustavsson and Bane in 1980 after collecting 65 1-7 day old embryos from 17 superovulated cows inseminated with semen from bulls heterozygous for the translocation. They were able to karyotype 29 embryos and of these, 18 were normal, nine were heterozygous for the translocation, and two were apparently trisomic for chromosome number 1. The authors suggest that the trisomic embryos resulted from non-disjunction of chromosome number 1 during meiosis in the translocation bull as reported in previous publications (Logue, 1977; Logue and Harvey, 1978[a]; Popescu, 1978).

Further investigations by King, Linares and Gustavsson, published in 1981, reported on the results of their cytogenetic study which involved 88 1-15 day old embryos sired by bulls heterozygous for the 1/29 Robertsonian translocation, and 28 7-15 day old embryos sired by a normal bull. They were able to karyotype 47.4 per cent of the embryos and found that all those in the control group had the normal complement of 60 chromosomes, although the 15 day old embryos also had up to 25 per cent

polyploid cells. However, two (2.3%) embryos in the translocation group were considered to be trisomic for chromosome number 1.

It is currently assumed that embryo death due to the 1/29 Robertsonian translocation does not occur in members of the British Friesian breed of cattle since more karyotypes have been examined from animals belonging to this breed than any other in the United Kingdom and, in spite of finding the translocation in other breeds [e.g. Charolais (Long , 1985)] to date, it has never been identified in a British Friesian animal. However, the study which follows describes members of the British Friesian breed in which the 1/29 Robertsonian translocation was identified using C- and G-banding techniques. It also investigates the effect of the translocation on fertility by examining the breeding records of carrier animals and the karyotype of embryos sired by bulls heterozygous for the translocation.

Furthermore, the possibility that the translocation may have developed de novo in a member of the British Friesian breed is investigated and karyotypic aberrations which develop during the prolonged culture of cells are identified in order to investigate the suitability of using in vitro culture as a possible method to study the formation of chromosome aberrations, such as the 1/29 Robertsonian translocation which occur in vivo.

CHAPTER TWO

THE IDENTIFICATION OF INDIVIDUAL CHROMOSOMES USING BANDING TECHNIQUES

2.1 INTRODUCTION

A method which enabled the identification of individual chromosomes was first described in 1969 by Caspersson and his co-workers. This technique used Quinacrine Mustard to produce a fluorescent banding pattern along human chromosomes and was called Q-banding. It was adapted for use in cattle chromosomes by Hansen (1973). However, the banding pattern produced on the smaller chromosomes of each set were very similar and slight differences were only evident in the less contracted metaphase spreads.

Drets and Shaw (1971) described a method for identifying individual chromosomes which did not require the relatively expensive fluorescent microscope necessary for examination of Q-bands. This technique, which involved treatment of fixed chromosomes with sodium hydroxide followed by staining in giemsa, produced a pattern resembling that of Q-banding and was called G-banding. G-bands were also produced on human chromosomes by Seabright (1971) who used trypsin prior to staining. Evans, Buckland and Sumner (1973) modified the technique described by Drets and Shaw (1971) for use in cattle chromosomes. However, more distinct bands were obtained by Gustavsson, Hageltorn and Zech (1976[a]) and Lin and his co-workers (1977) using modifications of the method described by Seabright (1971). These research workers identified each individual cattle chromosome and arranged them in pairs to form a karyotype. However, the order in which chromosomes were arranged in the karyotype varied slightly between each group of research workers. Consequently, an international conference was held which recommended a standard

description for the G-banded karyotype of cattle and other domestic species (Ford et al. 1980).

Dutrillaux and Lejeune (1971) described a staining technique which produced a banding pattern which was the reverse of Q- and G-banding and this was called R-banding. Gustavsson and Hageltorn (1976) modified it for use in cattle chromosomes but they found no advantage to using R-banding instead of Q-banding to identify individual chromosomes. Nevertheless, these research workers point out that, whereas the telomeric region of most G-banded cattle chromosomes is unstained, this region is darkly stained using the R-banding technique and, consequently, the latter technique may be of more use when specifically studying the telomeric region of certain chromosomes.

A technique which specifically stains the telomeric region of human chromosomes was first described by Dutrillaux (1973) and Gustavsson, Hageltorn and Zech (1976 [b]) demonstrated that a modification of this method stained the telomeric region of cattle chromosomes. A banding technique which specifically stains the centromeric region was first described by Pardue and Gall (1970) in mouse chromosomes. Popescu (1973) used a modification of this technique to produce C-bands on cattle chromosomes and C-banding was subsequently used by several research workers (e.g. Gustavsson et al. 1976 [a]; Masuda et al 1978) to determine whether various Robertsonian translocations were monocentric or dicentric in nature.

None of these banding techniques were used in this laboratory at the beginning of the study. However, since G-

banding is the internationally recognised technique for identifying individual cattle chromosomes (Ford et al, 1980), methods of producing G-bands were investigated in additon to the use of C-banding to stain the centromeric region specifically.

2.2 MATERIALS AND METHODS

2.21 Chromosome preparations used

Fixed chromosome preparations for banding were obtained from routine blood cultures as described in Section 3.23. The amount of colcemid added to blood cultures used for C-banding gave a final concentration of 0.67 µg/ml of culture medium. Various amounts of colcemid were added to blood cultures used for G-banding to give a final concentration of 0.67, 0.335, 0.167 and 0.084 µg/ml of culture medium.

2.22 G-banding

Chromosome preparations which were at least seven days old were examined under phase contrast (Vickers, Photoplan) and those with a high concentration of moderately contracted metaphase spreads were selected for G-banding.

Two methods of producing G-bands were used. In method 1 trypsin and versene were used at room temperature (18-27^{o}C) to treat chromosome preparations. In method 2, trypsin alone was used at 18^{o}C. At the beginning of the study, both methods were used concurrently. However, the method which produced more distinct bands was selected for further investigation.

Method 1:

This method was based on the procedure described by Lin, Newton and Church (1977). 2.5% stock trypsin (Flow Laboratories) was stored in 2 ml aliquots at -20^{o}C until use. Various

concentrations of working trypsin solution were prepared by diluting the stock trypsin with equal volumes of Hank's Balanced Salt Solution (BSS) and versene (0.02% disodium salt in 0.85% saline, Flow Laboratories). The concentrations of working trypsin used were 0.12, 0.06 and 0.03 per cent adjusted to pH 8.0 with 5% sodium bicarbonate.

A microscope slide containing fixed chromosome preparations was immersed in freshly prepared working trypsin at room temperature (18-27°C) for a measured length of time. It was then dehydrated through 70, 95 and 100 per cent alcohol, rinsed in deionised water and air dried. The slide was stained for 2-3 minutes in 1.5 ml Giemsa (Gurr R66, B.D.H. Chemicals) diluted with 40 ml Sorensen's buffer of pH 6.8.

The chromosomes were examined under a light microscope (Leitz, Ortholux). If no bands were present then further slides were treated for a longer period in the working trypsin solution. If the chromosomes were distorted and over-trypsinised then further slides were treated in trypsin for a shorter time. The stained slides were dipped in xylene after drying at room temperature and mounted (D.P.X., B.D.H. Chemicals) with a coverslip.

Method 2:

This method used the technique described by Seabright (1971) with minor modifications. The concentration of fresh working trypsin used was 0.03% and was prepared by diluting stock trypsin (2.5%, Flow Laboratories) with Hank's BSS. The working trypsin was then refrigerated at +4°C until use when it was warmed by

holding the container in the grasp of a hand until the temperature reached 18°C. Otherwise, the procedure for obtaining G-bands was the same as described in Method 1 except that the slides were stained for four minutes.

2.23 C-banding

The technique described by Sumner (1972) was used with minor modifications. Slides containing fixed chromosome preparations were treated with 0.2 N hydrochloric acid for one hour at room temperature (18-27°C). During this time, a fresh solution of 5% barium hydroxide was prepared by boiling 5 g barium hydroxide (octahydrate) in 100 ml distilled water. The supersaturated solution was filtered (Number 1, Whatman) to remove undissolved crystals, and collected in a Coplin jar. The layer of scum which collected on the surface of this solution was removed by drawing a lens tissue across the surface. The slides were removed from the acid solution, rinsed in deionised water and immersed in the barium hydroxide solution for 5-15 minutes at 50°C. After thorough rinsing in deionised water, the slides were incubated for one hour at 60°C in 2 x SSC (0.3 M sodium chloride containing 0.03 M trisodium citrate), rinsed in deionised water and stained for one hour and a half with 1 : 50 Giemsa solution (Gurr's R66 : buffer pH 6.5, B.D.H. Chemicals). Finally, the slides were again rinsed with deionised water, dried at room temperature and dipped in xylene before mounting (D.P.X., B.D.H. Chemicals) with a coverslip.

2.24 Preparation of karyotypes

Evenly spread chromosome sets were photographed and the film (Technical Pan, Kodak) was developed (HC110, Kodak) and fixed

(Hypam, Ilford) according to the manufacturers' instructions. The chromosome image was enlarged (Leitz, Wetzlar) as much as possible without losing definition and three prints were obtained of the chromosome spread onto high contrast photographic paper (Ilfoprint 4.1 P, Ilford). The prints were processed according to the manufacturers' instructions, dried on a glazer (Kodak model 15TC) and flattened by placing overnight between heavy text books. One print was retained for reference and heat activated mountant (Paterson) was applied to the reverse of the remaining prints. After the mountant was dry, a scalpel blade was used to cut out each individual chromosome from one of the prints. Any chromosomes which overlapped were cut out from the other print. G-banded chromosome sets were arranged on a mounting board in pairs using the sequence described by Ford, Pollock and Gustavsson (1980) as a guideline and C-banded chromosomes were arranged in pairs in order of decreasing size. A permanent karyotype was obtained by activating the mountant with a heat press.

2.3 RESULTS

2.31 G-banding

Metaphase chromosome spreads were obtained using each of the various colcemid concentrations. However, almost all the chromosome sets examined using 0.67 or 0.335 µg/ml were highly contracted. More elongated chromosome sets were obtained using the lower concentrations and, consequently, all cells cultured for G-banding in this study were treated with 0.084 µg/ml colcemid.

Method 1:

Twelve slides were treated with 0.12% working trypsin for 120-180 seconds and 30 slides were treated with 0.06% trypsin for 10-20 seconds. Each chromosome examined from these slides appeared as a distorted faint outline. This was considered to be due to overdigestion by trypsin and, therefore, the concentration of trypsin was reduced further.

More than 70 slides were treated with 0.03% working trypsin. Those treated for longer than 60 seconds contained overdigested chromosomes. However, three separate batches of slides treated on different days for less than 60 seconds contained chromosomes with an indistinct banding pattern. The most conspicuous patterns occurred on the elongated chromosome sets and consisted of faint bands separated by slightly lighter bands. However, the bands were not well defined and, therefore, it was not possible to count the number on each chromosome. Consequently, individual chromosomes were not identified.

Table 2 shows the time in trypsin which produced the most conspicuous bands on chromosomes from the three batches of slides used and demonstrates that there was no relationship between the age of the slides and the time in trypsin. These bands were obtained on slides treated during the morning. However, no bands were produced when fresh slides were treated later in the day for the same time in trypsin. The chromosomes on slides from batches A and C were all overdigested, whereas those from batch B were underdigested. This inconsistency with which bands were produced using method 1 caused difficulty in obtaining a large number of banded chromosome sets for examination. The results of

G-banding using method 2 were more consistent and, since distinct bands were produced using this method, further investigation into the G-banding pattern on individual chromosomes was made using method 2.

TABLE 2

THE TIME IN 0.03% WORKING TRYPSIN SOLUTION WHICH PRODUCED THE MOST CONSPICUOUS BANDS ON CHROMOSOMES FROM THREE DIFFERENT BATCHES OF SLIDES USING METHOD 1

Batch of slides Identification	Age (weeks)	Time (seconds)
A	2	35
B	5	15
C	7	30

Method 2:

Over 180 slides were treated with 0.03% working trypsin. These slides belonged to ten different batches prepared 2-9 weeks prior to treatment which ranged from 10-300 seconds.

All chromosomes treated for longer then 240 seconds were overdigested by the trypsin and appeared as distorted, faint outlines on the slide. Chromosomes treated less than 90 seconds were apparently unaffected by the trypsin and appeared evenly stained.

Banded chromosomes were obtained on slides treated between 90 and 240 seconds. However, the length of time required to

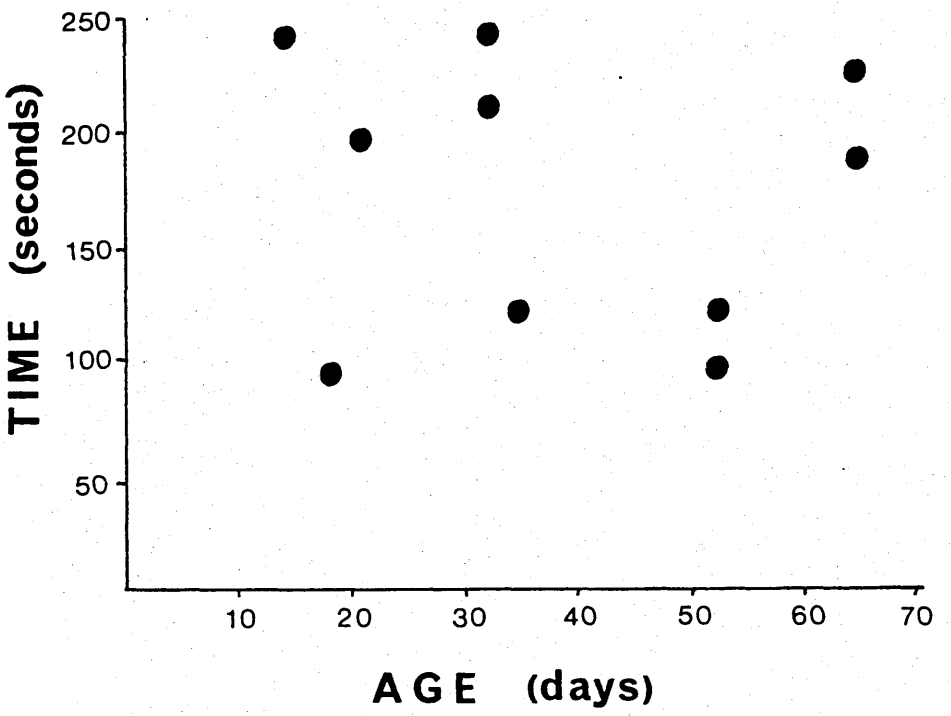

Figure 5. The age of slides related to the time required in 0.03% working trypsin to produce the most conspicuous banding pattern using Method 2

produce bands varied between batches. The time which produced the most distinct bands was called the optimal time and this is plotted for each batch on the scatter diagram in Figure 5. The most conspicuous bands occurred on the elongated chromosome sets with chromatids lying together. The banding pattern on these chromosomes consisted of a series of dark bands separated by light bands. These bands varied in thickness and were more numerous on the longer chromosomes. Elongated chromosomes treated for less than the optimal time were evenly stained, whereas those treated for longer tended to be distorted and contained faint bands which were indistinct.

There was no relationship between the age of the slides in each batch and the time required in trypsin to produce the most distinct bands since the correlation coefficient of these parameters (shown in Figure 5) is low ($r = -0.127$; $P > 0.05$).

The time required in trypsin to produce bands on slides from any particular batch remained constant throughout the day. Furthermore, fresh slides which were treated the following day required the same time in trypsin to produce bands. However, the length of time required in trypsin increased when slides aged several days between treatment. This is demonstrated in Table 3 which shows that when slides from batches D and E were first treated they required 90 and 195 seconds respectively to produce the most conspicuous bands although, when fresh slides were treated after ageing them further, the time required to produce distinct bands increased to 200 and 240 seconds, respectively.

TABLE 3

THE TIME IN 0.03% WORKING TRYPSIN SOLUTION WHICH PRODUCED THE MOST CONSPICUOUS BANDS ON CHROMOSOMES FROM TWO BATCHES OF SLIDES TREATED AT DIFFERENT AGES USING METHOD 2

Batch of slides Identification	Age (days)	Time (seconds)
D	18	90
	63	200
E	21	195
	28	240

G-banded karyotypes were prepared from 25 evenly-spread chromosome sets containing distinct bands. The length of chromosome 1 was measured in each karyotype and varied between 5.5 and 8.9 μ with a mean of 6.7 μ.

Bands tended to merge together in the more contracted chromosome sets and, consequently, fewer bands were distinguished on individual chromosomes from these sets. Furthermore, it was difficult to distinguish between the small chromosomes in the more contracted sets since the merged bands produced very similar patterns. Nevertheless, a different banding was produced on each of the small chromosomes in elongated sets which enabled them to be positively identified.

The banding pattern produced on each pair of chromosomes is described in Table 4. Chromosomes number 1, 5, 7, 10, 16 and 21 were particularly easy to identify due to their characteristic banding pattern. However, it was not possible to distinguish

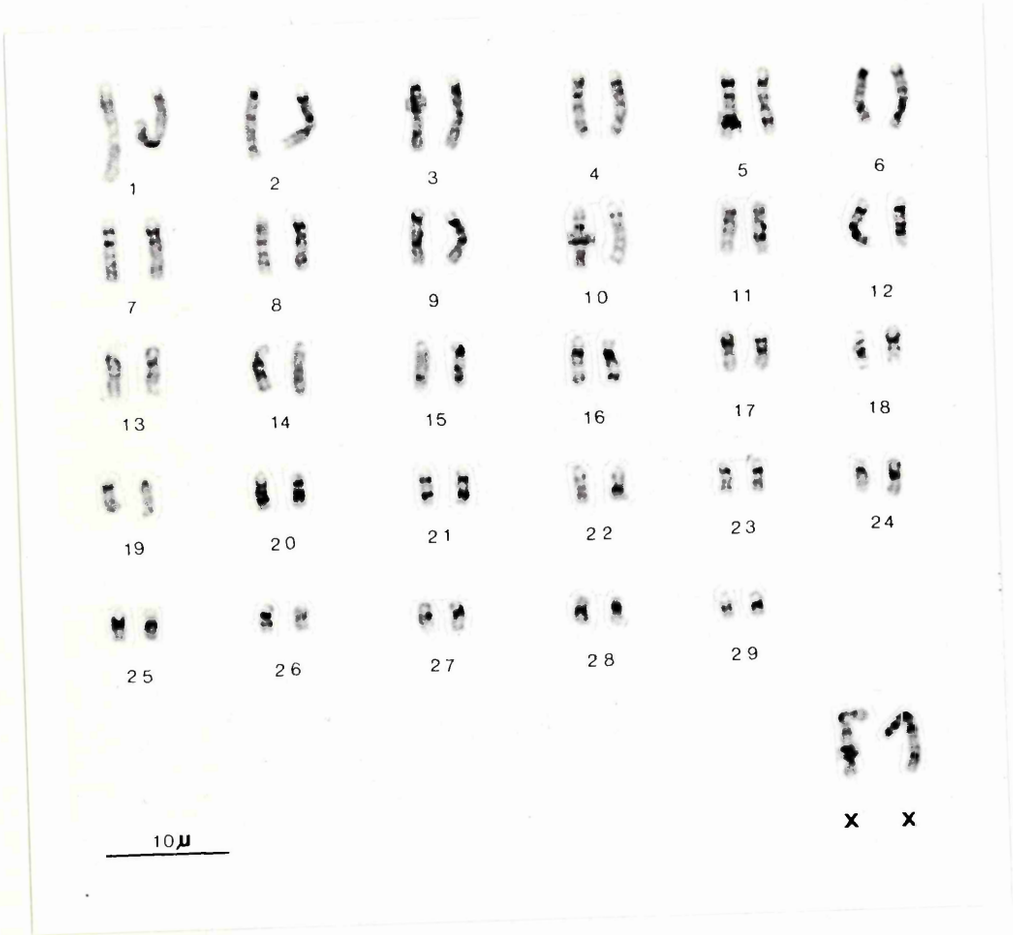

Figure 6

A representative normal G-banded bovine karyotype

between chromosomes 22 and 23 since their banding patterns were identical. Similar banding patterns were produced on chromosomes 4 and 6 which caused difficulty in distinguishing between them, particularly if the banding pattern was obscured by an overlapping chromosome. The banding pattern on chromosomes 25 and 27 were also similar. However, it was possible to readily differentiate between these two chromosomes in elongated sets where chromosome 25 had a faint terminal band which was absent in chromosome 27.

Figure 6 is a representative karyotype which demonstrates the G-banding pattern produced on each chromosome.

TABLE 4

THE G-BANDING PATTERN PRODUCED ON INDIVIDUAL CHROMOSOMES

Chromosome Number	Description
1	Identified as the longest autosome. There was a distinctive broad light band in the central region. This separated the proximal region, which had 2-3 dark bands, from the distal region. The distal region also had 2-3 dark bands, one of which was terminal in position.
2	A narrow light band was situated in the central region. There was a prominent dark band adjacent to the centromere followed by 2-3 dark bands. The distal region consisted of 3 dark bands and a light terminal band.
3	A distinct dark band was adjacent to the centromere followed by a pale band. There were two prominent central dark bands and the distal region contained 2-3 indistinct dark bands.
4	5-7 dark bands were evenly distributed along the chromosome. There was usually a broad pale band anterior to the distal two bands. This chromosome was sometimes difficult to differentiate from number 6.

(Cont'd)

TABLE 4 (Cont'd)

Chromosome Number	Description
5	The pattern was characteristically symmetrical. One prominent dark band was adjacent to the centromere followed by a light band. There were 2-3 dark bands in the central region followed by a dark band in the distal region and a light terminal band.
6	There were 5-7 dark bands evenly distributed along the chromosome. The distal band was usually less distinct. It was sometimes difficult to differentiate this chromosome from number 4.
7	Two prominent dark bands in the proximal region were separated by a prominent light band. There were 3-4 indistinct dark bands along the remainder of the chromosome.
8	The proximal half stained darkly and 2-3 dark bands were occasionally distinguished. This was followed by a broad pale band and two narrow dark bands in the distal half.
9	4-5 dark bands were evenly distributed along the chromosome. The darkest band was in the central region although the proximal part of the chromosome tended to be darker than the distal half.

(Cont'd)

TABLE 4 (Cont'd)

Chromosome Number	Description
10	There was a characteristic dark terminal band. A dark band was adjacent to the centromere followed by a light band. Two dark bands were in the central region. Each of these separated into two dark bands in elongated chromosome sets.
11	There were four evenly spaced dark bands. The third band was most prominent.
12	The proximal half consisted of a prominent dark band adjacent to the centromere followed by a pale band and a light band. The distal half contained two prominent dark bands followed by a pale terminal area.
13	There were two proximal dark bands which often fused to form one broad dark band. The central region contained a prominent dark band and the distal region stained pale.
14	This chromosome was pale adjacent to the centromere but became darker distally. There was a faint light band in the distal third followed by a dark terminal band.
15	There was a prominent dark band adjacent to the centromere followed by a narrow light band. The distal half contained 2-3 dark bands.

(Cont'd)

TABLE 4 (Cont'd)

Chromosome Number	Description
16	This was one of the easiest chromosomes to recognise. There was a prominent dark band adjacent to the centromere followed by another dark band. These often merged to form one broad dark band in contracted chromosome sets. The distal half consisted of a characteristic prominent light band followed by a dark band.
17	There were two dark bands in the proximal half which merged to form one broad dark band in contracted sets. The remaining part of the chromosome stained pale.
18	The central region contained a broad dark band which occasionally appeared like two dark bands merging together. There was a dark band adjacent to the centromere and a dark terminal band.
19	This chromosome was pale with a dark band adjacent to the centromere and a light terminal band.
20	The banding pattern on this chromosome varied according to the degree of contraction. Three dark bands were distinguished on elongated chromosomes with a faint light band separating the central and distal bands. Only two dark bands were visible on moderately contracted chromosomes.

(Cont'd)

TABLE 4 (Cont'd)

Chromosome Number	Description
21	This chromosome was easily recognised by its two prominent dark bands. One of these was adjacent to the centromere and the other was in the distal half. The remainder of the chromosome was pale.
22 & 23	It was not possible to distinguish between these chromosomes. Each had three dark bands which merged together in contracted sets.
24	There were two dark bands in the proximal half which merged to form one broad dark band in contracted sets. The distal half was pale with a faint dark terminal band.
25	The proximal half contained two dark bands which were not clearly separated and the distal half contained a faint dark terminal band. This chromosome was sometimes difficult to distinguish from number 27.
26	There were two dark bands separated by a prominent light band. The dark band adjacent to the centromere was more prominent than the other dark band.
27	There were two dark bands in the proximal region. The distal region was pale with no terminal dark band. This chromosome was sometimes difficult to distinguish from number 25.

(Cont'd)

TABLE 4 (Cont'd)

Chromosome Number	Description
28	There was one dark band in the proximal central region and a faint dark terminal band.
29	There was one proximal dark band. The remainder of the chromosome was pale without any dark terminal band.
X	The darkly stained centromeric region separated the submetacentric chromosome into two arms. The short arm contained a least two dark bands. The long arm contained a prominent light central band which divided the arm into a proximal and distal area. Each of these areas contained 2-3 dark bands.
Y	The centromeric region was darkly stained. The short arm was generally light with a narrow dark terminal band. The long arm contained two dark bands.

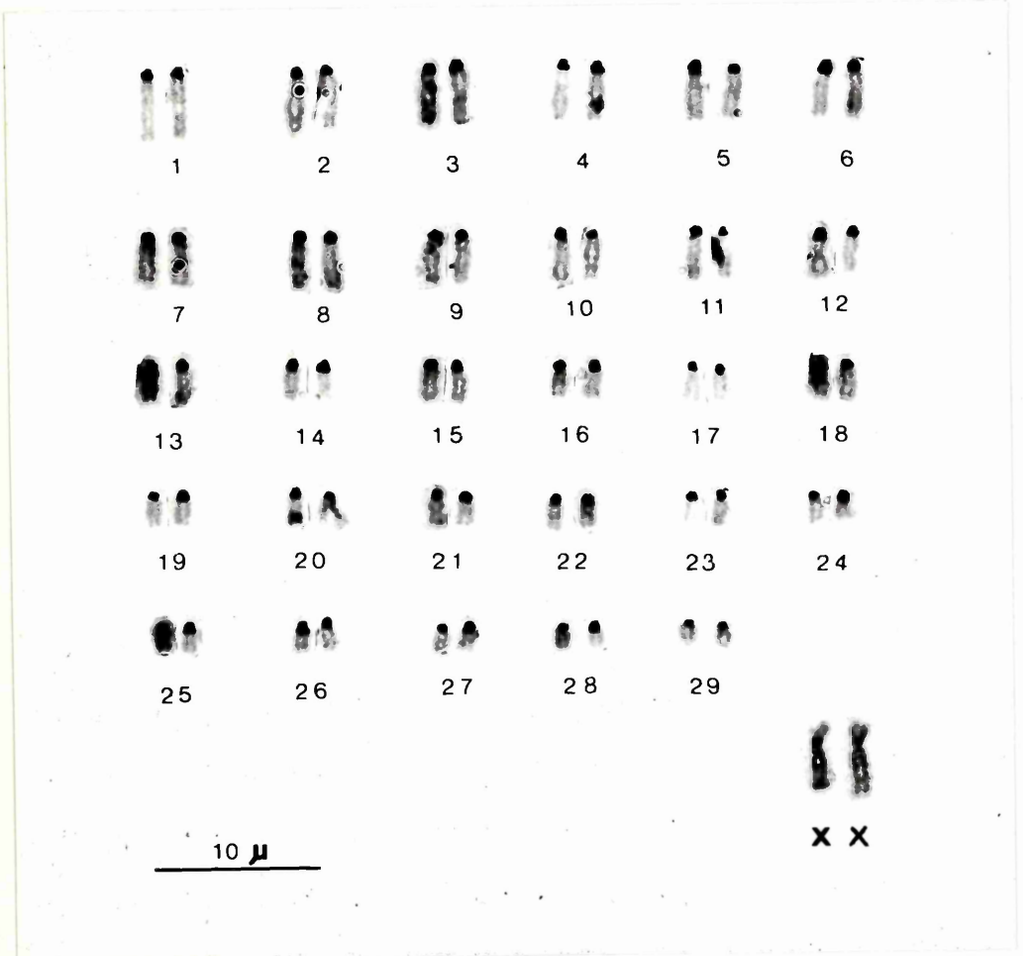

Figure 7

A representative normal C-banded bovine karyotype

2.32 C-banding

Barium hydroxide crystals formed on all slides treated to produce C-bands at the beginning of the study. These interfered with examination of the chromsomes. However, the formation of crystals was subsequently prevented by removing the scum which formed on the surface of the barium hydroxide solution with a lens tissue prior to immersing the slides.

The C-banded pattern was easier to produce on chromosomes than the G-banded pattern. Almost all slides treated with 5% barium hydroxide solution contained banded chromosomes. However, the bands were indistinct in slides treated less than ten minutes. Consequently, slides for banding were treated for 10-15 minutes.

C-bands were produced on both elongated and contracted chromosome sets. However, the bands were more distinct in the less contracted sets. Twenty elongated and moderately contracted sets belonging to female animals were examined. In each of these, the X chromosomes were stained pale although a slightly darker area was occasionally seen in the centromeric region. The arms of all autosomes were uniformly stained pale. However, the centromeric region of each autosome consisted of a prominent single dark band. The size and staining intensity of this band was similar in each autosome.

Figure 7 is a representative karyotype which shows the C-banding pattern produced on a complete set of chromosomes.

2.4 DISCUSSION

Although the range of colcemid concentrations used in this study stopped cells dividing during metaphase, sufficiently elongated chromosome spreads suitable for G-banding were obtained using the lowest concentration only. Inoue (1952) demonstrated that colcemid stops cell division by inhibiting the formation of spindle fibres. However, Taylor (1965) subsequently demonstrated that colcemid and its related compound colchicine, cause overcontraction and separation of the chromatid arms if either high concentrations are used or the period of exposure is prolonged. Consequently, all concentrations used in the present study apparently inhibited spindle formation although only the lowest concentration, giving a final concentration of 0.084 µg/ml, was sufficiently low to avoid overcontraction when used for 90 minutes.

Most research workers incubate cells for 90 minutes in colcemid to stop cell division and Evans, Buckland and Sumner (1973) showed that metaphase spreads suitable for banding are obtained from cattle, sheep and goats using the same concentration although the amount was not mentioned. Hageltorn and his co-workers used colchicine instead of colcemid and found that the same concentration (200-400 µg/ml) used for 90 minutes stopped mitosis in leucocytes from cattle (Gustavsson, Hageltorn and Zech 1976[b]), pigs (Hageltorn and Gustavsson 1973), cats (Hageltorn 1980) and rabbits (Hageltorn and Gustavsson 1979). The concentration used by Lin et al (1977) to obtain elongated chromosome spreads for G-banding was 0.1 µg/ml for 90 minutes, which is similar to that used in this study. However, Lee and

Kamra (1981) used a lower concentration of 0.05 µg/ml of colcemid to treat human leucocytes. Nevertheless, these research workers found it was necessary to increase the period of incubation to 120-180 minutes in order to obtain a suitable supply of elongated chromosomes for G-banding. It may be that elongated chromosomes could have been produced in the present study using a lower concentration of colcemid for a longer period. However, since an adequate supply was obtained using 0.084 µg/ml for 90 minutes, the concentration and time were not varied.

A method which, to date, has not been used in cattle to obtain elongated chromosomes suitable for G-banding involves the synchronising of cell division. This technique was first described by Yunis (1976) who synchronised the division of human leucocytes by adding amethopterin to the culture medium followed several hours later by thymidine. Amethopterin is a folic acid analogue which inhibits DNA replication by causing a thymine deficiency. Consequently, cells are unable to enter mitosis. However, the subsequent addition of thymidine enables DNA replication to proceed and the cells divide in synchrony. Cell division is then stopped by adding colcemid and a larger number of elongated chromosome sets are obtained than using colcemid alone. Synchrony of cell division prior to adding colcemid is now frequently used in the human to obtain chromosomes for G-banding (Ikeuchi 1984) and it may be that this technique could be adapted for use in cattle.

Although Lin et al. (1977) obtained distinct G-bands on cattle chromosomes treated with 0.12 mg/ml trypsin for 45

seconds, all chromosomes were overdigested when the same concentration was used to treat slides in the present study. Nevertheless, Seabright (1972) noted that different batches of trypsin vary in their efficiency and it may be that the trypsin used in this study was more potent than that used by Lin et al. (1977). Supportive evidence for this was provided when slides treated for less than 60 seconds in a two-fold dilution of the concentration used by Lin et al.(1977) contained chromosomes which were not overdigested.

A distinct banding pattern was obtained in this study on chromosomes treated with 0.03% trypsin dissolved in balanced salt solution alone, but not when trypsin was dissolved in balanced salt solution containing versene which tends to suggest that the presence of the chelating agent adversely affected the formation of bands. In contrast, however, Wang and Federoff (1972) and Lin et al.(1977) produced distinct G-bands on chromosomes treated with trypsin in balanced salt solution containing versene. However, whereas these research workers attached the chromosomes onto the microscope slide by passing it through a flame, the chromosomes were attached in the present study by drying in air and, since the molecular structure of chromosomes is altered by heat (Comings 1978), it would appear that the structure of flame-dried chromosomes is such that versene augments the action of trypsin, whereas, in air-dried chromosomes, the structure is refractory to the action of trypsin when versene is present. Nevertheless, Seabright (1972) advised against flaming slides since she found that heat adversely affects the banding pattern which is produced and most research workers banding human

chromosomes (e.g. Francke and Oliver 1978) now use air-dried slides to produce G-bands.

An alternative reason why satisfactory G-bands were obtained by Lin et al.(1977) yet not in the present study, might be because the giemsa stains used were manufactured by different companies. However, Curtis and Horobin (1975) produced satisfactory G-banding on chromosomes stained with several combinations of the dyes used to make up giemsa. Consequently, it is unlikely that any slight variation in giemsa stain produced by a different manufacturer is responsible for the poor banding obtained using Method 1. Furthermore, the Giemsa stain used in this study produced distinct bands when used to stain chromosomes which were treated using Method 2.

The balanced salt solution used by Lin et al. (1977) to dissolve trypsin was Puck's BSS, whereas Hank's BSS was used in the present study. Nevertheless, the only difference between these two solutions is that Hank's BSS contains 60 mg/ml KH_2PO_4 and 48 mg/ml Na_2HPO_4, neither of which is present in Puck's BSS, and it is unlikely that the presence of these salts interfered with the formation of G-bands in Method 1 of this study since other research workers (e.g. Trusler 1975) obtained distinct bands using Hank's BSS. Furthermore, satisfactory G-banding patterns were obtained in Method 2 of this study using trypsin in Hank's BSS.

It is of interest to note that, using Method 1, the action of trypsin on each batch of slides varied during the day. For example, chromosomes from batches A and C were overdigested when

treated in the afternoon for the same length of time which produced faint bands earlier in the day. In contrast, chromosomes from batch B were undertrypsinised when treated later in the day. No variation in the action of trypsin during the day was reported by Lin et al.(1977). However, Bender, Kezdy and Gunther (1964) demonstrated that the enzymatic activity of trypsin is maximal when used near body temperature and decreases as the temperature is reduced. Furthermore, whereas the room temperature probably remained fairly constant in the laboratory used by Lin and his co-workers (1977), this temperature fluctuated considerably throughout the day in the present study due to the intermittent use of a steam autoclave and, therefore, the effect of the trypsin treatment probably also varied accordingly.

The temperature of trypsin used to treat chromosomes in Method 2 did not vary with the laboratory temperature but remained constant at 18°C. Using this method, the time required to produce G-bands was always the same when slides from any particular batch were treated at different times of the day which supports the suggestion that varying laboratory temperatures affected the time required in trypsin to produce bands using Method 1. Further support comes from the results of research workers banding human chromosomes (e.g. Seabright 1972) which showed variations in the response to trypsin when the laboratory temperature was uncontrolled. In addition, Hageltorn and Gustavsson (1973) reported that trypsin should be used at a constant temperature in order to obtain consistent results in pig

chromosomes.

Sperling and Weisner (1972) demonstrated that storing slides for several days before treatment with trypsin improves the quality of G-bands produced on human chromosomes and this was also found to be true for rat chromosomes (Gallimore and Richardson 1973). In the present study, all slides were stored for at least seven days prior to banding. However, the length of time required in trypsin to produce the most distinct banding pattern varied between different batches of slides. The same observation was made in other species including the rat (Zimmerman and Sihuonen 1973) and the pig (Hageltorn and Gustavsson 1973). Furthermore, Wiscovitch, Singh and Osborne (1974) investigated the relationship between the age of human chromosome preparations and the time required in trypsin to produce banding and found that the time required in trypsin increased with the age of each slide, presumably due to the effect of environmental factors on the fixed chromosomes. However, in the present study, there was no correlation between the age of the slide and the time required to produce G-bands. Nevertheless, environmental conditions differ between laboratories and it may be that the environment remained stable in the laboratory of Wiscovitch et al. (1974) which resulted in a direct relationship between the length of storage and the time required in trypsin. However, in the present study, ambient temperature and relative humidity fluctuated considerably depending on the use of various laboratory equipment including a steam autoclave and therefore, each batch of slides was stored under different conditions.

The G-banding patterns produced in this study were observed in the elongated sets of chromosomes only and all highly contracted sets appeared to be evenly stained which tends to suggest that the highly contracted sets were refractory to the action of trypsin. However, the prolonged treatment of these chromosomes in trypsin provided clear evidence to the contrary since they appeared as faint outlines due to overdigestion by the enzyme. Furthermore, the bands on any particular chromosome examined in this study tended to merge together as the degree of contraction increased and consequently, it is more likely that G-bands were produced on the highly contracted chromosomes exposed to moderate trypsin treatment but that these bands were too close together to be distinguished separately.

In 1980, the International Conference on the Standardisation of Banded Karyotypes of Domestic Animals published a recommended description for the G-banding pattern on cattle chromosomes (Ford et al.1980). However, only the minimum of features required to identify individual chromosomes were mentioned and consequently, the descriptions of banding patterns produced in the present study are more detailed. Nevertheless, the number of bands distinguished on any particular chromosome varied slightly depending on the degree of contraction. For example, between two and three dark bands were counted on each of the proximal and distal regions of chromosome 1 in this study. The number of bands on this chromosome is not mentioned by Ford et al.(1980), although Lin et al.(1977) counted four dark bands on both the proximal and distal region. Nevertheless, the chromosome sets

examined by Lin and his co-workers tended to be less contracted than those examined in the present study (e.g. the average length of chromosome 1 examined by Lin et al. was 9.9 μ compared with 6.7 μ in the present study.) Gustavsson et al.(1976[a]) were unable to accurately count the number of bands in chromosome 1. However, this group of research workers examined G-bands on chromosomes which were more contracted than those examined in this study (chromosome 1 was approximately 5.8 μ long) and, therefore, the bands tended to merge together and were less distinct.

Chromosomes 1, 5, 7, 10, 16 and 21 were readily recognised in this study by their distinctive banding pattern. Distinct banding patterns were also found on these chromosomes by Gustavsson et al.(1976[a]) and Lin et al.(1977) and the descriptions correspond closely to those obtained in the present study. However, the chromosomes identified as 5 and 7 in this study were numbered as 4 and 6 by Gustavsson et al.(1976[a]), and those identified as 5, 7, 10 and 16 in this study were numbered 7, 5, 9 and 15, respectively, by Lin et al.(1977). Each group of research workers attempted to arrange the chromosomes in order of decreasing size. However, it is often not possible to distinguish any difference between the length of neighbouring chromosomes (Lin et al.1977) and consequently, the sequence of these particular chromosomes is arbitrary. This discrepancy emphasises the importance of following a standard system of numbering chromosomes such as that recommended by Ford et al. (1980) and used in the present study.

The banding pattern on chromosomes number 4 and 6 in this study were very similar with each having 5-7 dark bands, depending on the degree of contraction. However, chromosome 4 had a dark terminal band which was absent in chromosome number 6. Gustavsson et al.(1976[a]), Lin et al.(1977) and Ford et al. (1980) also recognised a dark terminal band in only one of these chromosomes and used it to distinguish between them. However, this identification feature is only useful if the terminal region of the chromosome is not obscured by an overlapping chromosome. In contrast, chromosomes with a distinctive banding pattern are usually recognisable even if they are partly obscured.

Ford et al.(1980) recognised that the banding pattern on chromosomes 22 and 23 are very similar and described the pattern as three dark bands of similar intensity with the distal two closer together in chromosome 22. This description corresponds to that given by Lin et al.(1977) although this group of research workers numbered them 23 and 24. However, in the present study, no difference was detected in the distribution of the three dark bands in chromosomes 22 and 23 and, consequently, it was not possible to distinguish between them. Nevertheless, the G-banded karyotypes used in this study contained chromosomes which were generally less elongated than those examined by Lin et al.(1977) and, therefore, any difference in the distribution of bands would be less conspicuous. Consequently, more elongated G-banded chromosome spreads are required in order to clearly differentiate between chromosomes 22 and 23. This conclusion is supported by Gustavsson et al.(1976[a]) who used chromosome sets which were even more contracted than those used in either this study or that

of Lin et al. (1977) and recognised two bands only in chromosomes 22 and 23, although the distal band sometimes appeared double indicating that it consisted of two bands merged together.

The importance of elongated G-banded chromosome sets for the identification of individual chromosomes was further highlighted when the pattern on the smaller chromosomes was examined since differences were apparent in the elongated sets only. Chromosomes 25 and 27 were particularly difficult to distinguish between in this study, and Gustavsson et al. (1976[a]) were unable to describe a clear difference between the banding pattern of these chromosomes (numbered 26 and 27 in their study). However, a faint dark terminal band was visible on chromosome 25 and absent on chromosome 27 when the elongated chromosome sets were examined in the present study, and Lin et al. (1977) also recognised a faint dark terminal band on chromosome 25. Furthermore Ford et al. (1980) recommended that the presence of this band be used to differentiate between these particular chromosomes.

The banding pattern which was most difficult to establish in this study was that belonging to chromosome 20. Three dark bands were visible on this chromosome in the elongated sets but only two dark bands were present in the moderately contracted sets. However, examination of the G-banded karyotypes prepared by Lin et al. (1977) tends to suggest that this group of research workers also had difficulty in determining the correct banding pattern on chromosome 20. The description of this chromosome, numbered 16

in their article, is brief and stipulates that the distinct dark band of the terminal region is a main feature for identification. There is no mention of the number of bands produced on the chromosome. However, the accompanying ideogram shows four dark bands, two of which are faint. Gustavsson et al. (1976[a]) described only three dark bands on chromosome 20 (numbered 19 in their study). However, the chromosome sets examined by this group of research workers were more contracted than those examined by Lin et al. (1977). Clearly, therefore, the banding pattern on chromosome 20 varies considerably depending on the degree of contraction. This is further supported by the standard description of this chromosome recommended by Ford et al. (1980) which details two dark bands but mentions that the proximal band may resolve into two. An accurate comparison of the banding pattern on this chromosome can only be made, therefore, between karyotypes with the same degree of contraction.

The numbering of the remaining chromosomes in this study differs slightly from that used by Gustavsson et al. (1976[a]) and Lin et al. (1977). However the banding patterns correspond closely and any difference can be readily explained by the mergence of bands which occurs in the less elongated sets. Consequently, it is unlikely that any chromosome aberration was present in the karyotypes prepared during this study, although certain structural rearrangements such as inversions which occur either between or within bands on the chromosome do not necessarily alter the banding pattern and would be undetected by G-banding. However, recent research shows that each dark G-band consists of several individual smaller bands which are visible

during late prophase when they produce a pattern called high resolution banding (Yunis, Sawyer and Ball, 1978). Consequently, this technique is used to identify aberrations which are not apparent in G-banded preparations although, to date, there is no report of its use in cattle chromosomes. Nevertheless, structural rearrangements which occur between the high resolution bands still remain undetected using this technique and, therefore, more precise methods of monitoring chromosome structure, such as genetic mapping (McKusick and Ruddle, 1977), are necessary in order to identify minor structural rearrangements.

The finding of a single dark band on all autosomes in the C-banded karyotypes in this study agrees with the results of other research workers (e.g. Hansen 1973; Evans, Buckland and Sumner 1973). Furthermore, each band was approximately the same size and stained with similar intensity which tends to suggest that there is little variation in the morphology of the centromere in cattle chromosomes. This is supported by the findings of Britten and Smith (1970) which show that the centromeric region of all cattle autosomes contains similar quantities of highly repetitive DNA sequences rich in guanine and cytosine. Similar DNA sequences also occur in the centromere of acrocentric sheep chromosomes (Britten and Smith 1971), and Evans et al.(1973) showed that the C-bands on these chromosomes are similar in size to those on cattle chromosomes.

Although a single dark centromeric band occurs on the centromeric region of most other mammalian species including the

mouse (Hsu and Arrighi, 1971), dark bands also occur on regions of the chromosome arm which are rich in highly repetitive DNA sequences called satellite DNA. However, to date, there is no report which describes the presence of satellite DNA on cattle chromosomes and the results of the present study provide further evidence of the absence of satellite DNA.

No distinct C-band was produced on the X chromosomes in this study which concurs with the results of other research workers (e.g. Hansen 1973; Evans et al.1973). However, the centromeric area of X chromosomes in this study tended to stain slightly darker than the chromatid arms. It may be that this is due to a technical artifact caused by the treatment of chromosomes in barium hydroxide solution. Support for this comes from the results of Sumner (1972) which show that chromosomes which are under-treated with barium hydroxide usually contain a dark stained area. However, in spite of under-treatment, similar dark stained areas did not occur on any other chromosome in this study which tends to suggest that the staining was specific to the centromere of the X chromosome.

Almost all other mammalian species contain a dark C-band in the centromeric region of the X chromosome due to the presence of highly repetitive DNA sequences (Hsu and Arrighi 1971) and, therefore, it may be that the centromere of X chromosomes in cattle also contain highly repetitive DNA sequences, although in insufficient amounts to produce a distinct C-band. Nevertheless, in order to demonstrate this, it would be necessary to determine the structure of DNA in this region which, to date, is unknown.

However, a slightly darker region occurs on the centromeric region of the X chromosome in C-banded karyotypes prepared from cattle by other research workers (e.g. Evans et al.1973) which tends to support the possibility that small quantities of high repetitive DNA sequences may be present.

CHAPTER THREE

THE IDENTIFICATION OF THE 1/29 ROBERTSONIAN TRANSLOCATION IN THE BRITISH FRIESIAN BREED OF CATTLE AND ITS EFFECT ON FERTILITY

3.1 INTRODUCTION

A Robertsonian translocation involving chromosomes in cattle was first reported by Gustavsson and Rockborn (1964) in the Swedish Red and White breed. Gustavsson (1969) investigated the translocation further and, by measuring the length of each individual chromosome, demonstrated that the Robertsonian translocation involved chromosomes number 1 and 29. However, he found it particularly difficult to identify these chromosomes using this method since their length closely resembled that of several other chromosomes. Later, he and his co-workers differentially stained individual chromosomes using the G-banding technique and confirmed that the Robertsonian translocation involved chromosomes 1 and 29 (Gustavsson, Hageltorn and Zech 1976[a]). These chromosomes were also identified using T- and R-banding techniques (Gustavsson, Hageltorn and Zech 1976[b]; Gustavsson and Hageltorn 1976 respectively) and the monocentric nature of the translocation was demonstrated using the C-banding technique (Popescu 1973).

The 1/29 Robertsonian translocation is also recognised in other breeds of cattle and a recent review of the literature (Long 1985) listed at least 35 breeds carrying the translocation. The incidence of the translocation varies considerably between these breeds. In the Romagnola and Blonde d'Aquitaine the reported incidence is as high as 32.0 and 20.6% respectively (Molteni et al.1977; Queinnec et al.1974 respectively). However, the incidence is lower in other breeds such as the Swedish Red and White in which 14.3% of cattle were found to be carriers (Gustavsson 1969). It is of particular interest to note that, to

date, the 1/29 Robertsonian translocation has not been identified in the British Friesian breed. This is in spite of more chromosomes being routinely examined in this country from Friesians than any other breed (Long 1985; Harvey 1987).

Gustavsson (1979) suggested two possible explanations for the occurrence of the 1/29 Robertsonian translocation in different breeds of cattle throughout the world. Firstly, the translocation may have originated in one animal of a primitive breed from which all affected breeds developed and secondly, the translocation may have arisen de novo in various animals belonging to different breeds. Gustavsson emphasised that neither of these explanations has been proven yet. However, he pointed out that the high incidence of the translocation in breeds from central Europe may indicate that the translocation is derived from a common stock which lived in this region. The translocation may then have disseminated into breeds of cattle in other parts of the world by the international movement of carrier animals. This is known to have happened in the case of Norwegian Red cattle which inherited the 1/29 Robertsonian translocation from imported Swedish Red and White cattle (Amrud 1969).

The karyotype of ancestors to animals carrying the 1/29 Robertsonian translocation in the Swedish Red and White and British White breeds were investigated by Gustavsson (1969) and Eldridge (1975) respectively. (The British White translocation was originally thought to involve chromosomes number 1 and 27, but these were subsequently identified as 1 and 29 (Eldridge, 1985)). These research workers demonstrated that, in each case,

the translocation was inherited from previous generations and did not arise de novo. However, cytogenetic studies were not carried out in ancestors to carriers in other breeds, e.g. Rahaji (Pathiraja et al. 1985) and, therefore, it is not possible to demonstrate whether the translocation was inherited or occurred de novo in these breeds.

Gustavsson (1969) examined the breeding records of Swedish Red and White cattle carrying the 1/29 Robertsonian translocation and demonstrated that the non-return to service rate was reduced by 3% at 56 days and 6% at 273 days after service. Similar results were also obtained by Refsdal (1976) when investigating the effect of the 1/29 Robertsonian translocation on fertility in Norwegian Red cattle. Gustavsson (1969) postulated that, during meiosis, the translocation chromosome forms a trivalent structure which occasionally undergoes non-disjunction to produce unbalanced gametes and subsequent studies clearly demonstrated that unbalanced products are formed during meiosis in bulls heterozygous for the 1/29 Robertsonian translocation (Logue and Harvey 1978[a]; Popescu 1978). Gustavsson (1969) further suggested that the unbalanced gametes are capable of fertilisation by a normal gamete but that the resulting aneuploid embryo is non-viable and dies early in gestation causing the animal to return to oestrus. To date, there are two reports in the literature of cytogenetic studies involving embryos from cattle carrying the 1/29 Robertsonian translocation. Popescu (1980) found two embryos apparently monosomic for chromosome number 1 and King and his co-workers (1980) found two embryos which were apparently trisomic for chromosome 1. These

chromosome complements do not occur in living animals which suggests that the embryos are non-viable. Consequently, these reports support Gustavsson's hypothesis (1969) that fertility is reduced in animals with the translocation due to the production of non-viable embryos.

3.2 MATERIALS AND METHODS

3.21 Animals Used

The proband was an 11 year old registered pedigree British Friesian bull heterozygous for a Robertsonian translocation between the largest and one of the smallest autosomes. He was bred in Herd A and used as a stock bull in Herd B. Herd A is situated in North West England and contains 124 adult pedigree British Friesian cattle and their offspring, some of which are related to the proband. Herd B, which was recently dispersed, was a pedigree British Friesian dairy herd situated in South West Scotland and contained progeny of the proband.

G- and C-banded karyotypes were also prepared from an aged Red Poll cow, belonging to Glasgow University Veterinary School, which was previously found to be heterozygous for a presumptive 1/29 Robertsonian translocation (Harvey, 1987).

3.22 Examination of Pedigree

The proband's pedigree was traced using the Herd Book of the British Friesian Cattle Society of Great Britain and Ireland and any non-registered ancestor was recorded.

The farm records from Herd A were examined and any living animals with an ancestor common to the proband were identified for chromosome analysis. A diagram was constructed to illustrate

the relationship of these animals to the proband.

The farm records from Herd B were also examined and all progeny of the proband were identified for chromosome analysis.

3.23 Chromosome analysis by conventional giemsa staining

The method used to obtain chromosomes for analysis was based on the technique originally described by Moorhead et al.(1960) and modified for whole blood by Basrur and Gilman (1964).

10 ml of heparinised blood was collected (Vacutainer, Becton-Dickinson) from the jugular vein in a sterile manner. Taking all reasonable precautions to maintain sterility in a laminar air flow cabinet (Pathfinder), 2 ml aliquots of the blood were transferred into universal bottles containing 10 ml of culture medium. The culture medium used (RPMl 1640, Flow Laboratories) was supplemented with 16% foetal calf serum, 75 iu/ml penicillin, 75 µg/ml streptomycin, 60 µl 200 mM L-glutamine (all Flow Laboratories) and 1.875 mg phytohaemagglutinin (Wellcome). The universal bottles were incubated at 37°C for 48 or 72 hours. Ninety minutes prior to the end of incubation 0.1 ml of 80 µg/ml colcemid solution previously prepared by dissolving 5 g colcemid (Demecolcine, Sigma) in 62.5 ml Hank's Balanced Salt Solution and storing in 5 ml aliquots at -20°C until use, was added to the culture medium. After completing incubation, the cell culture was poured into a labelled centrifuge tube and spun at 300 x g for ten minutes. The supernatant was aspirated off and the cells were resuspended in 10 ml of 0.022 Molar potassium chloride solution, using a fresh pipette for each animal, and incubated in a waterbath at 37°C for 15 minutes. Next, the supernatant was

discarded after spinning at 200 x g for ten minutes and the cells were suspended in 5 ml of freshly prepared fixative (methanol : glacial acetic acid, 3 : 1 at +4°C). The cell suspension was refrigerated at +4°C for 15 minutes and spun (ten minutes at 200 x g) before removing the supernatant and replacing it with 2 ml of fresh fixative. The cells were resuspended and refrigerated for at least 30 minutes after which they were spun (five minutes at 150 x g) and the supernatant removed. 0.5 ml of fixative was added to resuspend the cells and approximately 0.2 ml of the suspension was dropped from a height of 40 cm onto a microscope slide, previously cleaned with 70% alcohol, held at an angle of 45°. The slide was examined using a phase contrast microscope (Vickers, Photoplan) after drying at room temperature. If the concentration of cells was sufficient for chromosome analysis then further slides were prepared in the same manner. However, if the chromosome preparations were overcrowded on the slide, then more fixative was added to the cell suspension before preparing further slides. The chromosome preparations were stained with freshly prepared 10% giemsa solution (Gurr's R66 in buffer pH 6.5, B.D.H. Chemicals), rinsed under running tapwater, dried and mounted (D.P.X., B.D.H. Chemicals) with a coverslip.

Twenty-five evenly-spread chromosome sets were examined from each animal using a light microscope (Leitz, Orthrolux). The sex chromosomes were identified, the total number of chromosomes was counted with the aid of a manual trip meter, and any structural abnormality was recorded.

3.24 G-banding of chromosomes

Using the technique (Method 2) described in section 2.22, G-banded chromosome spreads were prepared from four daughters of the proband which were heterozygous for the translocation chromosome and from the Red Poll cow.

The banding pattern was examined on ten distinctly stained translocation chromosomes from each animal. This pattern was compared with that produced on normal chromosomes identified in G-banded karyotypes described in section 2.31. G-banded karyotypes were then prepared as described in section 2.24 from spreads in which all chromosomes were evenly spread and distinctly banded.

3.25 C-banding of chromosomes

Using the same animals as the previous section, C-banded chromosomes spreads were prepared as described in section 2.23.

The banding pattern was examined in at least ten distinctly stained spreads from each animal. The pattern on the extra submetacentric chromosome was compared with that of the normal chromosomes. Particular attention was given to the relative size and staining intensity of the C-bands produced. Photographs were taken of representative spreads and a C-banded karyotype was prepared from each animal as described in section 2.24.

3.26 Effect of the translocation on fertility

Unfortunately the farmer in Herd B kept scant breeding records which only occasionally contained service dates and were insufficient to calculate the non-return to service rate of individual animals. According to the farmer, the information in the herd records which was most accurate consisted of the

animals' date of birth and date of subsequent calvings and, since no other reliable information was available, these dates were used to study the effect of the translocation on fertility.

The proband's daughters were separated into two groups. One of these contained animals heterozygous for the 1/29 Robertsonian translocation and the other contained those with the normal karyotype. The age at first calving and the interval between subsequent calvings were calculated from the breeding records and the results were compared between each group using the two-sided t-test.

3.3 RESULTS

3.31 The Proband's Pedigree

Examination of the proband's pedigree showed that each ancestor for the previous six generations was a registered pedigree British Friesian. However, a great-great-great-granddam of the proband was the result of four generations of upgrading. This means that one ancestor which lived nine generations before the proband was not a pedigree animal but was of similar type to the pedigree British Friesians of that time and acceptable for upgrading into the breed.

Further examination of the proband's pedigree showed that the proband and his dam, granddam and great-granddam were all bred in Herd A. The herd records showed that the proband's granddam was one of two sister animals which formed part of the breeding nucleus after the herd was formed in 1954. These two sister progenators are identified in this study by the letters F and G and the proband was descended from animal F.

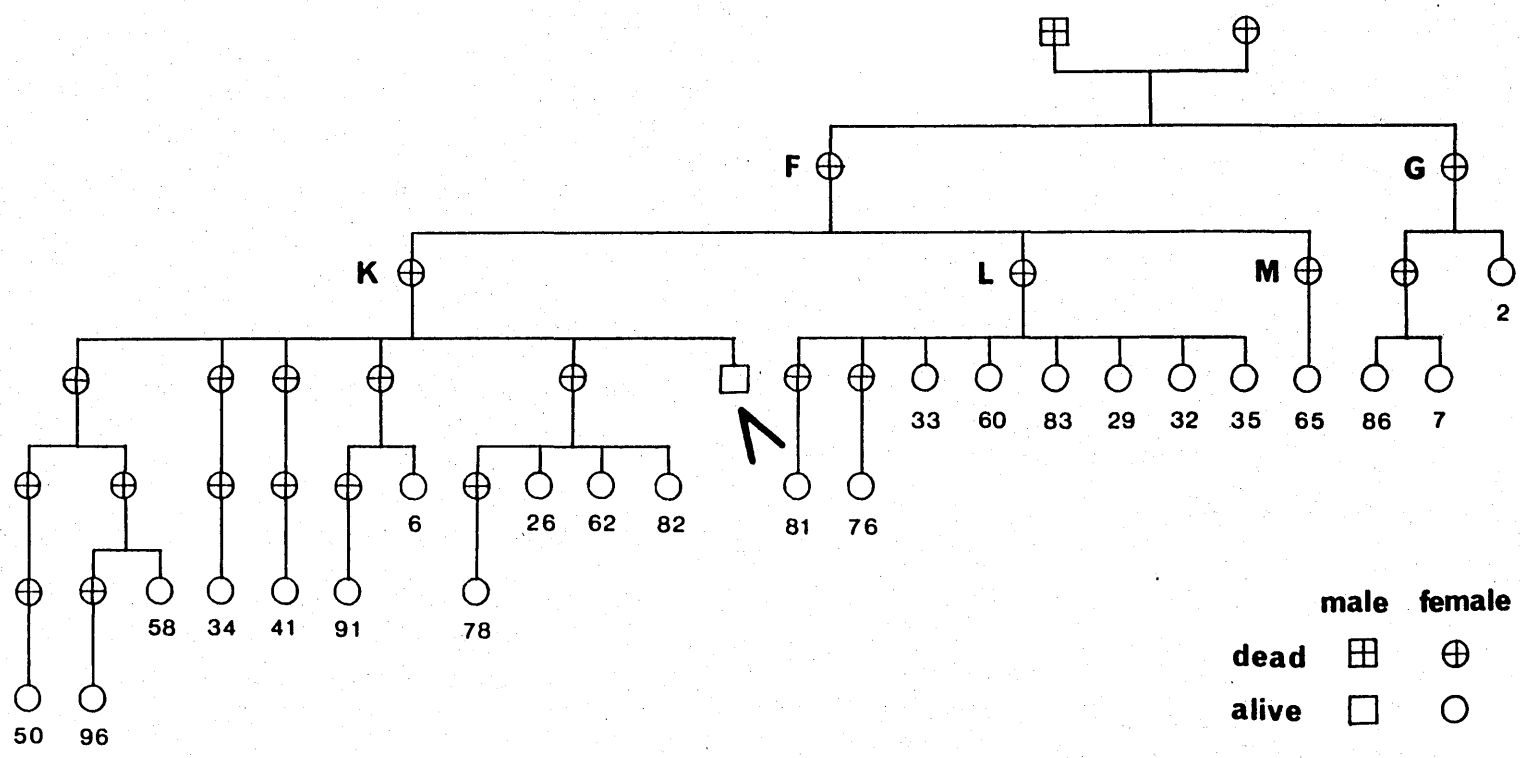

Figure 8. A diagram illustrating the relationship between relatives of the proband in Herd A (Proband indicated by arrow)

3.32 Relatives of the proband in Herd A

Herd records showed that there were 23 animals living in Herd A which were related to the proband. All bull calves were sold at birth and therefore, the animals related to the proband were female (see Table 5).

Examination of the herd records showed that the proband's relatives were all descended from either animal F or G. Animal F was the proband's granddam and animal G was her full sister. Animal G had two daughters. One of these, animal number 2, was living in the herd and a blood sample was taken for cytogenetic analysis. The second daughter of animal G was dead, but two of her offspring were in the herd and available for cytogenetic analysis. Their numbers were 86 and 7 and their relationship to the proband is shown in Figure 8.

Animal F had three daughters identified by the letters K, L and M in Figure 8. These animals were not alive at the time of this study. However, the herd records showed that 20 of the proband's relatives were descended from them. Eleven of the relatives were progeny from the proband's dam, animal K. However, none belonged to the same generation as the proband. Examination of their pedigree showed that four were granddaughters of animal K. Five were great-granddaughters and two were great-great-granddaughters. The different generations of these animals and their relationship to the proband are illustrated in Figure 8 where the great-great-granddaughters are identified as numbers 50 and 96; the great-granddaughters as numbers 58, 34, 41, 91 and 78; and the granddaughters as numbers 6, 26, 62 and 82. Animals 50 and 78 were also related to

the proband through his sire since the herd records showed that this animal was also the sire of number 78 and the grandsire of number 50.

Six relatives of the proband, identified in Figure 8 as 33, 60, 83, 29, 32 and 35, were daughters of animal L. Another two relatives, animals 81 and 76, were granddaughters of animal L and the remaining relative, animal 65, was the only daughter in the herd out of animal M.

The herd records showed that other animals related to the proband had been bred in Herd A. However, these animals had been sold and the farmer requested that their purchasers were not contacted. Consequently, no blood samples were available from them for cytogenetic analysis.

3.33 Cytogenetic analysis of the proband's relatives in Herd A

Giemsa stained chromosome preparations were obtained from each of the 23 animals related to the proband in Herd A. Twenty-five evenly spread chromosome sets were examined from each animal and, with the exception of animal 82, each set contained 60 chromosomes. All chromosome sets examined from animal 82 contained 59 chromosomes.

The sets which contained 60 chromosomes were composed of two submetacentric and 58 acrocentric chromosomes. They, therefore, had a total of 62 chromosome arms. The two submetacentric chromosomes were the largest, with one arm slightly longer than the other, corresponding to the normal X chromosome in cattle. The 58 acrocentric chromosomes varied in length according to the normal cattle autosomes. All animals related to the proband in

Herd A, with the exception of number 82, were therefore classified as having the normal female karyotype, 60,XX (see Table 5).

The 59 chromosomes found in all sets examined from animal 82 consisted of 56 acrocentric chromosomes and three submetacentric chromosomes. Each set, therefore, had a total of 62 chromosome arms. The 56 acrocentric chromosomes varied in length and two of the submetacentric chromosomes were identical and corresponded to the normal X chromosome. The other submetacentric chromosome was slightly larger and had a centromere situated near one end such that one chromosome arm was considerably longer than the other. The length of each individual chromosome was not calculated. However, by examining the less contracted chromosome sets, it was possible to see that the long arm of the abnormal chromosome was the same length as the longest autosome. The short arm corresponded in length to one of the smallest chromosomes. However, it was not possible to determine whether this was the smallest chromosome since their sizes were very similar. Consequently, the karyotype of animal 82 was classified as being heterozygous for a Robertsonian translocation involving the largest and one of the smallest autosomes. This description corresponded to that of the abnormal chromosome which was identified in the proband and presumed to be a 1/29 Robertsonian translocation.

TABLE 5

DETAILS OF ANIMALS RELATED TO THE PROBAND IN HERD A INCLUDING THEIR KARYOTYPE (all animals were Friesian and female)

Animal Identification Number	Age (years)	Karyotype
50	5	60, XX
96	4	"
58	5	"
34	6	"
41	3	"
91	4	"
6	13	"
78	4	"
26	7	"
62	5	"
82	4	59, XX, t
81	4	60, XX
76	4	"
33	6	"
60	5	"
83	4	"
65	5	"
86	4	"
7	13	"
2	18	"
29	3 months	"
32	3 months	"
35	6 weeks	"

3.34 Cytogenetic analysis of the proband's progeny in Herd B

Examination of the farm records showed that there were 35 progeny from the proband in Herd B. All of these animals were daughters of the proband since any male calves were sold for commercial beef production. Giemsa stained chromosome preparations were obtained from each of the progeny and 25 evenly spread chromosome sets were examined from each animal.

Details of the proband's progeny and their karyotype are shown in Table 6. Chromosome spreads containing 58 acrocentric chromosomes of varying size and two large submetacentric chromosomes were considered to be normal. This karyotype was found in chromosome sets from 16 animals. However, the remaining 19 animals had 59 chromosomes in each set examined. These sets comprised of 56 acrocentric chromosomes of varying size and three large submetacentric chromosomes. Two of these were identical with one arm slightly longer than the other corresponding to the normal X chromosome. The other submetacentric chromosome was larger with one arm considerably longer than the other. This chromosome corresponded to the presumptive 1/29 Robertsonian translocation identified in the proband and animal 82 from Herd A. Animals with this karyotype were classified as 59,XX,t.

There was no significant difference ($Chi^2 = 0.01$; $P>0.05$) between the number of normal progeny and the number heterozygous for the translocation.

3.34.1 G-banding

Slides containing fixed chromosome preparations from animals 10, 18, 73, 77 and the Red Poll cow were treated to produce G-banding. The G-banding pattern was not produced on chromosomes belonging to highly contracted sets. These chromosomes were generally dark with an occasional small light area. However, individual bands were distinguished in the less contracted sets of chromosomes. Ten of these chromosome sets which were evenly spread were selected from each animal for further investigation.

TABLE 6

DETAILS OF PROGENY FROM THE PROBAND IN HERD B INCLUDING THEIR KAROTYPE (all animals were Friesian and female)

Animal Identification Number	Age (years)	Karyotype
8	7	60, XX
10	5	59, XX, t
12	6	60, XX
14	6	60, XX
15	6	60, XX
18	6	59, XX, t
34	7	60, XX
38	8	60, XX
39	6	59, XX, t
43	5	60, XX
54	5	59, XX, t
58	6	60, XX
67	5	59, XX, t
71	7	59, XX, t
73	7	59, XX, t
77	7	59, XX, t
79	5	60, XX
85	6	59, XX, t
86	5	59, XX, t
101	6	59, XX, t
102	5	59, XX, t
106	5	60, XX
122	5	59, XX, t
C175	5	59, XX, t
L5	4	59, XX, t
J5	2	59, XX, t
B133	2	59, XX, t
P38	2	59, XX, t
A22	2	59, XX, t
B108	5	60, XX
P55	1	60, XX
P67	1	60, XX
B118	2	60, XX
C177	2	60, XX
B7	1	60, XX

Each spread contained 59 chromosomes composed of 56 acrocentric chromosomes of varying size and three large submetacentric chromosomes. Two of the submetacentric chromosomes were the same size, with one arm slightly shorter than the other. The centromeric region on these chromosomes stained darkly and the short arm had two dark bands. The long arm consisted of two dark bands adjacent to the centromere followed by a prominent light band and two dark bands. These were the only chromosomes with a dark centromeric region and the banding pattern was consistent with that of the X chromosome described in G-banded karyotypes from normal animals in section 2.31.

The remaining submetacentric chromosome was larger and had one arm considerably longer than the other. The centromeric region separating the two chromosome arms consisted of a single unstained band in all chromosomes examined. When examined under low power magnification (x 125), the long arm of this chromosome appeared darkly stained near the centromere. This was followed by a pale staining area and another darkly stained area terminally. When examined under high power magnification (x 675), the darkly stained areas were seen to consist of several dark bands. The number of bands varied slightly between different chromosome sets. In those which were more contracted, the bands tended to merge together and could not be distinguished separately, even under high power magnification. In less contracted chromosome sets, 1-2 dark bands were counted in the dark region of the long arm adjacent to the centromere and another two dark bands were counted in the terminal region. More

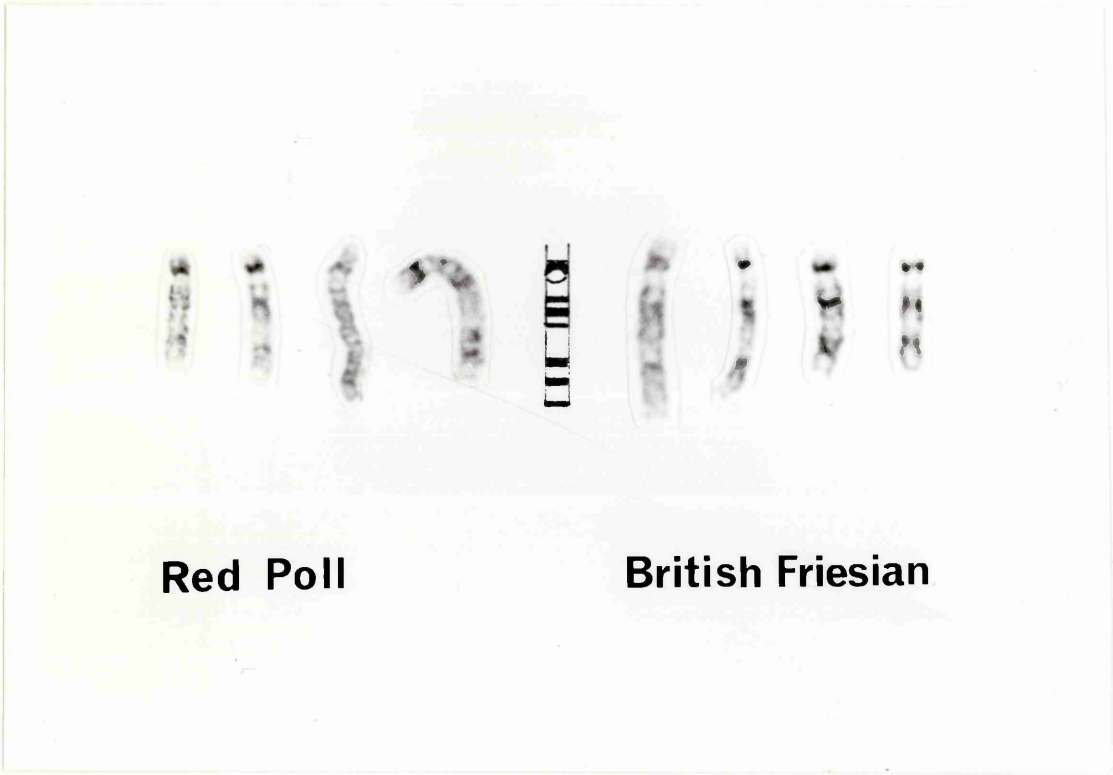

Figure 9

An ideogram illustrating the G-banding pattern on eight translocation chromosomes from the Red Poll and British Friesian animals

bands, however, were distinguished in the elongated chromosome sets. The pattern in these sets consisted of 2-3 narrow dark bands in the region next to the centromere followed by a distinctive broad light band in the central region and 2-3 dark bands, one of which always occurred at the very end of the long arm. This banding pattern corresponds to that found on chromosome number 1 in G-banded karyotypes examined from normal animals in section 2.31.

The banding pattern on the short arm of the extra submetacentric chromosome was more difficult to determine since it was less distinctive than that of the long arm. In moderately contracted chromosome sets, the short arm appeared as a single dark band. This pattern was also seen on several of the smaller acrocentric chromosomes in these spreads. When elongated sets of chromosomes were examined, discrete banding patterns were seen on the smaller chromosomes and on the short arm of the extra submetacentric chromosome. The short arm in these elongated spreads consisted of a dark band adjacent to the centromere followed by a faint staining area along the remainder of the chromosome corresponding to chromosome number 29 in the normal G-banded karyotypes examined in section 2.31. Figure 9 shows the banding pattern on the Robertsonian translocation. The pattern was identical in the Red Poll and British Friesian animals.

Photographs were taken of five elongated chromosome sets from each animal for karyotyping. Figure 10 is a representative G-banded karyotype and shows that the extra submetacentric chromosome is a Robertsonian translocation involving chromosomes

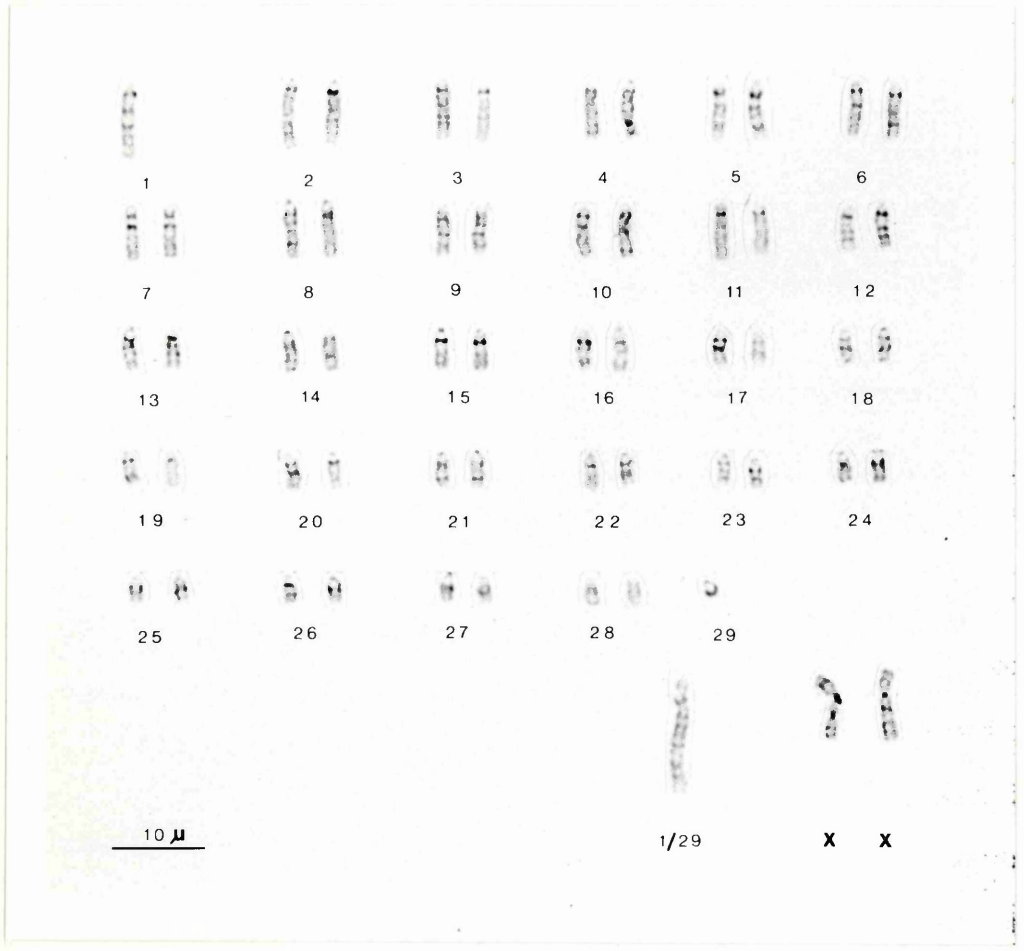

Figure 10

A representative G-banded karyotype heterozygous for the 1/29 Robertsonian translocation

number 1 and 29. The banding pattern on each homologous pair of chromosomes corresponds to the descriptions in section 2.31. However, care was required when arranging the smaller chromosomes in pairs since their banding patterns were similar. Nevertheless, only one chromosome in each set consisted of a dark band adjacent to the centromere followed by a pale staining area along the remainder of the chromosome which corresponds to the pattern identified on chromosome number 29 in section 2.31. However, this chromosome was occasionally confused with chromosome number 28 which also had a prominent single dark band. The band in chromosome 28, however, was further from the centromere. Furthermore, chromosome number 28 had a faint dark terminal band which distinguished it from number 29.

Occasionally, one of the longer acrocentric chromosomes or the long arm of the translocation chromosome was folded across itself or another chromosome causing the bands to merge together at the fold and causing difficulty in counting the precise number of bands. Nevertheless, the banding pattern on chromosome number 1 was so distinctive that there was no problem in identifying this chromosome.

3.34.2 C-banding

Although the C-banding pattern was produced in almost all the spreads, those in which chromosomes overlapped each other contained indistinct bands. The most distinct bands were seen in the moderately contracted and elongated chromosome sets and ten of these, which were evenly spread, were selected from each animal for further examination.

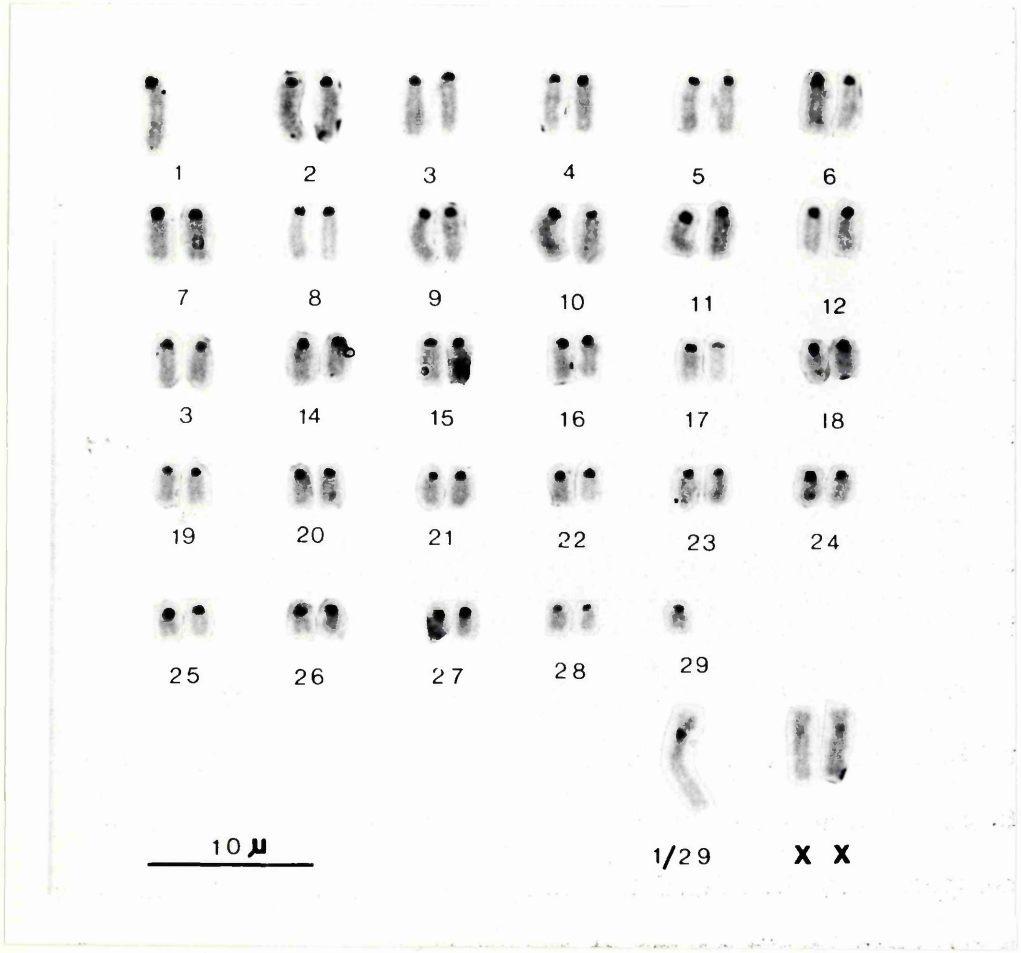

Figure 11

A representative C-banded karyotype heterozygous for the 1/29 Robertsonian translocation

Each of these spreads contained 59 chromosomes. Two of the chromosomes were identical, large submetacentric chromosomes which stained lightly along the length of each chromosome arm although an indistinct centromeric band was occasionally visible. This pattern corresponds to that of the X chromosome in C-banded karyotypes examined from normal animals in section 2.32.

Fifty-six of the chromosomes varied in size and were acrocentric. The arms of these chromosomes stained lightly and each chromosome contained a single dark centromeric band. In poorly stained spreads, the intensity of this band varied slightly between chromosomes. However, the C-bands were equally prominent and approximately the same size in the evenly stained spreads, which corresponds to the normal banding pattern on cattle autosomes (section 2.32).

The remaining chromosome was submetacentric and larger than the two X chromosomes, with one arm considerably longer than the other. These arms were faintly stained. The long arm corresponded in length of the longest autosome and the short arm was the same length as one of the smallest autosomes. In each of the extra submetacentric chromosomes examined, the arms were separated by one prominent dark C-band. Further examination of this band showed that it stained to the same intensity as the band on each acrocentric chromosome. Furthermore, the single band on the extra submetacentric chromosome was approximately the same size as the other monocentric C-bands. This is illustrated in Figure 11 which shows a C-banded karyotype from one of the British Friesian animals.

3.35 Effect of the 1/29 Robertsonian translocation on fertility

Examination of the farm records in Herd B showed that 27 of the proband's 35 daughters in the herd had calved. Service dates were not routinely recorded for these animals which usually ran with the bull. However, the breeding records contained calving dates for nine animals with the normal karyotype and 13 animals with the translocation.

The age at first calving for daughters with the normal karyotype ranged from 23-40 months as shown in Table 7. Animal 106 in this group, which calved at 40 months, did not thrive when young and was, therefore, put to the bull at a later stage than the other heifers. In the translocation group, the age at first calving ranged from 25-34 months as shown in Table 8.

The mean and standard deviation of the age at first calving is shown in Table 9 for the two groups of animals. When the two-sided t-test was used to analyse these figures, the test statistic, t, was found to be 0.56. From tables of the student t-distribution, the probability of obtaining this value is 0.59 and, therefore, there is no significant difference ($P > 0.05$) between the age at first calving of animals from these two groups.

The interval between subsequent calvings was calculated for each animal except number 71 whose records were misplaced. Table 7 shows that the intervals for animals with the normal karyotype ranged from 326-597 days, and Table 8 shows that the range for the translocation group was 326-625 days. The farm

records contained no reason for the prolonged interval between calvings in animals 8, 15, 18 and 73. Dystocia, retained placenta, metritis and anoestrus were occasionally diagnosed on the farm. However, the farmer was not able to recall whether any of these problems affected the animals with a prolonged calving interval.

The mean and standard deviation of the interval between calvings for animals in the two groups are shown in Table 9. The t statistic calculated using a two-sided t-test was 0.13. From tables, the probability of obtaining this value is 0.90 and, therefore, there is no significant difference ($P > 0.05$) between the calving intervals of these animals.

TABLE 7

DETAILS FROM THE BREEDING RECORDS OF THE PROBAND'S DAUGHTERS

WITH THE NORMAL KARYOTYPE IN HERD B

Animal Identification Number	Age at first calving (months)	Number of subsequent calvings	Interval between calvings (days)
14	33	1	362
15	23	1	481
79	29	1	347
12	26	2	335, 371
34	32	2	350, 360
58	29	2	350, 397
106	40	2	375, 368
8	29	3	597, 326, 449
38	32	3	337, 371, 336

TABLE 8

DETAILS FROM THE BREEDING RECORDS OF THE PROBAND'S DAUGHTERS HETEROZYGOUS FOR THE 1/29 ROBERTSONIAN TRANSLOCATION IN HERD B

Animal Identification Number	Age at first calving (months)	Number of subsequent calvings	Interval between calvings (days)
39	30	1	412
54	34	1	367
85	31	1	402
86	29	1	326
101	31	1	327
102	30	1	329
122	33	1	364
10	34	2	347, 390
18	34	2	391, 453
67	25	2	330, 369
73	31	3	625, 363, 389
77	31	3	439, 343, 362
71	34	Unknown	

TABLE 9

STATISTICAL ANALYSIS OF THE AGE AT FIRST CALVING AND INTERVAL BETWEEN CALVINGS OF THE PROBAND'S DAUGHTERS IN HERD A USING THE TWO-SIDED T-TEST

	Age at First Calving (months)		Interval between Calvings (days)	
Group	Normals	Carriers	Normals	Carriers
Number	9	13	17	19
Mean	30.3	31.3	382.8	385.7
Standard deviation	4.8	2.6	68.4	68.5
t	0.56		0.13	
p	0.59		0.90	

3.4 DISCUSSION

The finding of the 1/29 Robertsonian translocation in members of two pedigree British Friesian herds examined in this study is of particular interest since, to date, this chromosome aberration has not been recognised in the Friesian breed. This is in spite of more chromosome sets being routinely examined from Friesians than from any other breed in this country (Long 1985; Harvey 1987). Nevertheless, the 1/29 Robertsonian translocation has been reported in at least 35 other breeds throughout the world, although the incidence varies between breeds (Long 1985). In most affected breeds, the incidence is less than 3%, but in the Blonde d'Aquitaine and Romagnola breeds the incidence may be as high as 20.6% (Queinnec et al.1974) and 32.0% (Molteni et al. 1977) respectively. However, these figures must be considered with caution since relatively few animals were examined in each breed. In addition, carriers are not usually bred and, therefore, the incidence within breeds may vary. The most accurate figure for the incidence of the translocation within a breed was obtained for the Swedish Red and White breed. One thousand one hundred and seventy three animals distributed throughout Sweden were examined and the incidence in that population was found to be 14.3% (Gustavsson 1969). If the incidence of the 1/29 Robertsonian translocation in the British Friesian breed was within the range reported in other affected breeds then it is probable that the translocation would have been detected before now.

The occurrence of the 1/29 Robertsonian translocation in different breeds and countries may be due either to inheritance

of the translocation from a common ancestor or recurrent formation of the translocation in different animals. In each case, cytogenetic studies involving the Norwegian Red and Swedish Red and White breeds traced the 1/29 Robertsonian translocation to previous generations (Amrud 1969; Gustavsson 1969 respectively) and this tends to support the possibility that the translocation could be derived from a common source which lived prior to the differentiation of separate breeds. Furthermore, since the translocation is relatively common in Bos taurus breeds which developed in Central Europe (Gustavsson 1979), it could be that the translocation originated in stock from this region and was subsequently distributed into other areas by the trading of affected cattle. However, the 1/29 Robertsonian translocation also occurs in Bos indicus breeds including the Zebu (Pathiraja, Oyedipe and Buranendran 1985). Consequently, if the aberration originated in an ancestor common to all breeds of cattle then this animal must have lived over 10,000 years ago before the Holocene Period when separate Bos taurus and Bos indicus species are thought to have evolved (Friend 1978). Furthermore, all modern cattle breeds developed from the progenators of these two species (Friend 1978) and if the translocation was present at this early stage of evolution, then all breeds would be equally likely to inherit the translocation. However, the incidence of the translocation varies and the aberration is found in certain breeds only (Long 1985). Consequently, it is unlikely that the 1/29 Robertsonian translocation originated in one ancestor common to all cattle breeds.

It seems more likely, therefore, that the various incidences are the consequence of the translocation forming de novo in animals from several different breeds. If the translocation formed de novo relatively recently in the development of a breed then relatively few animals would be affected resulting in a low incidence in the breed. However if the translocation formed many generations ago then repeated breeding of affected animals would increase the proportion of carriers. This may explain the relatively high incidence in certain Central European breeds. However, the incidence of the translocation within a herd can also be influenced by the sex of carrier animals. Since the probability of inheriting the 1/29 Robertsonian translocation is 0.5 (Gustavsson 1969), half the offspring from an affected animal will also be carriers. A cow heterozygous for the translocation may, in her lifetime, therefore, produce between two and six calves carrying the translocation. However, a stock bull serving a large herd of cows could easily produce 50 or more affected calves in his lifetime. In the British White breed of cattle, the relatively high incidence of a translocation, originally thought to involve chromosomes number 1 and 27 (Eldridge 1975) but later identified as the 1/29 Robertsonian translocation (Eldridge 1985), was found to be due to two prolific stock bulls heterozygous for the translocation (Eldridge 1975).

In the present study, only two carriers of the 1/29 Robertsonian translocation were identified in Herd A but if the translocation had been inherited from an ancient progenator then, by repeated breeding of affected animals, the aberration would probably now be disseminated throughout the breed and affect a

large number of animals. However, despite more chromosome sets being routinely examined from Friesians than any other breed in this country (Long 1985; Harvey 1987), the translocation has, to date, not been recognised in this breed. Consequently, the translocation is unlikely to have been inherited from an ancient progenator. Again, if the translocation formed de novo in the British Friesian breed many years ago, then a large proportion of animals related to the proband would probably also be carriers. However, the translocation was present in only one relative in Herd A, animal 82, which tends to suggest that it formed de novo relatively recently and is still confined to a small proportion of animals.

Although the proband and animal 82 have different sires which are unrelated, both the carriers are related to each other through animal K which is the granddam of animal 82 and the dam of the proband. It is possible, therefore, that these carriers inherited the translocation from animal K. Alternatively, it may be that the translocation occurred de novo in the proband and animal 82 separately. This is unlikely, however, for the following reason. The incidence of the de novo formation of the 1/29 Robertsonian translocation is unknown. However, Herd A had 124 animals and if the translocation formed de novo in one of these animals then the incidence in that herd would be 1 in 124. Consequently, the probability of an animal in that herd having the translocation would be 1/124. Assuming, therefore, that one animal was already a carrier, the probability of the translocation forming independently in another animal would be

$(1/124)^2$ which is highly unlikely. Consequently, the translocation probably formed in only one animal which may have been either animal K or one of her predecessors.

One of animal K's half-sisters, animal L, had six daughters with the normal karyotype and since the probability of having six normal offspring from a carrier animal is very low [0.015, assuming the probability of inheriting the translocation is 0.5 (Gustavsson 1969)], animal L probably had the normal karyotype. Animal K's other half-sister, animal M, had only one offspring and, although it's karyotype was normal, analysis of chromosomes from other offspring would be necessary in order to establish whether animal M carried the translocation. If animals M and K were both carriers then they could have inherited the translocation from their dam. However, there was no other progeny from animal M on the farm for examination and consequently, it is not possible to demonstrate whether the translocation originated in animal K or was inherited from her dam.

There was no possible way of determining in this study whether animal K's sire carried the translocation since Herd A contained no other progeny from him for cytogenetic analysis. However, animal K's dam was animal F which, together with her full sister animal G, formed part of the herd's breeding nucleus after it was formed in 1954. Consequently, the herd contained progeny from animal G. If the translocation was identified in any of these animals, then it may have been inherited from animal G and such evidence would support the possibility that animal F was a carrier. Unfortunately, however, most progeny

from animal G had been sold leaving only one daughter and two granddaughters from another daughter and although their karyotype was normal, the probability that both animal G's daughters were normal if she was a carrier is too high (0.25) to suggest that animal G was not a carrier. More progeny would have to be examined, therefore, in order to determine her karyotype using statistical inference. However, this was not possible in the present study since the farmer did not wish their owners to be contacted.

Consequently, the results indicate that the translocation was probably inherited from the proband's dam, animal K, but cytogenetic studies involving related animals in other herds would be required in order to determine whether the translocation originated in animal K or in a recent predecessor.

All the proband's ancestors for the previous nine generations were pedigree Friesians which shows that, unless there was an error in the pedigree, the translocation was not introduced to the British Friesian breed by mating with a carrier animal belonging to another breed. The only possible exception might have occurred nine generations before the proband when a non-registered Friesian-type animal was upgraded into the Friesian Herd Book. If this animal was a carrier, then repeated breeding of affected progeny would probably have produced a large number of carriers in the breed by now. However, more chromosome sets are routinely examined from the Friesian than any other breed and, to date, the translocation has not been detected (Long 1985; Harvey 1987). Furthermore, examination of the British

Friesian Cattle Society of Great Britain and Ireland Herd Book showed that breeding lines which developed from the upgraded animal include bulls which have been selected for use in Artificial Insemination Centres. These animals are routinely examined for chromosome abnormalities and, with the exception of the proband, no Friesian bulls have been recognised carrying the 1/29 Robertsonian translocation (Isbister, 1985).

Robertsonian translocations between chromosomes other than numbers 1 and 29 have also been reported in various breeds of cattle. For example, the 2/8 Robertsonian translocation in the Friesian breed (Pollock 1974), the 14/20 in the Swiss Simmental breed (Harvey and Logue 1975), and the 3/4 in the Limousin breed (Popescu 1977). However, the incidence of these aberrations is much lower than that of the 1/29 Robertsonian translocation (Long 1985). Few progeny from affected animals have been studied and, therefore, the proportion of progeny which inherit these translocations is unknown. Nevertheless, a large proportion of children inherit the Robertsonian translocation if one of the parents is a carrier (Hamerton 1970). Similarly, Robertsonian translocations are inherited by approximately half the progeny from carrier mice (Ford and Evans 1973) and sheep (Bruere 1975), and the 1/29 Robertsonian translocation in cattle is inherited by 50% of progeny (Gustavsson 1969). It seems probable, therefore, that other Robertsonian translocations in cattle are also inherited by a large proportion of offspring. Further supportive evidence comes from the results of meiotic studies in cattle which showed that the 14/20 Robertsonian translocation segregated in a 1 : 1 ratio and that 63% of progeny from three carriers were

also affected (Logue and Harvey 1978[b]). However, the incidence of this translocation and all others, except the 1/29 Robertsonian translocation, is low (Long 1985). If these aberrations had been present in the cattle population many years ago then, by repeated breeding of affected progeny, the incidence would probably be higher by now. Consquently, it seems likely that these translocations formed relatively recently and are restricted to a few animals.

An alternative explanation for the low incidence might be the result of selection against carriers in a breed. However, several studies in cattle showed that carriers of various Robertsonian translocations are phenotypically normal (e.g. Gustavsson 1969; Logue and Harvey 1978[a]). This is also the situation in the mouse (Ford and Evans 1973), sheep (Bruère 1975) and in the human (Hamerton 1970). Furthermore, research involving the 1/29 Robertsonian translocation in cattle showed no correlation between the translocation and characters such as milk production, butter fat and body conformation (Gustavsson 1969). In addition, the quality, freezability and fertility of semen from bulls is unaffected by various Robertsonian translocations (Moustafa et al. 1983). Consequently, it is unlikely that any low incidence of carriers is a result of artificial selection on commercial grounds.

The translocation identified in the present study was monocentric. However, many of the Robertsonian translocations which have a low incidence in cattle are dicentric. These include the 6/16 Robertsonian translocation in the Dexter

(Eldridge 1974), the 3/4 Robertsonian translocation in the Limousin (Popescu 1977) and the 5/21 Robertsonian translocation in the Japanese Black (Masuda et al.1978). Unfortunately, the karyotype of all ancestors to these animals is not known and, therefore, it is not possible to determine whether the translocations occurred de novo or were inherited from a previous generation.

In Man, the de novo formation of a Robertsonian translocation was demonstrated in a child from parents of normal karyotype and C-banding studies showed the translocation to be dicentric (Gosden et al.1979). A de novo Robertsonian translocation has also been reported in the mouse and, although C-banding was not carried out, the translocation in G-banded preparations appeared dicentric (Harris et al, 1986). These reports support the suggestion that Robertsonian translocations which formed recently are dicentric in structure (Niebuhr 1972). However, a large proportion of sheep in New Zealand carry Robertsonian translocations which are dicentric (Bruère 1975). These animals developed from Romney Marsh sheep imported from England around 1853 and, since the same translocations also occur in English sheep (Bruère et al.1978), it is assumed that the translocations formed before 1853 (Bruère et al.1976). This provides strong evidence, therefore, that it is not possible to determine the age of a Robertsonian translocation from only the appearance of its centromeric region and it is more likely that the nature of the centromere is related to the position of the breaks which occurred on the chromosomes at the time of centric fusion. If breaks occurred at the extreme tip of the centromere

of two chromosomes then subsequent fusion would result in a dicentric Robertsonian translocation; whereas breaks which are either at the tip of the centromere of one chromosome and between the centromere and chromatids of the other or, in the middle of each centromere on both chromosomes, would result in a monocentric Robertsonian translocation. The formation of the 1/29 Robertsonian translocation in the present study, therefore, presumably occurred following breaks in one of the latter two sets of positions.

Cytogenetic studies in the mouse demonstrated that centric fusions usually involve one chromosome containing regions responsible for the organisation of the nucleoli (Miller, Miller, Tantravahi and Dev 1978). These regions are called the nucleolus organiser regions (NORs) and may, in some way, predispose the chromosome to centric fusion. Robertsonian translocations between human chromsomes also predominently involve the acrocentric chromosomes containing NORs (Gosden, Gosden, Lawrie and Buckton 1979). However, to date, there is no evidence that NORs occur in chromosomes 1 and 29 in cattle. Nevertheless, to date, the methods used to identify NORs in cattle involved silver staining techniques (Gustavsson 1979) and it has been shown that only NORs with actively transcribing ribosomal RNA are detected by this method (Miller, Dev, Tantravahi and Miller 1976). Consequently, further studies are required, using alternative techniques (e.g. Matsui and Sasaki 1973) in order to determine whether inactive NORs are present in cattle chromosomes 1 and 29, which may predispose them to centric fusion.

Cytogenetic studies in the human showed that particular chromosomes lie close to each other during cell division and it was suggested that reciprocal translocations such as the Robertsonian translocation are more likely to occur between these chromosomes than between chromosomes lying distant to each other (Ferguson-Smith 1964). Furthermore, a study of somatic cells from parents of a child in which the de novo 13/14 Robertsonian translocation was present showed a high frequency of close association between particular chromosomes (Gosden et al.1979). However, the closely associated chromosomes were not numbers 13 and 14 which tends to suggest that close association between chromosomes in somatic cells is not itself a predisposition to centric fusion in a germ line. Nevertheless, heritable chromosome aberrations such as the Robertsonian translocation may be the result of chromosome breaks during meiosis (Griffen and Bunker 1967) and it may be that cytogenetic studies of meiotic preparations would show a high frequency of close association between particular chromosomes, such as 1 and 29 in cattle or 13 and 14 in the human, which predisposes them to centric fusion. However, to date, there is no report in the literature of such studies.

Many exogenous agents such as X-rays, feed additives, drugs, pesticides and industrial waste cause chromosome aberrations (Fechheimer 1972) and, since animals are being increasingly exposed to these agents, it may be that some can cause heritable chromosome aberrations such as the 1/29 Robertsonian translocation to form de novo in live animals. For example, the antibiotic actinomycin-C induces Robertsonian translocations to

form in cattle cells *in vitro* (Hsu, Pathak, Basen and Stark 1978). However, actinomycin and other agents tend to induce multiple chromosome aberrations (Hsu *et al*.1978) and the results of research in humans exposed to irradiation shows that multiple aberrations cause various forms of clinical illness and physical deformities in that generation or the next (Brill, Tomonaga and Heyssel 1962). Similar observations were also made in mice and, furthermore, fertility was reduced due to the death of abnormal conceptuses (Griffen and Bunker 1967). Herd A, in which the proband and his recent ancestors were bred, is a well managed farm with excellent breeding records and there is no history of any unexplained illness or of abnormal calves which tends to suggest that the 1/29 Robertsonian translocation was not induced to form by an exogenous agent which induces multiple aberrations. Furthermore, exogenous agents tend to affect all individuals from a group (Fechheimer 1972) and since only two of the animals examined in Herd A carried the translocation, this is further evidence against it being formed by an exogenous agent.

The heritability of the 1/29 Robertsonian translocation in the Friesian animals was clearly demonstrated in Herd B since 54.3% of the proband's offspring were carriers. This figure is consistent with inheritance of the translocation in Swedish Red and White cattle (Gustavsson 1969) and corresponds to the normal 1 : 1 segregation of the translocation observed during meiotic studies in a Charolais bull (Logue and Harvey 1978[a]). Furthermore, it is evidence that the fertility of balanced spermatozoa is unaffected by the presence of the chromosome

aberration. Meiotic studies involving the 14/20 Robertsonian translocation in cattle also demonstrated the normal 1 : 1 segregation (Logue and Harvey 1978[b]) resulting in inheritance of the translocation by approximately half the progeny. The normal 1 : 1 segregation also occurs during meiosis in several sheep Robertsonian translocations (Chapman and Bruère 1975). However, in mice, there is evidence of preferential selection for at least one Robertsonian translocation (Harris et al.1986). Results in that study demonstrated a significantly higher proportion (Chi^2 = 11.4, $P < 0.001$) of carriers among progeny from female heterozygous mice and the authors suggest that, during the first meiotic division of the oocyte, the Robertsonian translocation chromosome may be preferentially retained in the egg nucleus rather than extruded in the first polar body. Consequently, the majority of ova contain the Robertsonian translocation and, therefore, the majority of progeny inherit the aberration. Nevertheless, the segregation pattern of other Robertsonian translocations in mice follow the normal 1 : 1 pattern (e.g. Gropp et al. 1975) and, to date, there is no evidence for preferential selection of Robertsonian translocations in cattle or other domestic species.

A significantly lower non-return to service rate was recorded in large populations of Swedish Red and White and Norwegian Red cattle heterozygous for the 1/29 Robertsonian translocation (Gustavsson 1969; Refsdal 1976 respectively) and Gustavsson (1969) postulated that, during meiosis, the 1/29 Robertsonian translocation forms a trivalent structure which occasionally undergoes non-disjunction to produce unbalanced

gametes. He further suggested that such gametes are capable of fertilisation by a normal gamete but that the resulting aneuploid embryo is non-viable and dies during early pregnancy causing the cow to return to oestrus. Subsequent studies clearly demonstrated that unbalanced products are formed during meiosis in bulls heterozygous for the 1/29 Robertsonian translocation (Logue and Harvey 1978[a]; Popescu 1978) and recent cytogenetic studies demonstrated aneuploid embryos in animals inseminated by carriers of the translocation (King et al.1980; Popescu 1980). These findings, therefore, support Gustavsson's hypothesis that fertility is reduced in animals with the translocation due to the production of non-viable embryos.

In the present study, service dates were not available for animals in Herd B. Nevertheless, it was hoped that any reduction in fertility would be reflected by increases in the age at first calving and interval between calvings. However, statistical analysis of these parameters showed no significant difference between animals with the translocation and their sisters with the normal bovine karyotype. This may suggest that early embryonic death did not occur at a higher frequency in the group of animals with the translocation. However, the heifers in Herd B were introduced to the bull at a time suitable for autumn calving. Consequently, their age at first calving was principally determined by their age when put to the bull. In addition, the interval between calvings is influenced by many factors such as unobserved oestrus and delayed oestrus after calving caused by retained placenta, metritis or poor body condition (Esslemont,

Baillie and Cooper, 1985). Consequently, any effect of early embryonic death on the age at first calving and the interval between calvings was probably concealed by the greater effects of these other factors which were not recorded in the farm records. Furthermore, previous studies showed that it is necessary to examine the breeding records from several hundred carrier animals in order to demonstrate a reduction in fertility (Gustavsson 1969; Refsdal 1976). Consequently, the number of animals available for investigation in the present study was insufficient to detect any reduction in fertility due to early embryo death.

Further evidence of reduced fertility in animals carrying a Robertsonian translocation is provided by the results of research in the mouse. Several Robertsonian translocations are recognised in this species and these all form trivalent structures during meiosis. However, the proportion of trivalent structures which undergo non-disjunction varies between different translocations (Ford and Evans 1973) and, since the unbalanced gametes result in the production of non-viable embryos (Gropp et al.1974), Robertsonian translocations in the mouse which have a higher rate of non-disjunction cause a greater reduction in fertility (Ford and Evans 1973). Several different Robertsonian translocations have been identified in cattle (Long 1985). However, fertility studies involving a large number of animals have only been investigated for the 1/29 Robertsonian translocation (Gustavsson 1969; Refsdal 1976) and, consequently, it is not possible to evaluate whether different Robertsonian translocations in cattle vary in their rate of non-disjunction and effect on fertility. Nevertheless, fertility was not reduced in a small group of

animals carrying the translocation originally thought to involve chromosomes number 2 and 4 (Pollock and Bowman 1974) but later identified as a 2/8 Robertsonian translocation (Eldridge 1985), although a larger number of animals would have to be studied in order to detect any statistically significant effect of the 2/8 Robertsonian translocation on fertility.

In contrast to results obtained from cattle carrying the 1/29 Robertsonian translocation, results of investigations into the fertility of sheep heterozygous for various Robertsonian translocations showed no reduction in fertility (Bruère 1974; 1975). As in cattle, however, trivalent structures are formed during meiosis and a proportion of unbalanced gametes are produced by non-disjunction (Chapman and Bruère 1975). However, since there is no apparent reduction in the fertility of carriers, it is assumed that the unbalanced gametes are not fertilised and therefore non-viable aneuploid embryos are not produced (Bruère 1975). Supportive evidence for this was obtained by finding no unbalanced karyotypes in 16-17 day old blastocysts sired by carrier rams (Long 1977). However, any non-viable embryos would probably be dead by this stage (Long and Williams 1980) and, therefore, be undetected. Consequently, further cytogenetic examination of early embryos is required in order to determine whether or not Robertsonian translocations in sheep result in the formation of aneuploid embryos which subsequently die.

As with cattle carrying the 1/29 Robertsonian translocation, the fertility of humans carrying Robertsonian translocations is

reduced (Hamerton 1970). This is also due to the formation of trivalent structures during meiosis which can result in the production of unbalanced gametes due to non-disjunction (Hamerton 1970). However, although the monosomic embryos are non-viable, embryos trisomic for chromosomes such as number 14 and 21 are viable (Hamerton 1970). Consequently, if the Robertsonian translocation involves either of these chromosomes, then trisomic embryos may form and survive gestation. The neonate, however, is phenotypically abnormal and described clinically as suffering from Down's Syndrome when trisomic for number 21. This syndrome is manifest by varying degrees of physical and mental retardation which are not recognised in the bovine. Neither has any trisomic karyotype containing a Robertsonian translocation been identified in liveborn cattle although such embryos are produced by carriers of the 1/29 Robertsonian translocation (King et al. 1980). This tends to suggest that trisomic embryos produced by cattle heterozygous for a Robertsonian translocation are non-viable. However, for commercial reasons, phenotypically abnormal calves are usually disposed of at birth without ascertaining their karyotype or that of the parents. Consequently, it may be that some of these abnormal calves are trisomic and derive from a parent carrying a Robertsonian translocation. Further cytogenetic investigation is, therefore, required in order to determine whether all trisomic embryos produced by cattle heterozygous for a Robertsonian translocation die in utero, as apparently occurs in the mouse, or whether some survive gestation as recorded in the human.

CHAPTER FOUR

THE MORPHOLOGY AND KARYOTYPE OF EMBRYOS SIRED BY NORMAL BULLS AND BULLS HETEROZYGOUS FOR THE 1/29 ROBERTSONIAN TRANSLOCATION

4.1 INTRODUCTION

Animals used in this study were induced to superovulate in order to obtain a large number of embryos for recovery and examination. However, since the history of these animals was unknown, preliminary investigations, including determination of uterine tube patency, were carried out in order to avoid the use of animals with any reproductive abnormality, such as occlusion of the uterine tube, from the study.

A number of research workers (e.g. Kessy and Noakes, 1979[a]) determined uterine tube patency by examining vaginal mucous for the presence of starch grains which were previously injected through the gluteal muscles and sacro-iliac ligament onto the surface of the ovary. However, other research workers (e.g. Kothari, Renton, Munro and MacFarlane, 1978) determined the patency by infusing phenolsulphonphthalein dye into the uterus followed by the examination of urine to detect any dye which had passed through a uterine tube before being absorbed by the peritoneum and excreted via the kidneys. Nevertheless, this technique suffered from the limitation that it only detected animals with bilateral occlusion of the uterine tubes. Consequently, a modification was developed, whereby the dye was distilled into each uterine horn separately (Kelly, Renton and Munro, 1981) and this method was used in the present study.

The earlier studies of superovulation in cattle used pituitary extracts containing follicle stimulating hormone and luteinising hormone (FSH-LH) injected over a period of several days (Dowling, 1949). However, superovulation was also produced using pregnant mare serum gonadotrophin (PMSG) which has a longer

half life and, therefore, requires only one injection (Dowling, 1949). Nevertheless, regardless of the preparation used, the timing of the gonadotrophin administration related to the occurrence of oestrus was found to be an important factor determining the ovarian response since acceptable levels of superovulation were only achieved when treatment closely preceded oestrus (Dowling, 1949). However, variations in the length of oestrus cycles cause difficulty in accurately predicting the day of natural oestrus and, therefore, an attempt was made to induce oestrus in the early superovulation studies by manual enucleation of the corpus luteum per rectum (Dowling, 1949). Nevertheless, enucleation was not always complete using this method and the problem of predetermining the day of oestrus in superovulation regimes was not overcome until the 1970's when the luteolytic prostaglandin F_2alpha (PG) become available. Currently, the generally accepted method of superovulating cattle involves the administration of a gonadotrophin during the mid-luteal phase of the oestrus cycle followed 48-72 hours later by a luteolytic dose of PG and although the gonadotrophin used by most research workers is either PMSG or FSH-LH (Betteridge, 1977) others use human menopausal gonadotrophin (e.g. Lauria, Oliva, Genazzani, Cremonesi, Crotti and Barbetti, 1982). Nevertheless, the superovulation response produced by these gonadotrophins is notoriously unpredictable (Betteridge, 1977; Lauria et al, 1982) and, therefore, in the present study, the response was monitored by counting the number of corpora lutea by rectal palpation and, in some animals, by laparoscopic examination of the ovaries.

Although it is possible to collect embryos by dissection of the reproductive tract after slaughter, research workers developed a surgical technique in order to enable the recovery of embryos on repeated occasions from individual animals (Rowson, Moor and Lawson, 1969). However, this method resulted in the formation of adhesions which interfered with embryo recovery and, therefore, emphasis was placed on the development of non-surgical methods for the collection of embryos. Sugie, Soma, Fukumitsu and Otsuki (1972) described a technique whereby the base of a uterine horn is occluded with a cuffed cannula through which a further tube is passed towards the uterotubal junction and fluid circulated from the base to the tip of the uterus. However, this basic technique was subsequently improved by Newcomb, Christie and Rowson (1978) who directed a 3-way catheter through the cervix, by means of a rigid cannula, into the tip of the uterine horn where the cuff was inflated and eggs were recovered by a circulation system of flushing. This technique is now used for commercial embryo recovery (Christie, 1987) and was also used in the present study.

The animals from which embryos were recovered in this study were inseminated with semen from either normal bulls or bulls heterozygous for the 1/29 Robertsonian translocation and, in this way, it was hoped to be able to demonstrate any differences in the viability, morphology or karyotype of embryos belonging to either group. Although several laboratory techniques including dye exclusion tests (Kardymowicz, 1972) and measures of glucose uptake (Renard, Philippon and Menezo, 1980) were developed to evaluate embryo viability, these are sometimes difficult to

interprete and require a prolonged in vitro culture period which, in itself, may reduce viability. Consequently, schemes for evaluating embryo viability based on morphological features alone were developed. For example, some research workers (e.g. Boland, Crosby and Gordon, 1978) used differences in the appearance of the inner cell mass as a guide to embryo viability, whereas others (e.g. Greve, Lehn-Jensen and Rasbech, 1979) used various parameters such as the presence of loose blastomeres and vesicles. However, a comprehensive system using the appearance of the trophectoderm, inner cell-mass, blastocoele and zona pellucida was developed to classify embryos as either normal, in the process of degeneration or degenerate (Linares and King, 1980) and this method was used in the present study.

4.2 MATERIALS AND METHODS

4.2.1 Animals used for Embryo Recovery

Thirty-seven cows were selected from non-lactating, barren, culled cows. Details of the cows are shown in Table 10.

The age of these animals was estimated from the appearance of their teeth. Animals with two, three and four permanent incisor teeth in wear were estimated to be two and a half, three and three and a half years old respectively. Those with a full complement of incisor teeth showing a moderate amount of wear were considered to be greater than four years old, and animals with worn teeth and exposed roots were considered to be aged.

4.2.2 Housing and Maintenance

The cows were maintained at grass during the summer. In the winter months, they were tied by the neck in a byre. Diet of hay

and concentrate was fed to maintain the animals in good condition.

4.2.3 Clinical Examination

A general physical examination including temperature, pulse, respiration and body condition was made and any abnormalities were recorded.

4.2.4 Rectal Palpation

The reproductive tract was examined every second or third day by rectal palpation. Ovaries, uterus and cervix were palpated. Parameters recorded were ovarian size and structures, uterine size and tone, consistency of cervix and vulval discharge.

4.2.5 Detection of Oestrus

Observations of oestrus behaviour were made twice daily. The animals were observed, in a group of six, in an uncovered yard, 95 m^2, for between 20 and 30 minutes. The specific signs recorded were: bellowing, restless activity, attempting to mount, and being mounted by others.

Heat detectors (Kamar) were used on cows at grass.

4.2.6 Uterine Tube Patency

The phenolsulphonphthalein (PSP) Dye test described by Kelly, Renton and Munro (1981) was used to select animals with patent uterine tubes for embryo recovery. Details of the 37 barren, non-lactating animals examined are shown in Table 12.

The first eight animals examined were sedated with 5 mg xylazine (Rompun, Bayer). Thereafter, 5 mg acetylpromazine (ACP, C-Vet) was administered by intravenous injection to any animal which required sedation.

TABLE 10

Details of animals used for embryo recovery and the number of times they were stimulated to superovulate

Identification	Breed	Age (years)	Number of super-ovulation treatments
4	Friesian	3 1/2	2
11	"	3 1/2	3
18	Ayrshire	3 1/4	3
23	Friesian	3 1/4	2
25	Shorthorn	Aged	1
27	Red Poll	Aged	4
29	Friesian	3 1/2	1
35	"	3	1
36	"	3 1/2	1
40	"	3 1/4	1
41	Hereford X	Aged	2
43	Friesian	3 1/2	2
47	"	> 4	1
50	Ayrshire	> 4	1
51	"	3	1
52	"	> 4	1
62	Friesian	Aged	3
63	"	2 1/2	1
74	"	2 1/2	2
80	"	> 4	1
81	"	3	2
103	Hereford X	> 4	1
104	Friesian	3 1/2	3
105	Ayrshire	3	1
138	Friesian	3	2
144	"	3	3
145	"	3	2
146	"	3 1/2	1
148	"	Aged	2
150	Ayrshire	3	1
152	"	> 4	4
164	"	> 4	1
165	"	> 4	2
166	Friesian	> 4	1
172	"	> 4	2
181	Ayrshire	> 4	2
186	Hereford X	Aged	1
		Total:	65

4.2.7 Karyotype of Animals used for Embryo Recovery

The karyotype of animals used in the study was obtained using the method described in section 3.23.

4.2.8 Superovulation

Cows were superovulated on up to four occasions as shown in Table 10, with an interval of at least six weeks between each. 2000 iu Pregnant Mare Serum Gonadotrophin (PMSG) (Folligon, Intervet) were administered by intramuscular injection once between days 7 and 13 of the oestrus cycle and 500 iu cloprostenol (Estrumate, ICI) were injected intramuscularily 48 hours later.

Cow number 27, which failed to superovulate when treated with 2000 iu PMSG, was injected with 3000 iu PMSG on subsequent occasions.

4.2.9 Ovarian Response to Superovulation

The numbers of corpora lutea were counted by rectal palpation at the time of embryo recovery. Each ovary was grasped between thumb and finger tips and gently rotated in order that every aspect was palpated. Firm structures, estimated to be between 8 and 20 mm in diameter which protruded from the surface of the ovary and occasionally had a small central depression, were classified as corpora lutea.

The two-sided t-test was used to demonstrate any statistically significant effect of certain parameters on the superovulation response. The parameters investigated were age and breed of animal used, batch of PMSG, day of oestrus cycle on which it was injected, and whether the animal had previously been superovulated.

During part of the study, ovarian structures were examined one or two days after embryo recovery by laparoscopy using the method described by Wishart and Snowball (1973).

4.2.10 Insemination

All cows were artificially inseminated at the time of oestrus and twelve hours later using the standard technique recommended by the Scottish Milk Marketing Board (Isbister, 1985). Unfortunately, semen from the proband was not available for use until the latter part of the study. Consequently, semen from another bull heterozygous for the 1/29 Robertsonian translocation was used during the earlier part of the study. In addition, two bulls with the normal bovine karyotype were used for artificial insemination. Details of these animals are shown in Table 11.

TABLE 11

Description of bulls used for artificial insemination

Identification	Breed	Karyotype
W.S.	British Friesian	60, XY
L.P.L.	British Friesian	60, XY
C.	Charolais	59, XY, t (1, 29)
Proband	British Friesian	59, XY, t (1, 29)

4.2.11 Embryo Recovery

Embryos were recovered from the cows sedated with 5 mg of acetylpromazine (ACP, C-Vet) between 6 and 8 days after oestrus

using the non-surgical technique described by Newcomb, Christie and Rowson (1978) with the following modifications. Firstly, movement of the 3-way plastic catheter (Franklin Medical) through the metal introducer was assisted by the application of a small amount of lubricant (K-Y Jelly, Johnson and Johnson) to the region of the cuff. Secondly, a mark was made on the catheter 90 cm from the tip in order to indicate how far the catheter was inserted into the uterus. Thirdly, in order to prevent the catheter tip folding back on itself during this procedure, the catheter was refrigerated at +4°C until immediately prior to use since this temperature provided the catheter with a moderate degree of rigidity. Fourthly, when the catheter was correctly positioned, 2 ml of air was used in addition to sterile saline solution in order to inflate the cuff and occlude the uterine horn lumen.

The flushing fluid used to recover the embryos consisted of freshly prepared phosphate buffered saline (Oxoid) containing 2% Heat Treated Foetal Calf Serum, 100 iu/ml pencillin + 100 µg/ml streptomycin (all Flow Laboratories). 250 ml was flushed into each uterine horn at 37°C using 50 ml plastic syringes (Terumo) and recovered in a sterile siliconised glass measuring cylinder. At the end of the recovery procedure, 3 g of penicillin (Crystapen, Glaxovet) were injected into the uterus of each cow before removing the catheter.

4.2.12 Physical Evaluation of the Embryos

Each measuring cylinder containing flushing medium recovered from the uterus was allowed to stand for at least 45 minutes in an incubator at 37°C so that all embryos settled to the bottom of

the cylinder. Taking care not to disturb the embryos, the flushing fluid was then aspirated off from the top of the cylinder, using a water-pump, until 60 ml remained at the bottom of the cylinder. A stereodissection microscope (Vickers) was used to locate embryos in this fluid by examining it in 20 ml aliquots poured into a sterile glass petri dish. If observation of the embryos was obscured by the presence of cell debris then the concentration of debris was reduced by adding fresh flushing medium to the measuring cylinder.

The stage of development of each embryo recovered was classified using the parameters described by Lindner and Wright (1983) as a guideline and embryos were further classified as being either normal, in the process of degeneration or degenerate based on the description of embryos recovered by Linares and King (1980).

4.2.13 Cytogenetic Analysis of the Embryos

After physical evaluation, each embryo was transferred, using a drawn pasteur pipette, to a multiwell plate (S.A.S., Flow Laboratories) filled with sterile culture medium which consisted of phosphate buffered saline (Oxoid) containing 20% Heat Treated Foetal Calf Serum (Flow Laboratories), 100 iu/ml penicillin + 100 µg/ml streptomycin (Flow Laboratories), and 50 µg/ml colchicine (Sigma). The embryos were then incubated at 37^{o}C for between two and five hours and microscopic preparations for cytogenetic analysis were prepared in the following manner using the technique described by Tarkowski (1966), with minor modifications.

The embryo was transferred, using a drawn pasteur pipette, from the culture medium into 1% trisodium citrate solution and incubated at 37° for 10-12 minutes. Next, the embryo was aspirated into the tip of a drawn pasteur pipette and expelled, suspended in as small a volume of hypotonic solution as possible, onto a microscope slide which was freshly cleaned with 70% alcohol. The hypotonic solution was encouraged to evaporate by placing the slide on the warm stereodissection microscope stage and, when almost all was evaporated, 10 µl of fixative (methanol : glacial acetic acid, 1 : 1) was dropped onto the embryo from a micropipette (Finnpipette, Labsystems). The fixative was encouraged to evaporate by blowing on it and another three to five 10 µl drops were added, allowing the fixative to almost evaporate between each drop. This process was repeated using a fresh microscope slide for each embryo.

Each microscope slide was placed in fixative (methanol : glacial acetic acid, 3 : 1) overnight then dried at room temperature. Ten per cent Giemsa stain was prepared by filtering 5 ml Giemsa (Gurr's R66, B.D.H. Chemicals) and 45 ml Giemsa buffer (pH 6.5, B.D.H. Chemicals) through a filter (No. 1, Whatman) and the slides were immersed in the stain for 30 minutes and then rinsed in fresh running tap water, dried at room temperature, and mounted (D.P.X., B.D.H. Chemicals) with a coverslip.

Chromosome spreads from each embryo were examined using a light microscope (Leitz, Ortholux). The number of chromosomes in each set was counted and the sex and 1/29 Robertsonian translocation chromosomes were identified. If two chromosome

spreads were intermingled then the total number of chromosomes was counted and divided by two. The result was considered to represent the number of chromosomes in each cell of that particular embryo with the proviso that cells and chromosomes were not generally overspread on the slide and that no loose chromosomes were present in the area surrounding the two intermingled sets.

4.3 Results

4.31 Uterine Tube Patency

Results of using the P.S.P. Dye Test on 37 animals are given in Table 12. Urine was recovered from these animals at five minute intervals and the appearance of dye in the urine within 20 minutes of instillation to the uterine horn was taken to indicate a patent uterine tube.

Twenty-six animals were diagnosed as having bilaterally patent uterine tubes and these animals were selected for embryo recovery. Animals 50 and 52 were also selected for embryo recovery, although patency of the left uterine tube was not determined in these animals.

One animal was diagnosed as having bilateral occlusion of the uterine tubes and eight animals had a unilateral occlusion. The patency of the contralateral tube in seven of these animals was not determined since the first side examined was found to be blocked and animals with non-patent uterine tubes were excluded from embryo recovery.

Although the volume of urine produced by animals in this study was not measured, animals sedated with xylazine appeared to

produce large quantities of very dilute urine which caused difficulty in detecting the presence of dye. The volume and concentration of urine produced by animals sedated with acetylpromazine resembled that of animals without sedation and caused no difficulty in detecting the presence of dye.

The PSP Dye Test was abandoned in eight animals, examined during the earlier part of the study, which are not listed in Table 12. In six of these, aged between 2 1/2 and 3 1/2 years, it was not possible to pass the introducer through the cervix which was small and tightly closed. The test was abandoned in the remaining two animals, each aged 4 years, when the introducer accidentally penetrated the uterine wall. This occurred in one animal as it unexpectedly coughed violently when the passing the introducer through the cervix. In the other animal, the effect of the epidural anaesthetic was wearing off and the return of rectal contractions forced the tip of the introducer through the uterine wall. Neither animal showed any adverse reaction to the injury and examination of one of the wounds during laparoscopy two days later showed that it was healing normally without adhesions.

4.32 The karyotype of animals used for embryo recovery

Each of the 37 animals used for embryo recovery had the normal bovine karyotype except for animal 27, the Red Poll in Table 10, which was heterozygous for the 1/29 Robertsonian translocation.

TABLE 12

The time dye first appeared in the urine of 37 animals examined using the PSP Dye Test

Animal Identification Number	Breed	Age (years)	Time (minutes) Left Uterine Tube	Time (minutes) Right Uterine Tube
3	Ayrshire	3	*	*
4	Friesian	3 1/2	15	15
11	"	3 1/2	15	15
18	Ayrshire	3 1/4	15	20
25	Shorthorn	Aged	15	15
27	Red Poll	Aged	20	15
40	Friesian	3 1/4	15	15
41	Hereford X	Aged	15	15
43	Friesian	3 1/2	20	20
47	"	> 4	15	15
50	Ayrshire	> 4	-	15
51	"	3	15	20
52	"	> 4	-	15
62	Friesian	Aged	15	15
63	"	2 1/2	20	15
68	"	3	-	*
74	"	2 1/2	15	15
80	"	> 4	20	20
81	"	3	15	15
83	Ayrshire	> 4	-	*
84	"	> 4	*	-
103	Hereford X	> 4	15	15
104	Friesian	3 1/2	15	20
107	"	Aged	*	-
108	Ayrshire	> 4	-	*
117	Friesian	3	*	15
132	Ayrshire	Aged	*	-
133	Friesian	3	*	-
144	"	3	15	20
145	"	3	15	15
146	"	3 1/2	15	20
148	"	Aged	20	15
152	Ayrshire	> 4	15	15
165	"	> 4	15	15
166	Friesian	> 4	15	20
172	"	> 4	20	20
181	Ayrshire	> 4	15	15

* no dye detected within 40 minutes
\- not examined

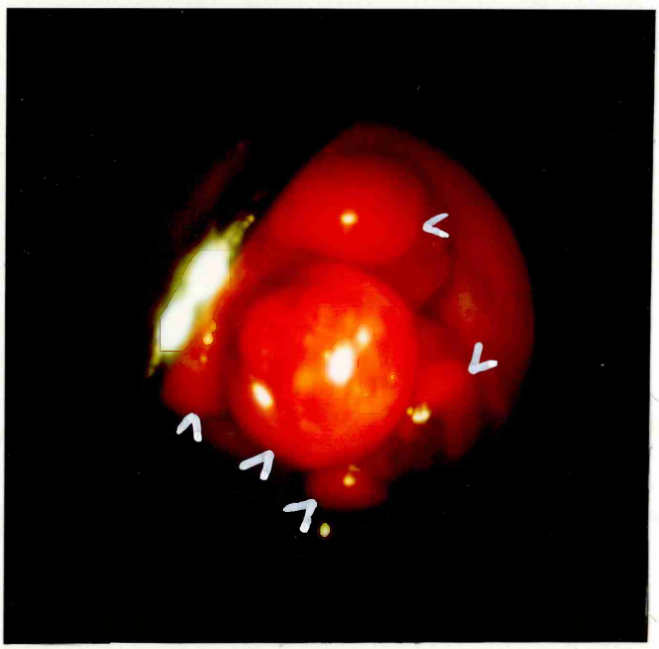

Figure 12

A laparoscopic view of five corpora lutea (indicated by arrows) protruding from the surface of an ovary

4.33 The ovarian response to superovulation

The number of corpora lutea was counted in each animal by rectal palpation at the time of embryo recovery and ranged between 0 and 20 as shown in Table 19. Although follicular structures, estimated to be between 2 and 50 mm in diameter, were palpated in each ovary, it was impossible to count them since they tended to overlap each other and could not be distinguished individually.

Laparoscopic examination of the ovaries 2-4 days after embryo recovery was successfully carried out on 24 occasions and the total number of corpora lutea which were identified ranged between 0-24 (see Figure 12) as shown in Table 13 which also gives the number counted by rectal palpation in these animals.

These figures corresponded with each other on 11 occasions. However, a greater number was counted during laparoscopic examination on 8 occasions due to corpora lutea which were situated closely together and could not be distinguished individually by rectal palpation.

On four occasions, fewer corpora lutea were counted during laparoscopy due to luteinised follicles which were wrongly classified as corpora lutea by rectal palpation. These structures were yellow/grey when compared with the yellow/orange corpora lutea and, although they bulged from the ovarian surface, there was no distinct neck.

In animal 52, one of the corpora lutea identified by rectal palpation was considered to be two conjoined corpora lutea during laparoscopic examination since, although only one neck was obvious where it protruded from the ovary, two distinct

depressions were seen on its surface.

Laparoscopic examination of the ovaries was unsuccessful in three animals. In two of these, despite frequent attempts to view them from different angles, the ovaries remained obscured by peritoneal fat suspended from the ovarian ligament, whereas the procedure was abandoned in the remaining animal due to gross rumen distention which obstructed the passage of the endoscope. This animal was accidentally fed prior to examination.

TABLE 13

The differing numbers of corpora lutea identified in superovulated ovaries by laparoscopy and rectal palpation

Animal Identification Number	Number of corpora lutea counted by	
	Laparoscopy	Rectal Palpation
4	9	9
11	8	5
	8	8
	6	6
18	0	2
	17	15
25	0	0
52	9	8
62	0	0
74	1	2
80	4	5
81	1	1
103	2	2
104	1	1
138	4	3
	24	20
144	11	9
145	8	4
	2	2
148	12	12
152	3	4
	9	9
	9	8
166	19	14
Total	167	149
Mean	7.0	6.2

4.34 Statistical analysis of various parameters which may have affected the response to superovulation

Whereas the number of corpora lutea was counted on 24 occasions using laparoscopic examination of the ovaries, the superovulation response was determined on all 65 occasions by rectal palpation and, therefore, these results were used for statistical analysis. The effects of age, breed, single or repeated treatment, batch of PMSG used, and day of oestrus cycle when PMSG was administered are shown in Tables 14, 15, 16, 17 and 18 respectively.

Since no history was available for the animals, it was necessary to estimate their age and Table 14 shows that there was no statistically significant difference ($P > 0.05$) between the number of corpora lutea counted in the three approximate age groups. Neither was there any significant difference between the Friesian and Ayrshire animals although the number of corpora lutea in the group of five beef animals was significantly less ($P < 0.03$) (Table 15).

The number of corpora lutea counted in the 37 animals after a single treatment was not significantly different ($P > 0.05$) from that produced in animals which were stimulated to superovulate on repeated occasions (Table 16). Neither was there any significant difference ($P > 0.05$) between the number of corpora lutea produced by the two most frequently used batches of PMSG (Table 17).

Table 18 shows that there was no significant difference ($P > 0.05$) between the number of corpora lutea produced on any particular day of PMSG treatment and the overall mean, although

there was a significant difference ($P < 0.02$) between the response of day 9 and 10 ($T = 2.516$) and days 9 and 13 ($T = 3.280$). However, the group treated on day 10 included all but one of the beef animals which produced significantly less corpora lutea than the other animals used in the study and only four animals were treated on day 13 of which two did not respond.

TABLE 14

The number of corpora lutea counted per rectum in animals of different age groups after the first superovulation treatment

Age (years)	Number of Animals	Number of Corpora Lutea		
		Total	Mean	Standard Deviation
< 4	20	110	5.5	3.8
> 4	11	73	6.6	4.7
Aged	6	19	3.2	3.3
Overall	37	202	5.5	4.1

< 4 v. overall T = 0.037

> 4 v. overall T = 0.751

Aged v. overall T = 1.519

TABLE 15

The number of corpora lutea counted per rectum in animals of different breeds after the first superovulation treatment

Breed	Number of Animals	Numbers of Corpora Lutea		
		Total	Mean	Standard Deviation
Friesian	22	139	6.3[a]	3.9
Ayrshire	10	55	5.5[a]	4.4
Beef	5	8	1.6[b]	1.5
Overall	37	202	5.5[a]	4.1

Values with different superscript differ significantly ($P < 0.02$).

Friesian v. overall	T = 0.801
Ayrshire v. overall	T = 0.026
Beef v. overall	T = 4.046

TABLE 16

The number of corpora lutea counted per rectum after single and repeated superovulation treatments

Superovulation	Number of Occasions	Number of Corpora Lutea		
		Total	Mean	Standard Deviation
Single	37	202	5.5	4.1
Repeated	28	150	5.4	5.3
Overall	65	352	5.4	4.6

Single v. overall T = 0.050

Repeated v. overall T = 0.051

TABLE 17

The number of corpora lutea counted per rectum after treatment with two different batches of PMSG

Batch Number	Number of Occasions Used	Number of Corpora Lutea		
		Total	Mean	Standard Deviation
313621	8	49	6.1	6.2
376541	10	60	6.0	4.9
Overall	18	109	6.1	5.3

313621 v. overall T = 0.028

376541 v. overall T = 0.028

TABLE 18

The number of corpora lutea counted per rectum in animals treated with 2000IU PMSG during different days of the oestrus cycle

Days of Oestrus Cycle	Number of Occasions	Numbers of Corpora Lutea		
		Total	Mean	Standard Deviation
8	4	19	4.8^{ab}	2.5
9	20	157	7.9^{a}	4.9
10	21	94	4.5^{b}	3.6
11	6	23	3.8^{ab}	5.7
12	3	11	3.7^{ab}	3.1
13	4	9	2.3^{b}	2.6
14	4	35	8.8^{ab}	6.1
Overall	62	348	5.6^{ab}	4.6

values with a different superscript differ significantly ($P < 0.02$)

4.35 Embryo Recovery Rate

Both uterine horns were flushed in all animals except for one horn of animal 18, in which the introducer penetrated the uterine wall, and animal 103 in which the catheter folded back on itself and could not be correctly reinserted.

The number of eggs recovered from each animal ranged between 0 and 24 as shown in Table 19 which also gives the number of corpora lutea counted by rectal palpation at the time of embryo recovery.

On 13 occasions, the total number of eggs recovered

corresponded with the number of corpora lutea counted by rectal palpation.

On six occasions, the number of eggs exceeded the number of corpora lutea, whereas fewer eggs than corpora lutea were recovered on 25 occasions, and no egg was recovered on 13 occasions when between 1 and 11 corpora lutea were palpated and on eight occasions where corpora lutea were absent from each ovary.

Embryos were recovered from both horns in 16 of the 26 animals determined by the PSP dye test to have bilaterally patent uterine tubes. In another four animals, embryos were recovered from one horn only and no embryo was recovered from the remaining six animals, three of which did not superovulate. Embryos were recovered from both horns of animals 50 and 52 in which only the right horn was examined using the PSP dye test.

All the eggs collected in this study were recovered from cows with the normal karyotype.

In total, 214 eggs were recovered during the study from animals with a total of 352 palpable corpora lutea which represents an overall recovery rate of 60.8%.

TABLE 19

The numbers of corpora lutea counted during rectal palpation of the superovulated animals and the numbers of eggs recovered from each uterine horn

Animal Identification Number	Corpora Lutea		Eggs	
	Left	Right	Left	Right
4*	6	3	0	2
	2	4	2	1
11*	3	2	1	2
	6	2	3	0
	4	2	3	2
18*	3	5	-	4
	1	1	0	0
	7	8	9	3
23	0	0	0	0
	1	0	1	0
25*	0	0	0	0
27*	0	1	0	0
	0	1	0	0
	0	0	0	0
	0	0	0	0
29	0	0	0	0
35	4	4	2	4
36	4	4	3	0
40*	5	6	0	0
41*	3	1	0	4
	2	6	2	3
43*	1	1	0	2
	4	0	4	0
47*	5	4	7	5
50	6	8	6	3
51*	0	0	0	0
52	6	2	3	0
62*	0	4	0	5
	0	0	0	0
	2	4	3	4
63*	1	2	2	0
74*	6	5	1	0
	1	1	0	0
80*	2	3	0	0

(Cont'd)

* determined by the PSP dye test to have bilaterally patent uterine tubes

TABLE 19 (Cont'd)

Animal Identification Number	Corpora Lutea		Eggs	
	Left	Right	Left	Right
81*	0	1	0	0
	0	1	0	1
103*	2	0	0	-
104*	5	4	6	4
	1	0	0	0
	0	0	0	0
105	2	2	2	2
138	1	2	2	1
	7	13	8	16
144*	3	6	1	2
	1	4	0	4
	1	0	0	0
145*	2	2	2	2
	2	0	0	0
146*	5	5	1	2
148*	6	3	3	4
	7	5	1	1
150	4	1	5	0
152*	1	1	0	0
	1	3	0	2
	4	5	1	0
	4	4	0	2
164	5	5	3	2
165*	0	3	0	4
	8	8	3	4
166*	7	7	7	7
172*	4	1	4	2
	2	5	0	5
181*	0	1	0	1
	2	3	0	0
186	0	1	0	1
Overall	352		214	

* determined by the PSP dye test to have bilaterally patent uterine tubes.

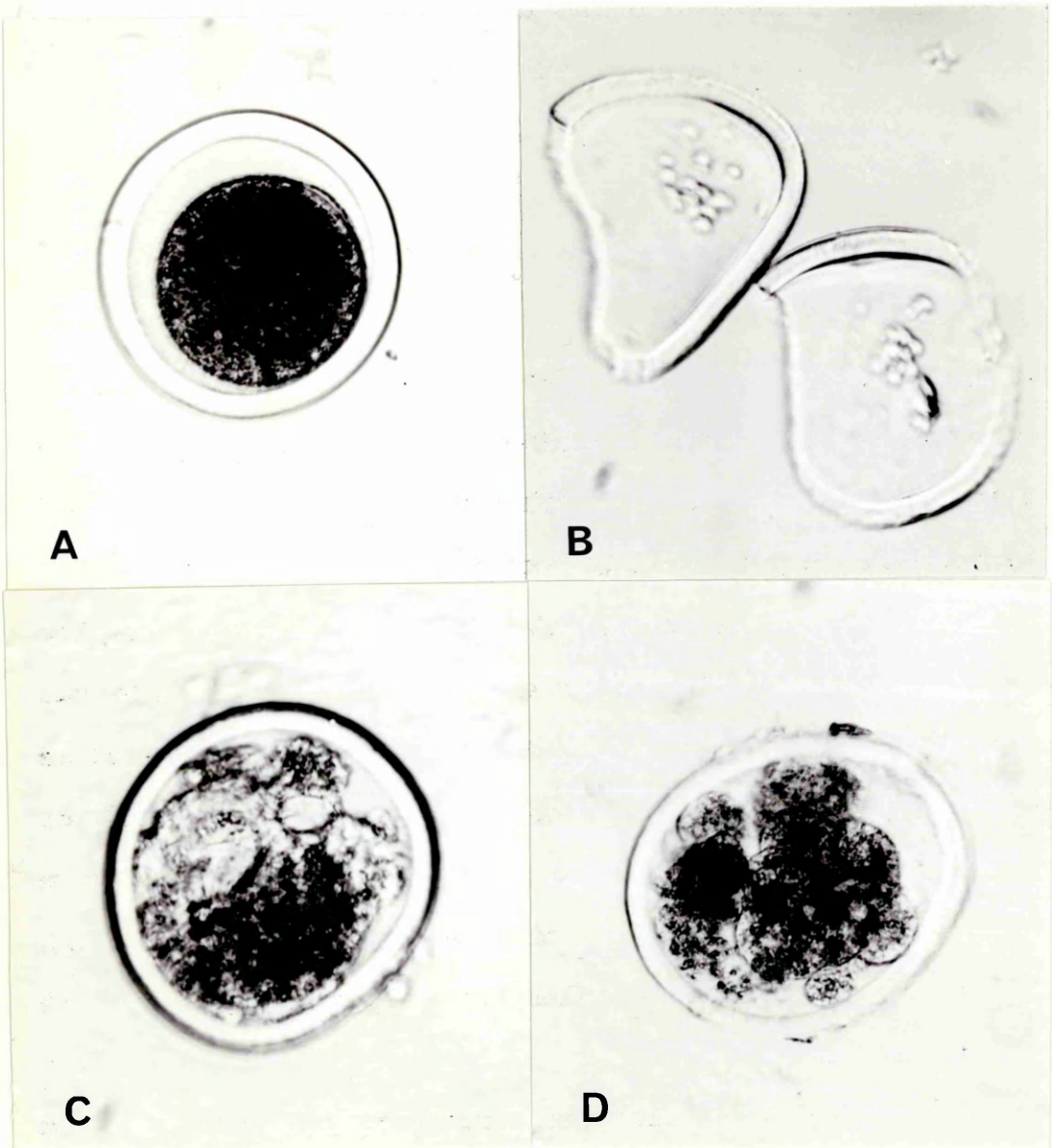

Figure 13

A. unfertilised egg. B. fragments of zona pellucida. C. blastocyst in the process of degeneration showing an indistinct blastocoele and inner cell-mass. D. degenerate morula containing a low number of cells of different sizes. (all x 120 approx.)

4.36 The Physical Evaluation of Embryos Recovered

A total of 214 eggs were recovered during the study as shown in Table 20. Forty of these eggs were classified as unfertilised since the contents of the zona pellucida appeared homogenous and there was no evidence of cells (Figure 13. A). Another nine consisted of fragmented pieces of zona pellucida (Figure 13. B) and two were lost before classification. The fragmented pieces of zona pellucida were all recovered from animals seven days after oestrus.

Of the remaining 163 embryos, 105 were classified as normal since there were no loose cells or detritus in the perivitelline space, the surface of the cell-mass was even and, if the inner cell-mass and blastocoele were developed, then these were clearly distinguished (Figure 14). Thirty-nine embryos were classified as being in the process of degeneration since either loose cells or detritus were present in the perivitelline space, or the inner-cell mass and blastocoele were not clearly distinguishable (Figure 13. C). The remaining 19 embryos were classified as degenerate since either the zona pellucida was broken (6), or there was a low number of cells (7), or the blastomeres were detached from one another and of different sizes (6) (Figure 13. D). Those embryos with a broken zona pellucida appeared to be normal otherwise.

There was no significant difference ($P > 0.05$) between either the number of fertilised and unfertilised eggs ($Chi^2 = 1.49$) or the number of normal and abnormal embryos ($Chi^2 = 0.003$) sired by bulls with the normal karyotype and bulls heterozygous for the 1/29 Robertsonian translocation.

TABLE 20

The morphology of eggs recovered from the superovulated animals inseminated with semen from different bulls

Sire	Lost Before Classification	Normal	In the Process of Degeneration	Degenerate	Unfertilised	Fragments of Zona Pellucida	Total
W.S.	2	34	12	6 $	5	1	60
L.P.L.	-	5	2	1	5	3	16
C.	-	42*	22	9 $	20	2	95
Proband	-	24	3	3	10	3	43
Overall	2	105	39	19	40	9	214
Percent	0.9	49.1	18.2	8.9	18.7	4.2	100

* This figure includes 2 embryos with an irregularly shaped zona pellucida.

$ These figures each include 3 embryos with a broken zona pellucida.

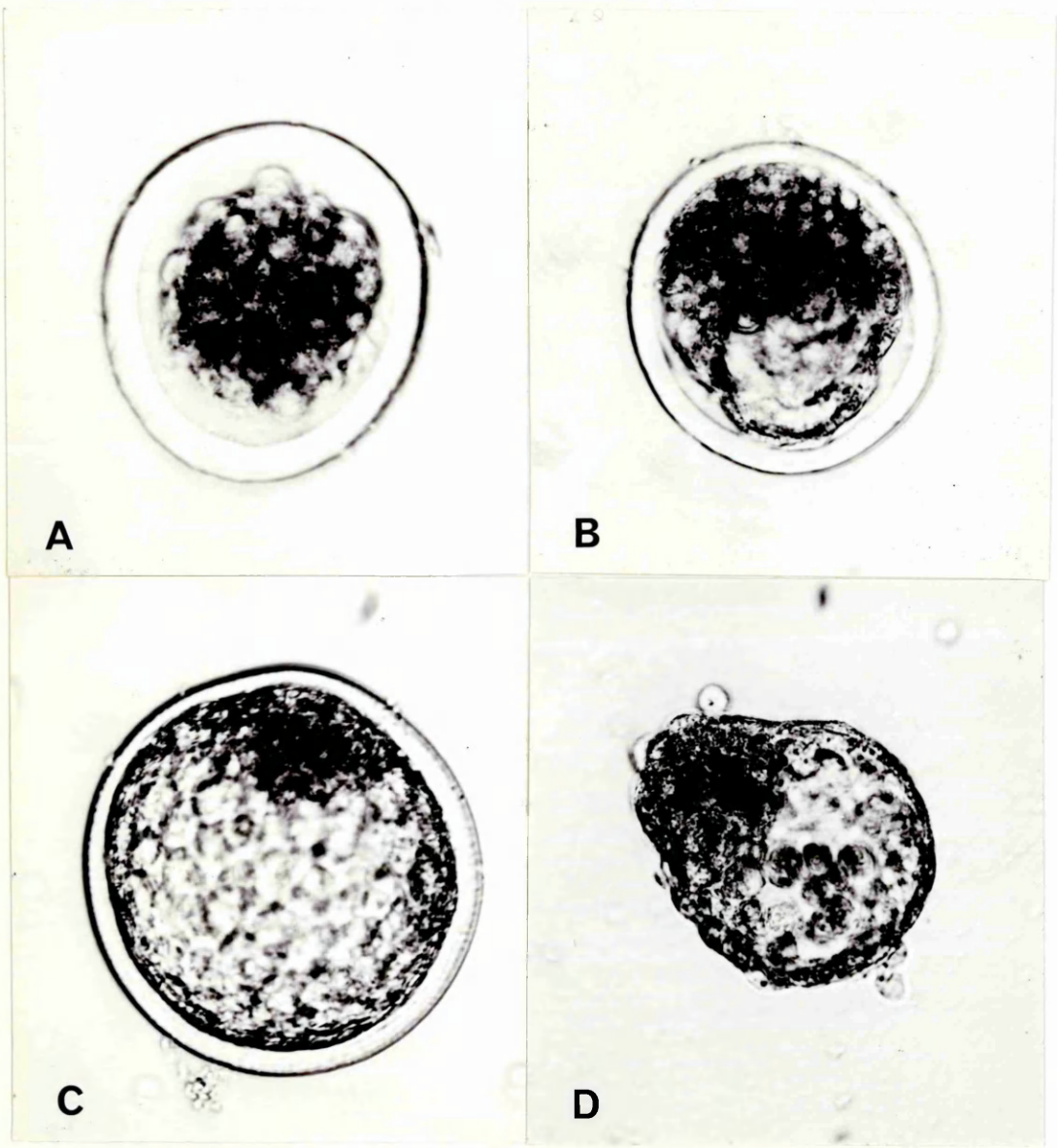

Figure 14

Four normal embryos at different stages of development.
A. compact morula. B. blastocyst.
C. expanded blastocyst. D. hatched blastocyst.
(all x 120 approx.)

The 163 fertilised embryos recovered during this study were at various stages of development as shown in Table 21. Thirty-nine of the embryos contained a cell-mass occupying most of the perivitelline space with a low number of blastomeres which were difficult to discern from one another and these were classified as morulae. In 52 embryos, the individual blastomeres were coalesced forming a compact mass occupying 60-70% of the perivitelline space corresponding to compact morulae (Figure 14. A). Twenty-three of the embryos showed early formation of the blastocoele, trophectoderm and inner cell-mass occupying 70-80% of the perivitelline space and these were classified as early blastocysts. The blastocoele was prominent in 41 blastocysts with clearly differentiated compact darker inner cell-mass and lighter trophectoderm occupying most of the perivitelline space (Figure 14. B). The diameter of seven embryos was increased up to 1.5 times with a thinning of the zona pellucida by approximately one third of its original thickness corresponding to the expanded blastocyst stage of development (Figure 14. C). One embryo, consisting of a distinct darker inner cell-mass and lighter trophectoderm, was recovered without a zona pellucida and classified as a hatched blastocyst (Figure 14. D).

Table 21 demonstrates that, although there was a range in the stage of development among embryos recovered on any specific day, the embryos recovered on day 8 after oestrus tended to be further developed than those collected on days 6 and 7.

TABLE 21

The stage of development of embryos recovered during different days after oestrus

Days after Oestrus	Morula	Compact Morula	Early Blastocyst	Blastocyst	Expanded Blastocyst	Hatched Blastocyst	Total
6	7	1	1	-	-	-	9
7	28	50	19	23	1	-	121
8	4	1	3	18	6	1	33
Overall	39	52	23	41	7	1	163

Three of the embryos were lost during manipulation in the culture medium. However, slide preparations were obtained from each of the remaining 160 embryos. In 14 of these, a proportion of cells spread over the edge of the slide and could not be counted. The cells in the remaining 146 slides varied in size and the number differed according to the stage of development as shown in Table 22. The number of chromosome sets was approximately the same at all stages of development except in the morulae which rarely contained chromosomes sets. In total, 12,597 cells were counted in addition to 529 chromosome sets giving an overall mitotic index of 4.0%.

TABLE 22

The mean number and standard deviation of cells and chromosome sets counted on 146 slides prepared from embryos at different stages of development

	morula	compact morula	early blastocyst	blastocyst	expanded blastocyst	hatched blastocyst
Number examined	27	42	23	41	7	1
Cells	25.8 (± 13.1)	82.4 (± 22.5)	94.4 (± 26.8)	119.5 (± 28.3)	144.2 (± 46.5)	175.0 (± 0.0)
Chromosome Sets	0.7 (± 1.1)	4.7 (± 3.1)	4.2 (± 2.9)	4.5 (± 3.1)	4.7 (± 3.5)	5.0 (± 0.0)

4.37 Cytogenetic analysis of embryos

A microscopic preparation for cytogenetic analysis was prepared from each of the 163 embryos incubated in culture medium except three which were lost during handling. Whilst metaphase chromosome spreads were absent in 34 slides (see Tables 23 and 27), between one and 14 sets of chromosomes were present in each of the remaining 126 slides.

Cytogenetic analysis was not possible in 27 of these slides due to excessive overspreading of chromosomes nor in 32 slides in which the chromosomes overlapped each other. However, slides prepared from the remaining 67 embryos each contained between one and five evenly-spread chromosome sets suitable for cytogenetic analysis.

In 53 embryos, the chromosome sets consisted of 58 acrocentric autosomes and two sex chromosomes corresponding to the normal karyotypes 60 XX and 60 XY (Table 24). Eleven embryos, each sired by a bull heterozygous for the 1/29 Robertsonian translocation, contained cells with 56 acrocentric autosomes, two sex chromosomes and one Robertsonian translocation, (Table 24). The remaining three embryos were recovered from different animals seven days after oestrus and each contained evenly-spread chromosome sets with an unbalanced complement of chromosomes (Tables 24 and 25).

Whilst embryo R3 contained seven chromosome sets, the chromosomes overlapped each other in three of these which

TABLE 23

The reasons which prevented karyotyping of embryos sired by different bulls

Sire	Embryos				
	No mitotic diploid cells	Chromosomes Overspread	Overlapping	Lost	Total
W.S.	14 $	11	5	1	31
L.S.C.	3	-	1	-	4
C.	15 $	12	17*	1	45
Proband	2	4	9	1	16
Overall	34	27	32	3	96

* This figure includes 2 embryos with an irregularly zona pellucida.

$ These figures each include 3 embryos with a broken zona pellucida.

TABLE 24

The karyotype of embryos sired by different bulls

Sire		Karyotype of embryo								
		59		59,t		60		60,t		
Identification	Karyotype	XX	XY	XX	XY	XX	XY	XX	XY	Total
W.S.	60,XY	-	-	-	-	9	12	-	-	21
C.P.L.	60,XY	-	-	-	-	3	1	-	-	4
C.	59,XY,t(1,29)	2	-	2	5	10	8	-	1	28
Proband	59,XY,t(1,29)	-	-	1	3	1	9	-	-	14
Overall		2	0	3	8	23	30	0	1	67

There is no significant difference ($Chi^2 = 3.028$, $P > 0.05$) between the number of normal and balanced heterozygous embryos sired by bulls heterozygous for the 1/29 Robertsonian translocation.

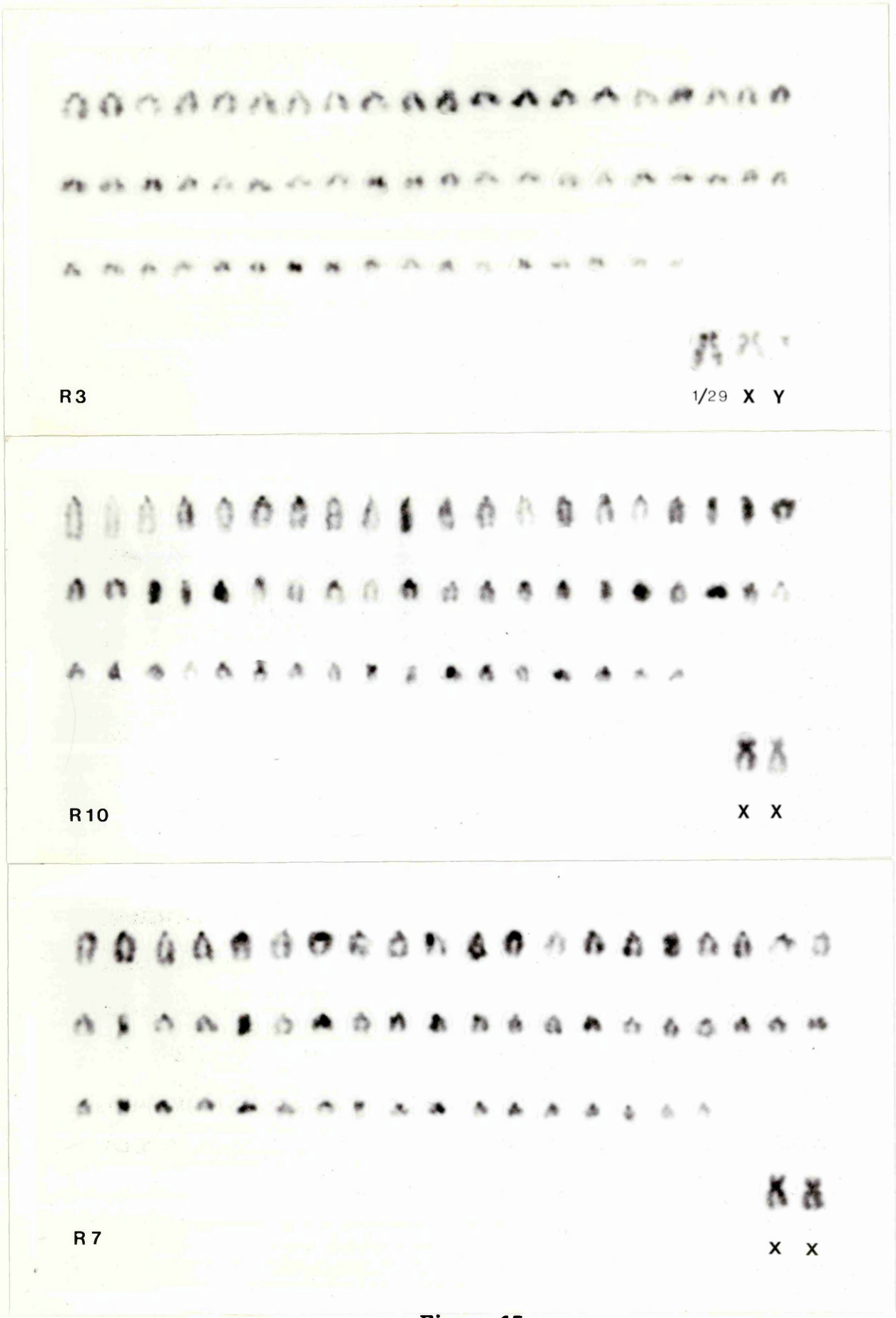

Figure 15

Representative karyotypes prepared from the three aneuploid embryos

prevented cytogenetic analysis. The remaining four sets were evenly spread and each contained 57 acrocentric autosomes together with one X, one Y and one Robertsonian translocation. This embryo was considered to be trisomic for one of the autosomes.

Embryo R10 contained four chromosome sets, of which two were incomplete due to overspreading on the slide. The other two were evenly spread with no loose chromosomes present in the vicinity of either set. Each set consisted of 57 acrocentric autosomes and a pair of X chromosomes corresponding to the karyotype 59 XX which is deficient in one autosome.

There were four chromosome sets in the slide prepared from Embryo R7. However, one of these was partly obscured by a cell and it was impossible to count the chromosomes in another since they overlapped themselves. The remaining two chromosome sets were each evenly spread over a small area of the slide and each contained 57 acrocentric autosomes and two X chromosomes. There were no loose chromosomes on this slide and the embryo was classified as being monocentric for one of the autosomes.

Photographs were taken of each unbalanced set of chromosomes identified in the three aneuploid embryos and the chromosomes were cut out individually. Unfortunately, the chromosome sets were highly contracted which prevented arrangement of the autosomes in distinct pairs according to decreasing size. Consequently, it was not possible to identify the unbalanced chromosome in these karyotypes (see Figure 15).

TABLE 25

Details of the 3 embryos with an unbalanced chromosome complement

Identification	Stage of Development	Morphology	Number of cells	Number of Diploid Chromosome Sets	Karyotype
R3	blastocyst	normal	130	7	60,XY,t
R10	compact morula	normal	110	4	59,XX
R7	morula	normal	35	4	59,XX

The sire of each embryo was heterozygous for the 1/29 Robertsonian translocation and identified as animal C in Table 11.

A single polyploid chromosome spread was identified in the slides prepared from five embryos described in Table 26. However, although each spread appeared to be tetraploid, it was not possible to count the exact number of chromosomes per set due to either overspreading or overlapping. In slides prepared from embryos 138 R5 and 166 L4, diploid chromosome spreads with the normal 60 XY karyotype were also present. However, whereas diploid chromosome spreads were seen in embryos 41 L1 and 164 L3, it was not possible to determine the karyotype due to either overspreading or overlapping. No diploid chromosome spread was found in the slide prepared from embryo 62 R2. However, in this embryo, four distinct X chromosomes were seen in the polyploid spread.

Of the 102 embryos classified as normal at the time of recovery 57 (55.9%) were successfully karyotyped, whereas only ten (25.6%) of those considered to be in the process of degeneration and none of those classified as degenerate were karyotyped. These figures are shown in Table 27 which gives the reasons which prevented cytogenetic analysis of the remaining embryos.

TABLE 26

Details of the 5 embryos containing a polyploid cell

Identification	Sire	Stage of Development	Morphology	Number of Cells	Number of Diploid Chromosome Sets	Karyotype
138 R5	C.	early blastocyst	normal	105	11	60 XY
166 L4	W.S.	blastocyst	normal	100	6	60 XY
41 L1	Proband	compact morula	normal	52	3	unknown
164 L3	Proband	hatched blastocyst	normal	175	5	unknown
62 R2	C.	early blastocyst	IPD	106	0	unknown

IPD In the process of degeneration

TABLE 27

The results of cytogenetic analysis of slides prepared from 160 embryos of different morphology

Morphology	No mitotic diploid cells	Chromosomes overspread	Chromosomes overlapping	Successfully karotyped	Total
Normal	3 (2.9)	20 (19.6)	22 (21.6)	57 (55.9)	102+ (100.3)
In the process of degeneration	12 (30.8)	7 (17.9)	10 (25.6)	10 (25.6)	39 (97.9)
Degenerate	19 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	19 (100.0)
Overall	34	27	32	67	160

+ a further three embryos were lost prior to preparing slides

( ) percent.

4.4 Discussion

The results of the PSP Dye test which showed that dye appeared in the urine within 15-20 minutes of instillation in the uterine horn of animals considered to have a patent uterine tube is consistent with those of a previous study (Kelly et al, 1981). However, the animals in that study were pretreated with the spasmolytic hyoscine (Buscopan Compositum, Boehringer-Ingelheim) and, therefore, although this drug causes relaxation of the uterine muscle (Product Data Sheet), it does not appear to have any measurable effect on the rate at which dye passes through the uterine tube.

The increased production of urine in animals sedated with xylazine (Rompun, Bayer) in the present study is of particular interest since this observation is neither mentioned in previous studies using the PSP dye test nor in the product data sheet. Nevertheless, xylazine can have hypertensive side effects (Product Data Sheet) and the subsequent increase in renal blood supply was evidently sufficient to cause an increase in the urinary output of the animals treated in the present study. Consequently, acetylpromazine (ACP, C-Vet), which is not hypertensive and which caused no apparent increase in urine production (which, because of its dilution effect, caused difficulty in recognising the presence of dye), appears to be better suited for use in the PSP dye test when sedation is required.

Since each animal in which the PSP dye test was abandoned after repeated unsuccessful attempts to pass the introducer through the cervix was examined during the earlier part of the

study, failure to penetrate the cervix was probably a consequence of inexperience, especially since the two occasions in which the introducer accidently penetrated the uterine wall also occurred early in the study. However, these animals were only 2 1/2 - 3 1/2 years old so it may be that the lumen of their cervix was insufficient to accommodate the introducer. Nevertheless, the results show that the cervix in other animals of the same age was successfully penetrated later in the study when the technique was more familiar which indicates that the introducer was not too large for use in heifers and supports the suggestion that the problem was the result of inexperience.

Although it is possible that the PSP dye test may result in misdiagnosis if dye refluxes from the uterine horn into the vagina to contaminate urine or if the uterine mucosa is accidently damaged resulting in the absorption of dye by the submucosal vessels, attempts were made to avoid these situations by leaving the inflated catheter cuff in position until the test was completed and by careful manipulation of the uterus at all times. Nevertheless, despite the presence of copora lutea, no embryos were recovered in this study from 8 uterine horns which were classified as patent using the PSP dye test and, although it is conceivable that embryos in these horns were lost during the recovery procedure, the dye test suffers from the limitation that it indicates whether the uterine tube is patent only to fluid and not necessarily to embryos. Consequently, it may be that the uterine tube lumen of these animals was sufficiently narrow to block the transport of embryos but permit the passage of dye and,

therefore, a more accurate procedure to determine uterine tube patency, in animals to be used for embryo recovery might be the particle test which detects whether starch or pollen grains pass through the uterine tube (Kessy and Noakes, 1979[a]). Support for the use of this procedure comes from the results of a study (Kelly et al, 1981) which showed that a proportion of uterine tubes (11%) collected at an abattoir allowed the flow of PSP dye but not the passage of pollen grains.

Since no history was available for the cows used in this study, it is not known why they were culled from the national herd. However the results of the PSP dye test show that at least one uterine tube was blocked in 24.7% of the animals examined and, since a patent uterine tube is necessary in order for ova and spermatozoa to meet and for the fertilised ova to pass on into the uterus and establish pregnancy (Hunter, 1977), it is reasonable to assume that a large proportion of the animals were culled due to the problem of repeat breeding. Furthermore, this assumption is supported by the result of another study (Kessy and Noakes, 1979[b]) in which five repeat breeders were examined using the starch grain and PSP dye tests and each was found to have at least one blocked uterine tube.

Although the paralumbar approach provided good access for the laparoscopic examination of most ovaries in this study, there were two animals in which the ovaries were not visible due to peritoneal fat suspended in the ovarian ligament and, since this problem was also encountered by other research workers using the same approach (Sirard, Lambert, Beland and Bernard, 1985) it may

be that a different approach, such as the midventral one which allows any fat to fall away from the ovaries (Holland, Bindon, Piper, Thimonier, Cornish and Radford, 1981) , would have been more successful in these animals. However, the midventral approach requires general anaesthesia which was not considered to be justifiable in the present study.

A comparison of the results obtained by laparoscopy and rectal palpation shows that the number of corpora lutea counted by both methods was similar when the superovulation response was small. However, when the response was high, the number of corpora lutea estimated by rectal palpation was frequently less than that observed during laparoscopy, especially if the corpora lutea were situated so close to each other that they were difficult to distinguish individually per rectum. This observation was also made by other research workers (e.g. Newcomb, Christie and Rowson, 1978) and tends to suggest that laparoscopic examination of the ovaries is a more accurate method of determining the superovulation response. This is further supported by results of the present study which showed that luteinised follicles were occasionally wrongly classified as corpora lutea during rectal palpation and by the results of another study (Guay and Bedoya, 1981) which showed that, other than histological examination, direct observation was the most accurate method of identifying ovarian structures. Consequently, it is important to be aware of the limitations of rectal palpation when using this method to evaluate the superovulation response.

The finding of a corpus luteum with two pinpoint depressions on its surface during laparoscopic examination is particularly interesting since it is generally accepted that the corpus luteum in cattle has only one, if any, depression (Arthur, 1975). Although the depression is less distinct than the ovulatory fossa produced in the horse, the depression in cattle is also believed to represent a point of ovulation (Arthur, 1975) and it is possible, therefore, that the corpus luteum identified in the present study with two depressions was produced by the rupture of two follicles which were situated so close together that the developing corpora lutea conjoined to form one structure. However, such a situation would be a coincidence and unlikely to occur in cattle which are not stimulated to superovulate since normally only one or two follicles rupture during oestrus (Arthur, 1975).

Although knowledge of the endochrinology of superovulation is fragmented and disputed, there is general agreement (e.g. Booth, Newcomb, Strange, Rowson and Sacher, 1975) that PMSG, and other drugs used to induce superovulation, have an effect similar to Follicle Stimulating Hormone (FSH) which is reflected by a prodigious increase in oestrogen level following treatment. Prior to commercially available prostaglandins, PMSG was administered when animals move undergoing natural luteolysis to coincide with the fall in progesterone level and subsequent luteinising hormone surge leading to ovulation (Gordon, 1975). However, since improved ovulation rates were obtained by administering PMSG or FSH during mid-cycle followed 48 hours later by prostaglandin to induce luteolysis (Booth et al, 1975),

this regime is now generally followed. Nevertheless, as demonstrated in the present study, there is considerable variation in the superovulation response. Sreenan and Gosling (1977) suggested that the variation depends on the type of follicle population in the ovaries at the time of gonadotrophin injection. However, it was not until 1983 that Monniaux, Chupin and Saumande produced experimental evidence of this, which was further supported by the results of Moor, Kruip and Green (1984). Monnieux and co-workers demonstrated that normal developing follicles greater than 1.7 mm diameter at the time of PMSG injection have their growth rate stimulated and mostly ovulate; whereas early atretic follicles of the same size also grow but mostly become luteinised. If these observations are applied to the results obtained in the present study, then those animals which produced a large number of corpora lutea probably contained a large proportion of developing follicles at the time of treatment; whereas those which did not superovulate probably contained no follicles at a suitable stage of development. Nevertheless, the situation appears to be complex and there are various parameters which might influence the response to superovulation. These include the time of year, the breed and age of animals treated, the day of the oestrus cycle on which treatment is given, the batch, dose and type of drug used and the interval between successive treatments.

In the present study, embryos were recovered at all times of the year. However, an insufficient number of animals were superovulated to determine whether the season influenced the

response. Nevertheless, Church and Shea (1976) reported that fewer ovulations occurred in animals treated during autumn and this was also reported recently by Hasler, McCauley, Schermerhorn and Foote (1983). However, other research workers (Critser, Rowe, Del Campo and Ginther, 1980; Massey and Oden, 1984; Shea, Janzen and McDermand, 1984) found no seasonal effect on the response to superovulation. The effect of the time of year is, therefore, unclear and may be influenced by several interrelated factors such as sunshine, rainfall and temperature in addition to the nutritional status of the animal.

The results in the present study indicate that, after superovulation, there was no statistically significant difference between the mean number of corpora lutea palpated in the Friesian and Ayrshire breeds of animal. However, there were significant differences ($P < 0.03$) between the group of beef animals and the dairy breeds. It is not possible to draw a general conclusion from this, however, since the number of beef animals used in the study was low (5) and involved different breeds. However, a significant variation between breeds was reported by other research workers using a large number of animals (Shea, Hines, Lightfoot, Ollis and Olson, 1976; Donaldson, 1984[c]) although various drugs were used to induce superovulation in these studies and there was no agreement on which breed responded best.

Although the number of corpora lutea produced by animals of different age groups in this study were not significantly different, there was a tendency for fewer corpora lutea to be produced in the older animals which is consistent with the results of other research workers (Ozil, Heyman and Cassou, 1980;

Donaldson, 1984[b]; Hasler et al, 1983). Increasing the dose of PMSG given to one aged animal (number 27) in this study from 2000 IU to 3000 IU did not improve the superovulation response which tends to suggest that failure to respond was not a consequence of insufficient PMSG, especially since adequate superovulatory responses were produced in the majority of animals in this study using 2000 IU PMSG. However, the results of research by Monnieux et al (1983) clearly demonstrate that the number of corpora lutea produced after treatment with PMSG is limited by the proportion of developing follicles greater than 1.7 mm in diameter at the time of treatment and, therefore the poorer response obtained in older animals tends to suggest that fewer developing follicles were present. This is further supported by histological evidence which shows that the total number of follicles declines as animals become older (Ericksson, 1966).

Unfortunately, statistical analysis of the effect of the day of the oestrus cycle on which PMSG was administered in the present study was biased since all but one of the beef animals (which produced significantly fewer corpora lutea than animals from other breeds) were treated on the same day (Day 10). Furthermore, only four animals were treated on day 13 and, although two did not respond, a larger number of animals would need to be studied before attaching any significance to this result. Nevertheless, the mean number of corpora lutea produced by treatment with PMSG on any particular day between days 8 and 14 was not significantly different from the overall mean and

this finding is consistent with the results of other research workers (Hasler et al, 1983; Donaldson, 1984[a]) who analysed data from hundreds of animals. However, if animals are treated during either the early or late stages of the oestrus cycle, then significantly fewer corpora lutea are produced due to the smaller number of developing follicles present in ovaries during these stages of the oestrus cycle (Monniaux et al, 1983).

Research by Jainudeen, Hafez, Collnich and Moustafa (1966) demonstrated that, in cattle, antibodies form against exogenous gonadotrophins which might make the ovaries refractory to subsequent injections of PMSG and further supportive evidence was obtained by other research workers (Saumande and Chupin, 1977) who demonstrated that repeated superovulation of animals resulted in decreased ovarian responses. However, in later studies in which cows were allowed at least one natural oestrus between treatments, there was no significant reduction in the number of corpora lutea (Christie, Newcomb and Rowson, 1979; Nelson, Seidel and Elsden, 1979; Lubbadeh, Graves and Spahr, 1980), although Newcomb, Christie, Rowson, Walters and Bousfield (1979) recorded a slight decline. In the present study, 19 animals were superovulated on between two and four occasions and the results indicate that there was no significant difference between the mean number of corpora lutea palpated in animals after single and repeated treatments with PMSG. This tends to suggest that any antibodies which formed against PMSG in these animals were not effective after the interval of at least six weeks between successive treatments.

In 1976, Newcomb and Rowson suggested that varying FSH : LH ratios between different batches of PMSG may affect the superovulation response. However, Stewart, Allen and Moor (1976) found no statistically significant difference in FSH : LH ratio among several batches from one manufacturer and there was no difference in the mean ovulation rates of groups of animals treated with these batches. Comparison between the number of corpora lutea produced using two different batches of PMSG in the present study showed no statistically significant difference ($P > 0.05$) and, therefore, it is unlikely that different batches used during this study affected the response to superovulation.

In the present study, 2 ml of diluent were used to dissolve 2000 IU of PMSG. If a fraction of the solution remained in the syringe after injection then animals could have received several hundred units of PMSG less than calculated. Although care was taken to ensure that each animal received the correct dose, it is possible that a few animals did not and may have, consequently, produced fewer ovulations.

The incorrect storage of gonadotrophins has been shown to reduce the response to superovulation (Wollen, Schultz and Newkirk, 1985). However, the drugs used in the present study were stored according to the manufacturer's instructions and, therefore, unlikely to have contributed towards the variation in superovulatory response.

Lastly, although only one product was used in the present study, several different types of drug can be used to induce superovulation and there is increasing evidence that higher responses are obtained using daily injections of FSH instead of a

single injection of PMSG (Elsden, Nelson and Seidel, 1978) and that Human Menopausal Gonadotrophin (HMG) may produce even better responses (Lauria, Oliva, Genazzani, Cremonesi, Crotti and Barbetti, 1982). However, each of these drugs suffers from the limitation that the superovulation response varies unpredictably and to date there is no reliable product which guarantees a standard level of superovulation.

Research showed that approximately 7% of ova are still in the uterine tubes of superovulated cattle between six and eight days after oestrus (Newcomb, Rowson and Trounson, 1976). Consequently, using the technique employed in the present study, these ova would not be available for recovery. However, this does not account for the 60.8% recovery rate obtained in this study or the similar rates of 59-67% and 53.1% reported by other research workers (Shea et al, 1976; Newcomb et al, 1978). Since the uterine tube patency of animals used by these groups of research workers was not determined, it is possible that a proportion of uterine tubes were blocked which would prevent the recovery of embryos. However, in the present study, it is unlikely that the recovery rate was affected by blocked uterine tubes since bilateral tube patency was already demonstrated in 26 of the animals used and, in each of the remaining animals, eggs were recovered from all horns with a superovulated ovary.

In most cases disparity between the number of eggs recovered and the number of corpora lutea counted was probably the result of technical difficulties which were encountered during the recovery procedure. For example, if the catheter cuff was under-

inflated then the lumen of the uterine horn was not occluded and flushing fluid, possibly containing suspended embryos, leaked past and was not recovered by the catheter. To prevent this, it was necessary to ensure that the cuff was sufficiently inflated to occlude the lumen. However, the degree of cuff inflation was often difficult to determine by rectal palpation, especially at the beginning of the study. If the cuff was accidentally over-inflated then, despite the use of air which is more compressible than fluid, this resulted in damage to the uterine mucosa and the subsequent recovery of mucosal debris and erythrocytes in the flushing fluid. Embryos were usually obscured by the mucosal debris and, although the recovered fluid was carefully and repeatedly examined, it is possible that some embryos were not isolated from the debris. It is also possible that, at the beginning of the study, embryos were lost in mucosal debris caused by the catheter folding back on itself when being advanced along the uterine horn. However, this source of embryo loss was subsequently successfully prevented by refrigerating the catheter immediately prior to use since this provided it with a degree of rigidity sufficient to prevent kinking.

A further source of embryo loss may be due to the elevated oestrogen levels which occur in superovulated cattle (Booth et al, 1975). Harper and Chang (1971) demonstrated that, in several species, high oestrogen levels increase the rate at which ova are transported through the reproductive tract and, therefore, it is possible that a proportion of ova in the present study were situated posterior to the catheter cuff and,

consequently, not available for recovery. The relatively low fertilisation rate (81.1%) obtained in this and other studies involving superovulated cattle, for example 78% (Shea et al, 1976) and 61% (Hasler et al, 1983) is further evidence that the rate of ova transport is increased since, under normal circumstances, the ova are held in the uterine tubes until fertilisation is completed (Hunter, 1977). However, since hormone concentrations were not measured in this study, it is not possible to determine the relationship between the oestrogen levels of individual animals and the recovery rate.

Beaumont and Smith (1975) demonstrated that, in mice, some ova are trapped in apparently normal corpora lutea after superovulation and, although, to date, there is no report of this in cattle ovaries it is a possible cause of disparity between the numbers of corpora lutea counted and eggs recovered. Another possible cause may be due to the considerably large ovaries which develop after superovulation and which may interfere with the recovery of ova by the fimbrae, particularly if the response is high. However, in order to demonstrate the efficacy of fimbrae in collecting ova from superovulated ovaries, it would be necessary to carry out ovaroscopy at the time of ovulation and, to date, this has not been done. Nevertheless, in the present study, 24 ova were recovered from one animal in which 24 corpora lutea were observed by laparoscopy which tends to suggest that, in this animal at least, the fimbrae were capable of gathering all ova produced by a large superovulation response.

Whereas 105 (64.4%) of the embryos recovered in the present study were classified as being normal, 58 (34.5%) were considered to be either in the process of degeneration or degenerate which is slightly higher than the figure obtained in a large study involving 783 embryos of which 25.4% were considered to have poor viability (Lindner and Wright, 1983). However, although it is possible that some normal embryos were wrongly classified in the present study as being in the process of degeneration since it was frequently difficult to differentiate between normal developing blastomeres and loose degenerate cells in the early blastocysts, the animals used by Lindner and Wright (1983) were all healthy, highly productive cattle belonging to commercial herds, whereas in the present study all animals were cull-cows. Furthermore, recent research in Sweden demonstrated that the proportion of degenerating embryos recovered from repeat breeder cows is higher than that of normal animals (Gustafsson, 1985), and since infertility is the most common reason for culling (Esslemont, Baillie and Cooper, 1985), the higher proportion of degenerating embryos recovered in the present study was probably caused by the presence of repeat breeders among the animals used.

The cause of the higher incidence of embryo degeneration in repeat breeders is unknown. However, Gustafsson (1985) suggested that it may be due to the effect of an inapparent infection such as bovine viral diarrhoea which is known to adversely affect embryo viability (Grahn, Fanning and Zemjanis, 1984). Nevertheless, it may also be due to other factors such as genetic immunological and hormonal irregularities which could affect both repeat breeders and apparently normal animals. For example, a

genetic complex is identified in mice which, if present, results in poor development of the embryo (Warner, Gollnick and Goldbard, 1984). The genes involved are located on the major histocompatibility complex (MHC) and are thought to act by interfering with the immunological response between embryo and uterus (Warner et al, 1984). Furthermore, since all animals possess MHC complexes (Gotze, 1977) it is possible that similar genes are present in cattle chromosomes.

In sheep, there is evidence that oocytes matured under improper endochrine conditions are liable to undergo fertilisation and early cleavage but that some subsequently degenerate (Moor and Trounson, 1977) and consequently, another possible cause of degeneration in embryos recovered from superovulated animals is the adverse environment created by the elevated oestrogen and progesterone levels which occur after superovulation (Booth et al, 1975).

Research by Takeda and Hasler (1986) demonstrated that medium transferred in plastic disposable syringes may be harmful to the normal development of mouse embryos and other research workers (Bondioli and Hill, 1986) demonstrated that medium stored in a specific make of syringe (Monojet, Sherwood Medical) adversely affected the ability of bovine embryos to develop in culture. However, although this type of syringe was not used in the present study, it is possible that other makes may adversely effect embryo viability. Nevertheless, any adverse effect was not major since the majority of embryos showed no sign of degeneration.

Toxic residues, present for 36 hours after ethylene oxide sterilisation, are also reported to reduce embryo viability (Schiewe, Schmidt, Bush and Wildt, 1984). However, plasticware sterilised by ethylene oxide in the present study was allowed to aerate for at least 72 hours before use and, therefore, it is unlikely to have contained residues toxic to embryos.

A recent cytogenetic study (Linares, King and Gustavsson, 1980) showed that a higher proportion of embryos which were either degenerate or in the process of degeneration was recovered from animals inseminated by bulls heterozygous for the 1/29 Robertsonian translocation than from animals inseminated by normal bulls. This may have been due to the presence of non-viable aneuploid embryos produced by carriers of the translocation. However, in the present study, there was no significant difference between the number of morphologically abnormal embryos produced by the carrier and normal bulls. Nevertheless, it was often difficult to differentiate between the loose blastomeres of degenerating embryos and cells which were differentiating normally into the trophectoderm and inner cell-mass and, consequently, it is possible that some degenerating embryos produced by carrier animals were wrongly identified as being normal and that some of the normal embryos produced by the normal bulls were wrongly classified as being in the process of degeneration. However, although this possibility is supported by other research (Shea, 1981) which found that microscopic evaluation of embryo viability was highly subjective, the anticipated incidence of aneuploid embryos produced by carrier animals is small [less than 7% (Gustavsson, 1969)] and therefore

it would be necessary to examine several hundred embryos from both carrier and normal animals before any difference in the proportion of degenerate embryos could reasonably be attributed to the death of non-viable aneuploid embryos.

Linares and King (1980) demonstrated that embryo viability is dependant on the integrity of the zona pellucida and, although there was no evidence of reduced viability in the two embryos recovered with an irregularly shaped zona pellucida in the present study, no mitotic cell was present in any of the six embryos recovered with a broken zona pellucida which tends to suggest that these embryos were no longer viable. However, since these embryos were also at the expected stage of development, it is reasonable to assume that the cessation in cell proliferation occurred recently and it may be that the broken zona pellucidae were the result of damage caused to normal embryos during the recovery procedure. This is supported by the finding of nine fragmented pieces of zona pellucidae in flushing fluid recovered seven days after oestrus which, since this is earlier than embryos hatch under normal circumstances (Lindner and Wright, 1983), probably represent the remains of embryos which were accidentally destroyed during manipulation.

In the present study, karyotypes were successfully prepared from 41.1% of the embryos recovered which compares favourably with the results of other research workers who karyotyped 23.7% of sheep embryos (Long and Williams, 1980) and 42.9% and 47.4% of cattle embryos (Popescu, 1980; King et al, 1981, respectively). However, whereas an average of between 4.2 and 5.0 mitotic

spreads were available for cytogenetic analysis from embryos in the compact morula and blastocyst stages of development, mitotic spreads were rarely seen in the morulae and although this was probably partly due to the relatively low number of cells present in normal embryos at this stage of development it also reflects the higher proportion of degenerate embryos belonging to this group.

In addition to recognising diploid and aneuploid embryos in this study, five embryos were identified with a tetraploid cell. However, whereas it was possible to clearly demonstrate that four of these embryos were mixoploid since diploid spreads were also present, this was not possible in the remaining embryo since the only mitotic spread was tetraploid. If the embryo was truly tetraploid then it may have been produced by the simultaneous fertilisation of an ovum by three spermatozoa, although in this situation each would have to bear the X chromosome since four of these were identified in the tetraploid cell. Alternatively, a tetraploid embryo may arise if both the ovum and spermatozoon had an unbalanced chromosome number produced by restitution in either the first or second meiotic division. However, it is highly unlikely that a rare unreduced spermatozoon would by chance fertilise an equally rare diploid ovum. The reuniting of two polar bodies with an ovum nucleus to form a triploid egg might also result in a tetraploid embryo. However, tetraploidy causes a considerable increase in the size of the nucleus (Therman, 1951) and since this was not observed among the cells belonging to the embryo with the lone tetraploid spread, it is highly probable that these were diploid and that the embryo was,

therefore, mixoploid.

The tetraploid cells in the mixoploid embryos were unlikely to be a consequence of superovulation since, although there is evidence of an increase in the incidence of certain chromosome aberrations in mouse (Takagi and Sasaki, 1976) and rabbit embryos (Fujimoto, Pahlavan and Dukelow, 1974), these did not include polyploidy. Furthermore, polyploid cells were recognised among cells belong to embryos recovered from pigs under conditions of natural ovulation (Long and Williams, 1982). However, it may be that the tetraploid cells were formed by the fusion of two diploid cells since this is supported by the finding of binucleate cells in embryos from cattle (Staples, McEntee and Hansel, 1961) and other species including the pig (Long and Williams, 1982), sheep (Boshier, 1969) and mouse (Barlow and Sherman, 1972). In addition, aberrations in cell division such as endomitotsis whereby the duplicate chromosomes do not separate into two daughter cells but remain in one nucleus instead (Geitler, 1939), or endoreduplication, in which the chromosomes replicate twice instead of once between two mitoses (Levan and Hauschka, 1953), also cause polyploidy. Nevertheless, regardless of the process by which the tetraploid cells were formed, these cells do not appear to adversely affect the embryo's viability since successful pregnancies were obtained from embryos in which biopsied fragments showed that 25% of the cells were polyploid (Hare et al, 1980).

Although, to date, the function of the polyploid cells is unknown, these cells predominate in highly differentiated regions

of the plant root tip (Therman, 1951) and, therefore, it may be that polyploidy in the embryo is, in some way, also associated with cell differentiation. Support for this idea is provided by the observation that the incidence of polyploid cells rapidly increases during the period when embryonic cells differentiate into the inner cell-mass and trophectoderm (Hare et al, 1980; Long and Williams, 1982). Furthermore, since the vast majority of these cells occur in the trophectoderm (Long and Williams, 1982) it may be that they are specifically associated with structures which develop from trophoblasts and this is supported by the finding of polyploid cells in placental tissue (Nagl, 1978). Nevertheless, embryonic cytogenetics is still in its infancy and further research is required in order to fully understand the true role of polyploid embryonic cells.

If an individual chromosome becomes accidentally separated from a complete set during fixation onto the slide then that cell would appear deficient in one chromosome and, in this way, embryos could be wrongly classified as being monosomic. However, two monosomic sets of chromosomes were present in each of the two embryos considered to be monosomic in the present study. Furthermore, each of these sets was spread evenly on the slide and loose chromosomes were absent from the surrounding area. Consequently, it is unlikely that these embryos were wrongly classified. It is also unlikely that the trisomic embryo was the result of a technical artifact since it contained four sets of chromosomes which were evenly spread and each of these contained an extra chromosome.

Each aneuploid embryo was sired by a bull heterozygous for

the 1/29 Robertsonian translocation and, since no abnormal karyotype was recognised among the embryos sired by normal bulls, this tends to suggest that the unbalanced chromosome complement was associated with the presence of the translocation. This is further supported by the results of other studies which identified aneuploid embryos among only those produced by carriers of the 1/29 Robertsonian translocation and not among those recovered from normal animals (Popescu, 1980; King, Linares and Gustavsson, 1981). However, several research workers (e.g. Logue and Harvey, 1978[a]; Popescu, 1978) demonstrated that unbalanced secondary spermatocytes are produced by animals heterozygous for the 1/29 Robertsonian translocation and, since the karyotype of all the dams used in the present study was normal, the monosomic embryos were probably the product of fertilisation between normal haploid ova and hypomodal spermatozoa produced by the carrier bulls, whereas the trisomic embryo probably resulted from the fertilisation of a normal ova by a hypermodal spermatozoon. This is further supported by the fact that the finding of two monosomic and one trisomic embryo is consistent with the anticipated 1 : 1 ratio of hypomodal and hypermodal spermatozoa produced by carrier bulls (Logue and Harvey, 1978[a]).

Previous research showed that the production of aneuploid embryos by animals heterozygous for the 1/29 Robertsonian translocation is not specific to any particular animal or breed since aneuploid embryos are produced by several animals of different breeds including the Swedish Red and White (King et al,

1981) and an unidentified French breed (Popescu, 1980) and, furthermore, reduced fertility caused by the death of unbalanced embryos was clearly demonstrated in large populations of Swedish Red and White (Gustavsson, 1969) and Norwegian Red (Refsdal, 1976) animals. Consequently, it is unfortunate that none of the aneuploid embryos identified in this study were produced by the British Friesian bull heterozygous for the 1/29 Robertsonian translocation. Nevertheless, the overall incidence of aneuploidy among the 42 embryos sired in this study by bulls heterozygous for the translocation was 7.1% and, since only 14 embryos sired by the proband contained chromosome spreads suitable for analysis, the probability that any of them was aneuploid is only 0.0233. Consequently, more embryos would have to be examined from this animal in order to have a reasonable chance of identifying aneuploidy and, unfortunately, this was not possible in the present study since only a small amount of semen from the proband was available for insemination.

Although individual cattle chromosomes cannot be identified in conventional giemsa stained mitotic preparations (Gustavsson, 1969), it is occasionally possible to recognise chromosome number 1 in elongated sets where it is conspicuous as the longest autosome (Ford et al, 1980). However, the mitotic preparations obtained from the three aneuploid embryos in the present study were too contracted to enable identification of this chromosome and, consequently, it was not possible to determine whether chromosome number 1 or 29 was unbalanced. Nevertheless, Popescu (1980) considered that the mitotic preparations obtained from the two monosomic embryos sired by a bull heterozygous for the 1/29

Robertsonian translocation were sufficiently elongated to show that one member of the pair identified as number 1 was absent from each and another study (King et al, 1981) demonstrated that the surplus chromosome in two trisomic embryos, also sired by carrier bulls, appeared to be number 1 which tends to suggest that aneuploidy involving chromosome 1 may be more common than that involving chromosome 29. However, this is a tenuous inference based on the examination of relatively few embryos and it may be that one or more of the aneuploid embryos recovered in the present study was unbalanced for chromosome 29. Furthermore, individual chromosomes cannot be identified with certainty by measuring their length alone (Ford et al, 1980) and, therefore, further cytogenetic studies using differential staining techniques such as G-banding are required in order to positively identify the unbalanced chromosomes of aneuploid embryos recovered from animals heterozygous for the 1/29 Robertsonian translocation.

The finding of unbalanced embryos in the present study is evidence that at least some aneuploid embryos produced by animals carrying the 1/29 Robertsonian translocation remain viable during the first week after fertilisation and this is supported by the studies which recovered two trisomic embryos 3 and 7 days after oestrus (King et al, 1981) and two monosomic embryos 13 days after oestrus (Popescu, 1980). Research in the mouse demonstrated that the majority of aneuploid embryos die around the time of implantation or shortly thereafter (Gropp et al, 1974) and this may be the time of death for aneuploid embryos

produced by carriers of the 1/29 Robertsonian translocation in cattle since fertility studies involving these animals showed a marked reduction of between 3% and 7% in the 28 day non-return to service rate (Gustavsson, 1969; Refsdal, 1976; Dyrendahl and Gustavsson, 1979). However, there is also evidence of a reduction in the 56 and 273 day non-return to service rates (Gustavsson, 1969) which tends to suggest that there is a further loss of embryos during this period of gestation. It may be, therefore, that a proportion of the aneuploid embryos are capable of survival longer than others and this possibility is supported by the observation that monosomic mouse-embryos tend to die much earlier than trisomic ones and that embryos trisomic for particular chromosomes may survive until late in gestation (Gropp et al, 1974). Nevertheless, in order to determine the time of death for aneuploid embryos produced by cattle carrying the 1/29 Robertsonian translocation it would be necessary to examine cytological preparations at various stages after fertilisation and this was not attempted during the present study.

The presence of aneuploid embryos among the translocation group is evidence that at least some of the unbalanced spermatozoa produced by bulls heterozygous for the 1/29 Robertsonian translocation (Logue and Harvey, 1978[a]; Popescu, 1978) are capable of fertilisation. However, although this observation is consistent with research involving carrier mice, it contrasts with the results of research in sheep which, in spite of demonstrating the production of unbalanced secondary spermatocytes during meiosis (Chapman and Bruère, 1975), showed no evidence of embryo death since neither the conception rate at

first service nor the lambing percentage was reduced in animals heterozygous for either of three Robertsonian translocations (Bruère, 1974; Bruère, 1975; Bruère and Chapman, 1974). Consequently, Bruère (1975) concluded that the unbalanced gametes were incapable of fertilisation and, therefore, did not produce non-viable aneuploid embryos. This conclusion was supported by the results of another study which showed no evidence of aneuploidy among 16-17 day old embryos recovered from animals carrying one of the translocations (Long, 1977).

Alternatively, however, it may be that unbalanced embryos were produced by the carrier animals studied by Bruere and his co-workers but that the anticipated small reduction in fertility caused by their death was concealed by the influences of other factors such as poor nutrition or infection. Furthermore, it is also possible that any aneuploid embryos may already have died before being recovered by Long (1974) on day 17 and, consequently, cytogenetic analysis of earlier embryos is required in order to determine whether the behaviour of unbalanced gametes produced by non-disjunction of Robertsonian translocations in the sheep differs from that observed in other species including cattle and mice.

In the present study, the overall incidence of unbalanced embryos (7.1%) is consistent with the decreased non-return to service rates (3-7%) reported in animals inseminated by bulls heterozygous for the 1/29 Robertsonian translocation (Gustavsson, 1969; Refsdal, 1976; Dyrendahl and Gustavsson, 1979) and the increased rate of non-disjunction (6%) recorded in carrier bulls

(Logue and Harvey, 1978[a]). Consequently, although the number of embryos examined was relatively small, the results support the suggestion made by Gustavsson (1969) that fertility is reduced in animals inseminated by translocation bulls due to fertilisation of normal ova by unbalanced spermatozoa resulting in the production of aneuploid embryos which subsequently die.

CHAPTER FIVE

THE USE OF LONG-TERM LYMPHOCYTE CULTURE TO DETECT THE *In vitro* FORMATION OF CHROMOSOME ABERRATIONS SUCH AS THE 1/29 ROBERTSONIAN TRANSLOCATION

5.1 INTRODUCTION

In 1954, Chang observed that changes develop in the growth pattern of human cells during prolonged *in vitro* culture and Levan (1956) later demonstrated that chromosome aberrations develop in these transformed cells. Similar observations were also made in cells from other species including the mouse (Hsu, Billen and Levan, 1961), cattle (Nelson-Rees, Kniazeff and Darby, 1964) and sheep (Nelson-Rees, Kniazeff and Darby, 1967) where the chromosome aberrations always involved changes in chromosome number and morphology. For example, Hsu *et al.* (1961) found that the number of chromosomes per cell ranged from 64 to 73 in cells from the mouse and that there was an increase in the number of non-acrocentric chromosomes. However, these studies were made before banding techniques were available to identify individual chromosomes and, therefore, it was not possible to determine whether chromosome aberrations which are found *in vivo* also develop *in vitro*. Nevertheless, Nelson-Rees *et al.* (1964) demonstrated that large submetacentric chromosomes were among the morphological aberrations which develop during the long-term culture of bovine kidney cells and suggested that these may be the same as Robertsonian translocations which occur *in vivo* between two non-homologous acrocentric autosomes.

Now that various banding techniques are available for cytogenetic use, research workers are beginning to karyotype cells during long-term culture although most groups concentrate on neoplastic cells (Nielsen and Granzow, 1983) and, to date, there is no report which describes banded karyotypes prepared from bovine cells during long-term culture. Nevertheless, this

is probably partly due to the excessively long periods of culture which are necessary before chromosome aberrations develop in relatively slow growing cells. For example, abnormal submetacentric chromosomes only developed in the bovine kidney cells used by Nelson-Rees et al.(1964) after at least 20 months of culture.

However, Hulliger, Wilde, Brown and Turner (1964) demonstrated that bovine lymphocytes infected with Theileria proliferate rapidly in vitro and, furthermore, Brown (1985) observed that chromosome aberrations develop in these cells after culturing them for several weeks. Nevertheless, to date, these aberrations have not been identified (Brown, 1985) and, consequently, karyotypes were prepared from infected lymphocytes belonging to cytogenetically normal animals and animals heterozygous for the 1/29 Robertsonian translocation in order to identify the aberrations which develop and investigate whether these are the same as those which occur in vivo and whether certain chromosomes are involved more than others.

5.2 MATERIALS AND METHODS

5.21 Animals used

Leucocytes for long-term culture were obtained from six pedigree cows belonging to the British Friesian herd in South West Scotland described in Section 3.21. Four of these animals were half sisters sired by the proband, of which two were heterozygous for the 1/29 Robertsonian translocation and two had the normal bovine karyotype. The other two animals were unrelated and had the normal bovine karyotype. Details of these

animals are shown in Table 28.

TABLE 28

Details of cows used for long-term lymphocyte culture

Animal Identification Number	Sire	Age (years)	Karyotype
10	Proband	5	59, XX, t (1;29)
18	"	6	"
8	"	7	60, XX
106	"	5	"
1	AHA	12	"
21	CR	10	"

5.22 Isolation of leucocytes

Taking all reasonable aseptic precautions, 10 ml of heparinised blood (Vacutainer, Becton-Dickinson) were mixed with 10 ml of phosphate buffered saline (P.B.S.A., Oxoid), layered on top of 8 ml Ficoll-Paque (Pharmacia Fine Chemicals) in a universal bottle and spun for 30 minutes at 310 x g. The leucocytes suspended in the layers of plasma and Ficoll-Paque were then removed by transferring 5 ml of the fluid into 20 ml P.B.S. and spinning for ten minutes (310 x g). This process was repeated using fresh P.B.S. and the cells were transferred into a tissue culture flask (Nunc) containing 5 ml of culture medium (RPMl 1640) supplemented with 16% Heat Treated Foetal Calf Serum, 30 µl 200 mM L-glutamine, 75 iu/ml penicillin and 75 µg/ml streptomycin (all Flow Laboratories).

5.23 Stimulation of Rapid Cell Proliferation

Each tissue culture flask containing the isolated leucocytes was inoculated with 1 ml of freshly prepared Ground Up Tick Supernate (see Appendix) containing Theileria parva sporozoites, using a fresh sterile pipette for each animal in order to prevent cross-contamination of cells, and incubated at 37°C in an atmosphere of 5% carbon dioxide in air. 5 ml of fresh culture medium was added to each flask after 24 hours incubation.

5.24 Assessment of Cell Proliferation

A stereodissection microscope (Vickers) was used to examine the fluid in each culture flask on alternate days and the cells were classified as growing either singly or in clumps and in suspension or on the substrate. In addition the cell concentration was classified as either sparse, moderate or dense.

If the concentration of cells did not increase between successive examinations, then the percentage of non-viable cells was calculated by adding 0.5 ml of 0.2% trypan blue (B.D.H. Chemicals) to an equal volume of culture medium and counting the proportion of stained dead cells in a haemocytometer slide (Improved Neubauer).

The accurate concentration of cells and the proportion of non-viable cells was also calculated in each animal at the beginning and the end of the study.

5.25 Passage of cells into fresh culture medium

Using a fresh sterile pipette for each animal, 10% of the lymphocytes were transferred in a sterile manner into fresh culture medium whenever the cell concentration became dense. At this time, any cells growing in clumps or on the substrate were

brought into suspension by aspirating the flask contents into a pipette and forcefully expelling the fluid onto the substrate several times. Then, 1 ml of the cell suspension was transferred into a new flask containing 9 ml of fresh culture medium and 8 ml was discarded to leave 1 ml in the old flask. By adding 9 ml of fresh culture medium to the old flask, a duplicate cell culture was obtained for use if the other was accidentally contaminated.

5.26 Cryopreservation of cells

A large number of lymphocytes for cryopreservation was obtained by transferring 2 ml of culture medium containing a dense growth of cells into 18 ml of fresh culture medium and incubating at 37^{o}C for 48 hours. Then the cell culture was centrifuged at 300 x g for five minutes, the supernatant was discarded, the cells were resuspended in 1 ml of ice-cold medium containing 10% dimethyl sulphoxide (DMSO, B.D.H. Chemicals) and transferred into a vial (Cryopreservation Vial, Nunc) which was stored in liquid nitrogen.

5.27 Chromosome analysis

Mitotic cells for chromosome analysis were obtained by transferring 2 ml of culture medium with a dense cell concentration into 9 ml of fresh culture medium, incubating at 37^{o}C for 24 hours, and adding 0.1 ml of 5 µg/ml colcemid solution (Demecolcine, Sigma) 90 minutes prior to the end of the incubation period. Thereafter, the method described in section 3.23 was followed except that 0.75% sodium citrate solution was used for 35 minutes instead of 0.022 Molar potassium chloride.

Conventional giemsa staining

Slides containing fixed mitotic cells were stained with 10% giemsa as described in section 3.23. Then, the chromosome complement was counted in up to 26 evenly spread mitotic sets from each animal and any chromosome aberrations were recorded.

G-banding

The G-banding technique described in section 2.22 (Method 2) was used to differentially stain mitotic spreads on slides prepared from each animal at the end of the study (passage 90). Karyotypes were then prepared from the most clearly stained and evenly spread sets of chromosomes as described in section 2.24.

C-banding

If an aberration involving the centromeric region of a chromosome was recognised among the G-banded karyotypes prepared from any particular animal, then fresh slides containing fixed mitotic spreads from that animal were differentially stained using the C-banding technique described in section 2.23 in order to demonstrate the C-banding pattern of the aberration.

5.3 RESULTS

5.31 Cell proliferation

After 48 hours incubation, the lymphocytes from each animal increased in number to form a dense suspension in each flask and 10% (1 ml) of the cells were transferred into fresh culture medium. Thereafter the cells continued to proliferate and 10% (1 ml) were passaged into fresh medium every 48-72 hours by which time the cell concentration had again become dense and the colour of the medium had changed from red/pink to orange/yellow, indicating a reduction in pH from 7.4 to 6.8.

The only exception to this growth pattern involved animal 1 in which the cell concentration was only moderate 72 hours after the 33rd passage and the culture medium was still pink in colour. There was no visible evidence of microbial contamination and the cell viability was normal (Table 30). However, the haemocytometer slide showed that the cell concentration was approximately one third of normal (Table 29) and, therefore, instead of transferring 1 ml of old culture medium into fresh medium, the correct cell concentration was re-established by transferring 3.3 ml into 6.6 ml. Thereafter, the cell concentration increased as normal in this animal.

TABLE 29

The concentration of cells calculated at passages 5, 33 and 89 during the long-term culture of cells from each animal

Animal Identification Number	Cell Concentration (x 10^6 cells/ml)		
	Passage 5	Passage 33	Passage 89
10	1.3	1.2	1.1
18	1.3	1.3	1.1
8	1.3	1.0	1.0
106	1.3	1.3	1.0
1	1.3	0.4	1.0
21	1.3	1.3	1.1

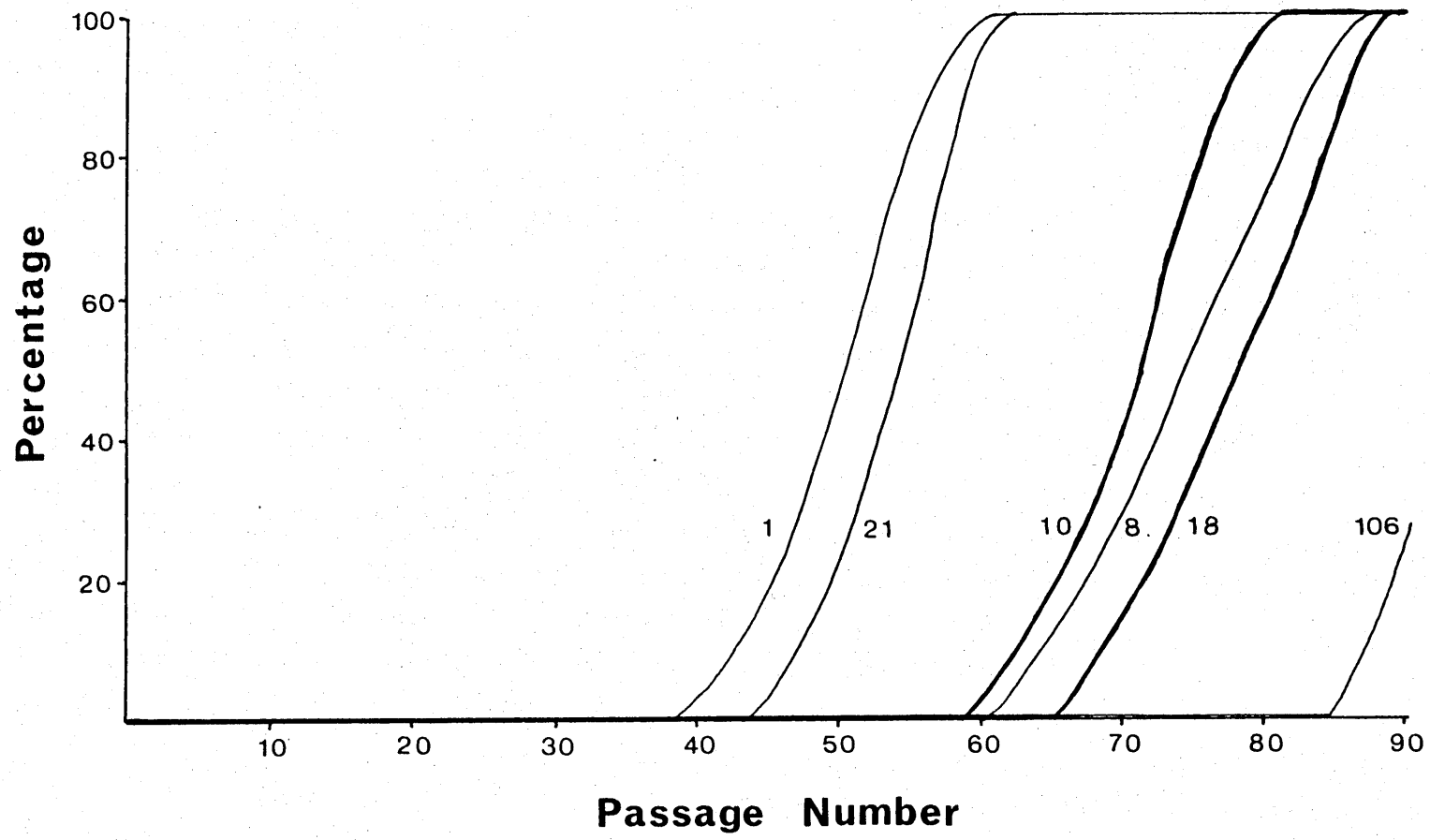

Figure 16. A graph showing the percentage of cells, from each animal, growing in clumps during the period of long-term culture. (Thick lines represent animals heterozygous for the 1/29 Robertsonian translocation)

TABLE 30

The percentage of viable cells present at passages 5, 33 and 89 during the long-term culture of cells from each animal

Animal Identification Number	Viable Cells (%)		
	Passage 5	Passage 33	Passage 89
10	> 99	> 99	93.0
18	"	"	93.4
8	"	"	95.6
106	"	"	95.8
1	"	"	88.5
21	"	"	94.7

Whereas the cells from each animal grew singly in a monolayer loosely attached to the substrate during the first three months of the study, this pattern subsequently changed with an increasing proportion of cells growing in clumps either loosely attached to the substrate or suspended in the culture medium. However, Figure 16 demonstrates that the passage at which cells were first seen in clumps differed in each animal.

Cells from each animal were stored by cryopreservation every 3-6 weeks. However, microbial contamination was seen in only four flasks during the study and, since these were discarded and replaced with the corresponding duplicate flasks which were free from contamination, it was not necessary to revive any of the cryopreserved cells.

5.32 Chromosome analysis of conventional giemsa stained preparations

Mitotic spreads for chromosome analysis were obtained from each animal every two weeks for 15 weeks, by which time chromosome aberrations were recognised in cells from all but one

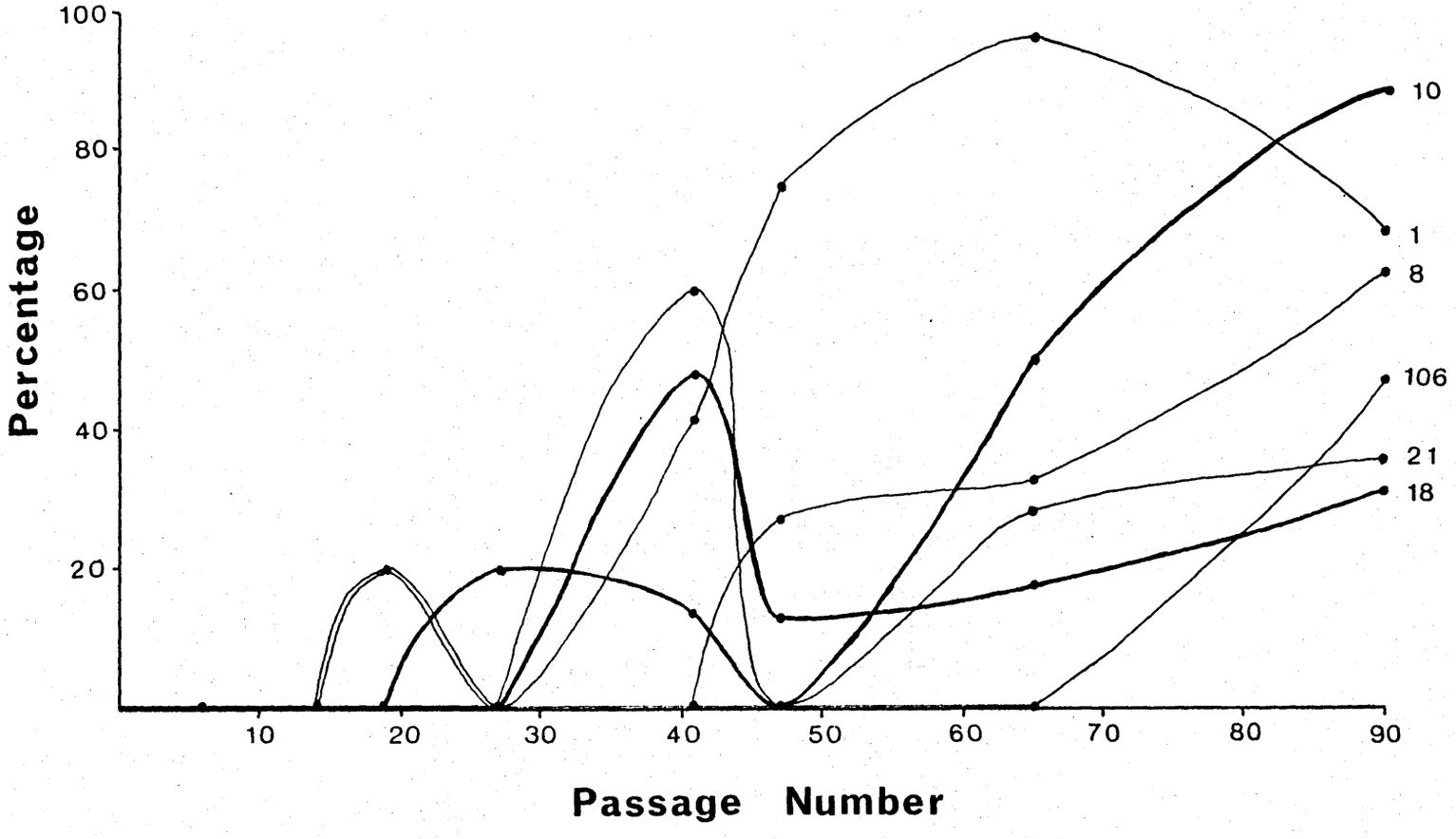

Figure 17. A graph showing the percentage of conventional giemsa stained mitotic spreads, from each animal, which contained chromosome aberrations. (Thick lines represent animals heterozygous for the 1/29 Robertsonian translocation)

animal (106) and, thereafter, every six weeks until aberrations were present in cells from the remaining animal. Consequently, giemsa stained mitotic spreads were analysed during passages 6, 14, 19, 27, 41, 47, 65 and 90.

The karyotype of each mitotic spread was unchanged from the original until Passage 19 when aneuploid and metacentric chromosomes were recognised in 20% of spreads from animals 1 and 8. However, these aberrations were no longer present by Passage 27 although both aneuploid and metacentric chromosomes were recognised at this stage in 20% of spreads from animal 10. Subsequently, aneuploid and metacentric chromosomes developed in cells from every animal although the passage at which they were first recognised varied as shown in Figure 17. Nevertheless, there was no relationship between the onset of chromosome aberrations and the presence of the 1/29 Robertsonian translocation. The proportion of cells with chromosome aberrations tended to increase relatively steadily in each animal during the second half of the study although there was a marked reduction during Passage 47 in animals 10, 18 and 21 as shown in Figure 17.

Although the metacentric chromosomes varied in size, the centromere was always central in position and no submetacentric chromosome was seen except for a pair of X chromosomes which was recognised in every cell and the 1/29 Robertsonian translocation which was present in each cell examined from animals 10 and 18.

Polyploid cells were rarely seen during the earlier passages of the study. Nevertheless, between 2 and 6% of mitotic spreads

examined during the second half of the study were polyploid and, although it was not possible to accurately count the chromosomes due to either overlapping or overspreading, these cells appeared to be near tetraploid.

Each mitotic spread contained between 5 and 10 small dark staining bodies 3-10 μ in diameter which were also present in the cytoplasm of non-dividing cells and represent the nuclei (Koch's bodies) of the Theileria schizonts.

5.33 Analysis of G-banded karyotypes

G-Banded mitotic spreads were obtained from each animal at Passage 90 and, although the bands tended to merge together on the chromosomes belonging to contracted spreads, distinct bands were produced on 24 evenly-spread elongated chromosome sets which were selected at random for karyotyping.

The banding pattern produced on each chromosome pair identified in these karyotypes was found to be the same as that described in section 2.31 with chromosomes 1, 5, 7, 10, 16, 21 and X being particularly easy to identify due to their characteristic banding pattern. However, as in section 2.31, it was not possible to distinguish any difference between the banding patterns of chromosomes 22 and 23, and difficulty was encountered in differentiating between the small chromosomes with similar banding patterns, especially in the more contracted mitotic spreads. The 1/29 Robertsonian translocation was readily identifiable in each karyotype prepared from the carrier animals and had the same pattern as described in section 3.34.1.

Eight of the karyotypes were apparently normal. However, metacentric chromosomes, aneuploidy and structural rearrangements

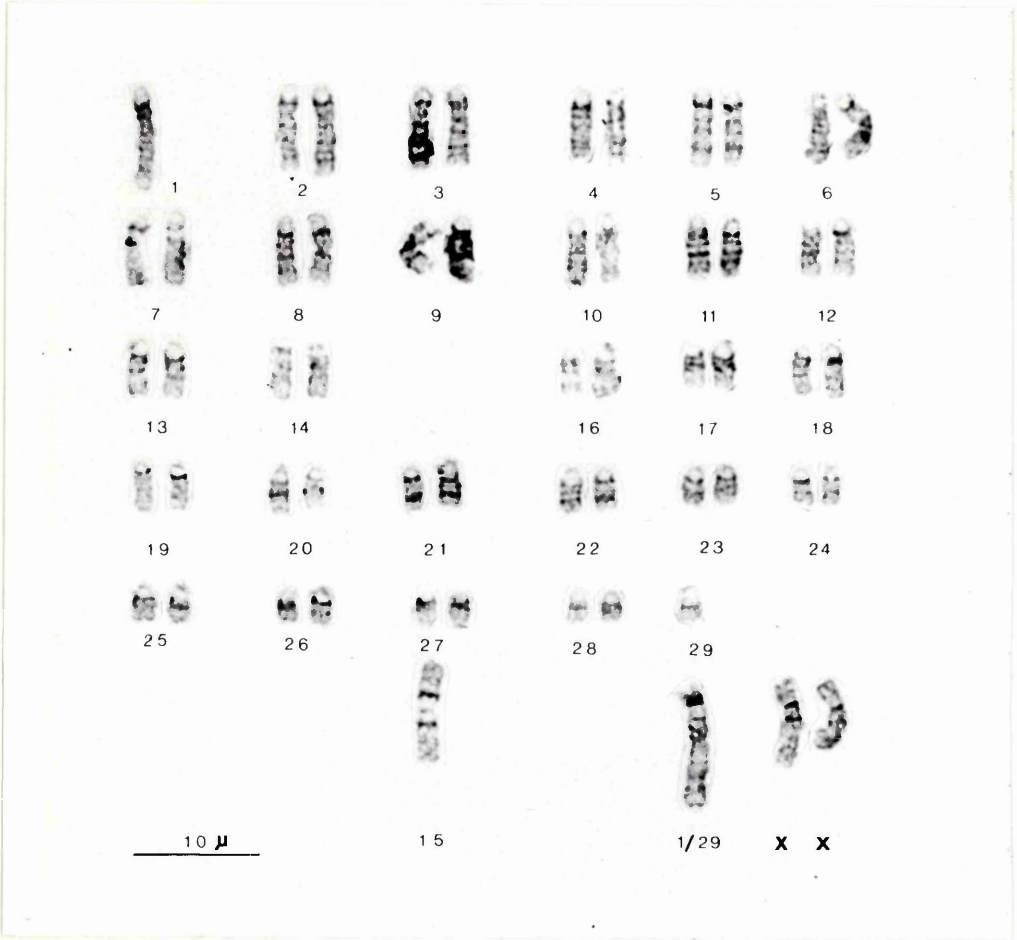

Figure 18

A representative G-banded karyotype containing a dicentric fusion between the chromosome pair number 15

were recognised in the remaining 16 karyotypes of which five contained more than one aberration (Table 31). Seven karyotypes contained one metacentric chromosome and two metacentric chromosomes were identified in another karyotype. However, the same banding pattern was produced on each of these chromosomes and consisted of an unstained double centromere which separated the chromosome into two arms of equal length. These arms each had an identical banding pattern composed of a prominent dark band adjacent to the centromere followed by a narrow light band and two dark bands in the distal half and, since this pattern corresponds to that produced on chromosome number 15 in the normal karyotypes examined in this chapter and sections 2.31 and 3.34.1, the metacentric chromosomes were identified as a dicentric fusion between a homologous pair of chromosomes number 15 (see Figure 18).

Chromosomes were either missing or surplus in ten of the karyotypes described in Table 31. Of these, two were monosomic for chromosome number 28 (since only one member of this pair was present), one was trisomic for chromosome 14 and another was trisomic for chromosome 25 (since each karyotype, respectively, contained three of these chromosomes). One karyotype was tetrasomic for chromosome number 20 since four of these chromosomes were identified, and another four contained six chromosomes corresponding to chromosomes number 22 and 23. However, since there was no appreciable difference between the banding pattern of these particular chromosomes, it was impossible to determine which were polysomic. Similarly, it was

TABLE 31

A list of G-banded karyotypes prepared from 24 mitotic spreads at Passage 90 giving details of the aberrations identified

Karyotype Identification Number	Animal Identification Number	Chromosome Aberration	
		Description	Chromosome Involved
1	10	nil	
2	"	2n + 2	Group 22 - 23
3	"	2n + 2	Group 22 - 23
4	"	2n + 2	Group 22 - 23
5	18	dicentric fusion	15
6	"	nil	
7	"	nil	
8	"	dicentric fusion	15
		trisomy	14
9	"	dicentric fusion	15
		tetrasomy	20
10	"	dicentric fusion	15
		2n - 2	Group 22 - 23
11	8	dicentric fusion	15
		dicentric fusion	15
12	106	nil	
13	"	nil	
14	"	nil	
15	"	nil	
16	1	2n - 2	Group 22 - 23
17	"	monosomy	28
18	"	monosomy	28
19	"	nil	
20	21	dicentric fusion	15
21	"	dicentric fusion	15
22	"	dicentric fusion	15
23	"	inversion	2
24	"	inversion	2
		trisomy	25

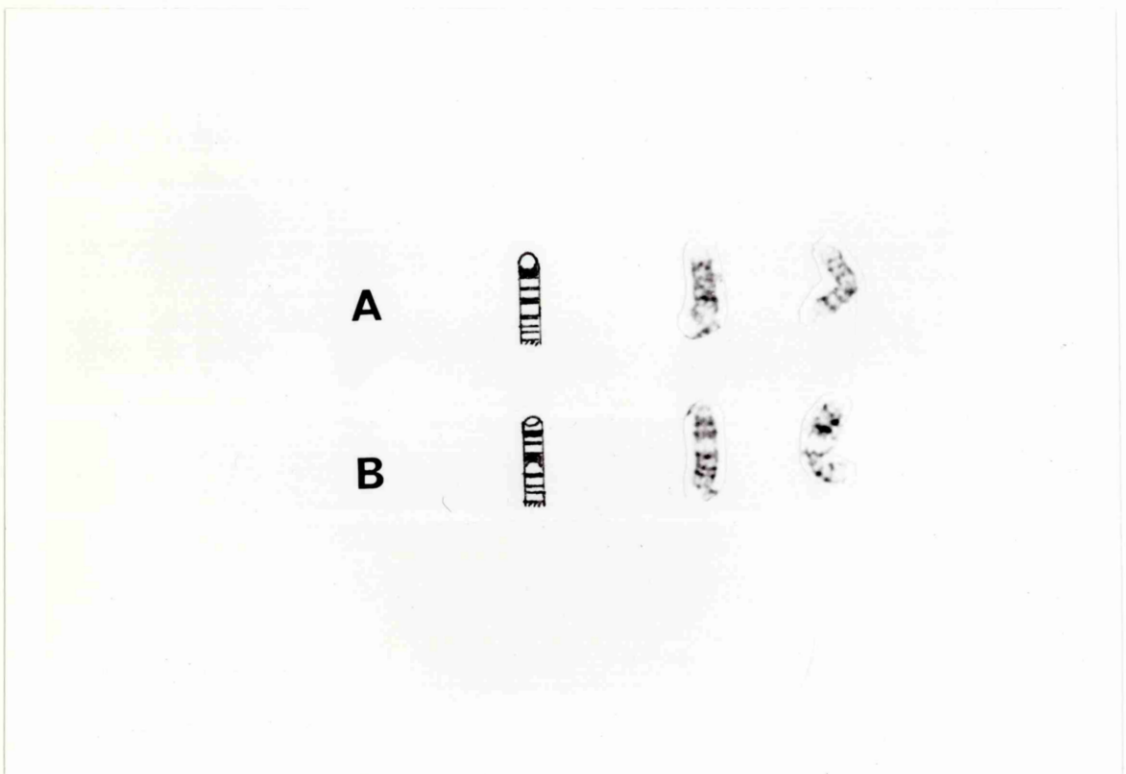

Figure 19

Ideograms illustrating the normal (A) and abnormal (B) G-banding pattern on chromosomes number 2 in karyotypes 23 and 24

not possible to identify the particular chromosomes missing from karyotype number 16 in which only two chromosomes were identified in the group 22-23 and, consequently, this karyotype was classified as being either monosomic for both pairs 22 and 23 or nullisomic for one of these pairs.

Karyotypes number 23 and 24 (Figure 19) each contained one large chromosome with an abnormal banding pattern consisting of a small centromere, 2-3 dark bands in the proximal half, a prominent light central band, three dark bands in the distal half and a light terminal band. This pattern corresponds to that of chromosome number 2 in which a section of the proximal half including part of the centromere is inversed and, therefore, the aberration was classified as an inversion. The banding pattern was normal on the homologous chromosome of this pair (Figure 19).

5.34 C-banding

Fresh slides containing fixed mitotic spreads from animals 18, 8 and 21 were treated to produce C-bands since chromosome aberrations involving the centromeric region were recognised among the G-banded karyotypes prepared from these animals at Passage 90 and, although bands were indistinct in contracted chromosome sets, distinct C-bands were produced on 20 elongated and moderately contracted chromosome sets which were examined from each animal.

Each chromosome set contained a pair of large submetacentric chromosomes which corresponded to the X chromosomes since they were uniformly stained with an occasional slightly dark area in the centromeric region, and a large submetacentric chromosome with a single prominent dark centromeric band corresponding to

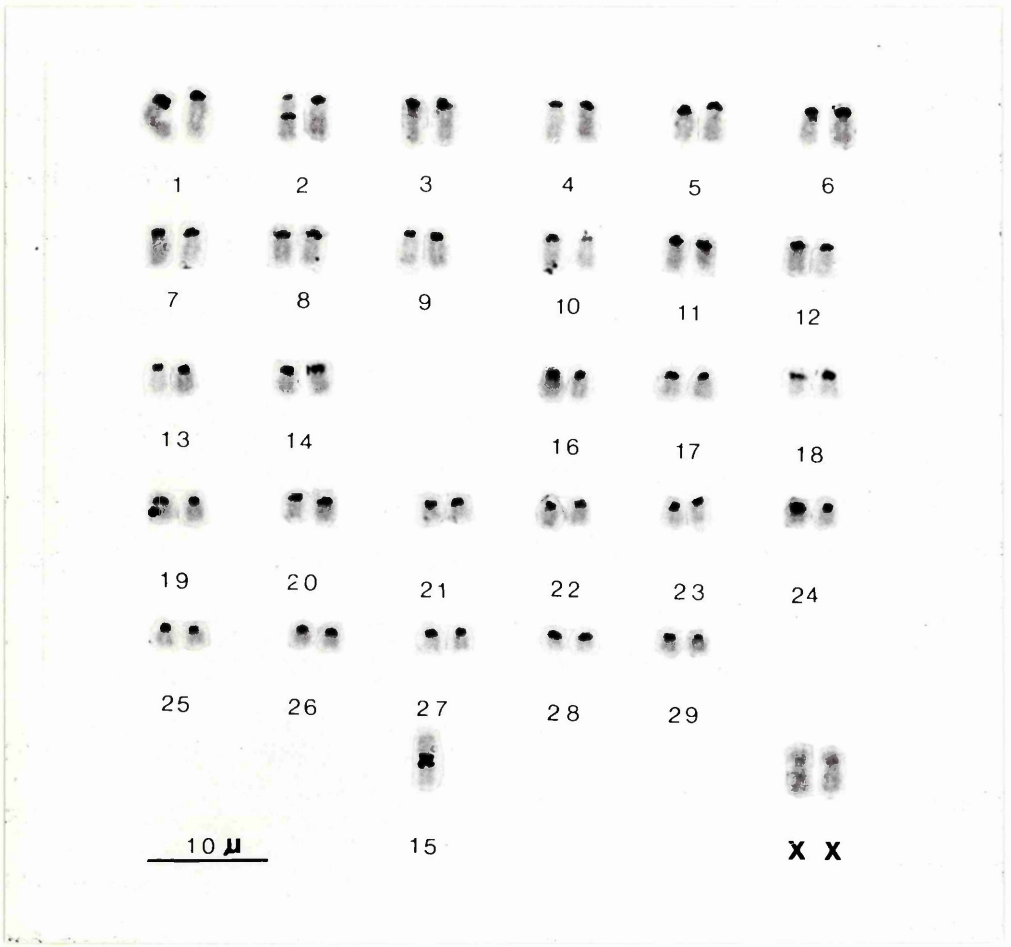

Figure 20

A representative C-banded karyotype containing a dicentric fusion between the chromosome pair number 15 and an abnormal banding pattern on one member of pair number 2

the 1/29 Robertsonian translocation was present in all chromosome sets examined from animal 18.

In addition, metacentric chromosomes with two centrally positioned prominent dark centromeric bands and lightly staining chromosome arms were recognised in spreads from each animal and classified as dicentric fusions. These particular chromosome aberrations were of different sizes. However, Figure 20 shows one which corresponds to a dicentric fusion involving a homologous pair of chromosome number 15.

A large chromosome with a small centromeric band and a prominent dark central band was identified among mitotic spreads from animal 21 and Figure 20 is a representative karyotype which shows that the chromosome with the abnormal banding pattern corresponds to number 2.

5.4 DISCUSSION

The results of this study clearly demonstrate that stimulation of cell proliferation by the infection of isolated lymphocytes with Theileria parva is a suitable method for obtaining an adequate supply of rapidly dividing bovine cells for cytogenetic analysis. However, to date, the precise mechanism by which Theileria stimulates cell division is unknown (Brown, 1985). Nevertheless, the intracellular schizonts which develop from the sporozoites are apparently treated as if they are chromosomes since both schizonts and chromosomes are pulled apart into daughter cells by the spindle fibres during anaphase (Stagg, Chasey, Young, Morzaria and Dolan, 1980) and, consequently, each daughter cell is infected with Theileria and will subsequently

divide. Furthermore, the results of this and other studies (e.g. Brown, 1983) demonstrate that, providing the cells are properly maintained in a suitable environment, then the cell population will increase tenfold every 48-72 hours for an apparently indefinite period although changes develop in both the karyotype and growth pattern of the cells. The only possible exception to this rapid rate of cell proliferation concerns animal 1 in this study since the proportion of cells present at passage 34 was considerably less than that which was usually seen after 72 hours incubation. Nevertheless, the rate of cell proliferation continued as normal in this animal after the correct cell concentration was established during passage 34 and, since there was no visible evidence of microbial contamination and since cell viability was normal and the pH of the medium did not fall, the lower concentration was probably caused by an error in pipetting during the previous passage which resulted in the transfer of fewer cells than usual into the fresh culture medium.

Although relatively little is known about the mechanisms involved in stimulating the division of lymphocytes which, under normal circumstances, do not divide (Moorhead et al, 1960), the duration of cell proliferation obtained in the present study is considerably longer than that produced by any other mitotic agent such as concanavalin A or phytohaemagglutinin which cause only a few cell cycles to occur (Beckman, 1962). This tends to suggest that the action of Theileria on lymphocytes is probably different from that of the other mitogens and this is supported by the fact that, whereas the theilerial schizonts are intracellular (Stagg et al, 1980) mitotic agents such as phytohaemagglutinin remain in

the culture medium (Forsdyke, 1973). Consequently, these compounds probably act on the cell membrane and there is evidence to show that changes in the cell surface can cause initiation of cell proliferation (Burger, 1970). However, it is not known how this is brought about although an increased transport of compounds such as phosphate, nucleotides and amino acids across the surface membrane appears to be a common observation in cells treated with concanavalin A (van den Berg and Betel, 1974).

The rapid rate of cell proliferation obtained in the present study tends to suggest that the interval during which individual cells prepare for DNA synthesis, but no DNA synthesis occurs [called the G1 phase by Cowdry (1950)], is very short or non-existent. Consequently, it seems reasonable to assume that the action of Theileria is continuous in which case infected cells will probably divide indefinitely, providing the environment is suitable. However, in spite of providing an apparently suitable and stable environment, most cell cultures appear to have a limited life-span after which the cell concentration and rate of proliferation decline as the cells die (Hsu, 1961). This phenomenon is called ageing (Hayflick and Moorhead, 1961). However, it is unlikely that the slight reduction in viability recorded in all animals at passage 89, in the present study was an indication of ageing since both the cell concentration and rate of proliferation remained high. Nevertheless, between 32 and 87% of the mitotic spreads examined from each animal at passage 90 contained chromosome aberrations and, since these were virtually absent in the earlier passages when viability was

determined, a more plausible explanation for the slight reduction in viability might be that some of the aberrations were incompatible with life. Alternatively, it may be that the reduction was a consequence of damage to the cells caused by the vigorous pipetting required to break down cell clumps during passage 89. However, this seems unlikely since the same pipetting technique was used throughout the study.

Transformation in growth pattern and karyotype occur in virtually all kinds of cell during prolonged periods of in vitro culture (Hsu, 1961) and the results of the present study clearly demonstrate that bovine lymphocytes are no exception. However, opinion is divided as to whether or not changes in growth pattern are linked to the development of chromosome aberrations. For example, some research workers observed a close correlation between the appearance of karyotypic abnormalities and the onset of altered growth pattern characteristics (e.g. Hsu, 1961) and suggest that the transformed growth patterns are a direct consequence of the chromosome aberrations, whereas others (e.g. Levan, 1956) point out that changes in growth pattern do not always coincide with the onset of karyotypic aberrations and argue that the altered growth patterns are a result of genetic mutations.

In the present study, the development of karyotypic aberrations preceded the onset of changes in growth pattern by between 4 and 13 weeks in every animal except 106 which tends to suggest that the two phenomena were unrelated. However, in animal 106, changes in the growth pattern and the appearance of karyotypic aberrations occurred within 11 days of each other.

Nevertheless, since cytogenetic analysis was not carried out during the passage when these changes were first seen, it is not possible to determine whether they were brought about by the development of chromosome aberrations. However, it is probably more likely that the changes in growth pattern observed in this study were the result of genetic mutations which occurred independently from the karyotypic aberrations, since, whereas the growth pattern was transformed in the entire population of cells from five of the animals by passage 90, karyotypic aberrations were present in only 32-88% of the cells. Furthermore, the fact that the growth pattern was transformed in the entire cell population tends to suggest that several mutations occurred during the study in cells from each animal since a single mutation would affect daughters of that cell only. Also these mutations affected the cells differently since some formed clumps in suspension whereas others became attached to the substrate.

Various agents are known to cause the formation of chromosome aberrations among cells grown in vitro and, of these, the most common are probably viruses and mycoplasma associated with certain neoplastic conditions such as murine leukaemia and Rous sarcoma (Nichols, 1969). However, it is unlikely that any infective agent was responsible for the chromosome aberrations which developed in the present study since all reasonable precautions were taken to maintain sterility and, in any case, infective agents were not handled in the laboratory. Furthermore, there was no history of viral or mycoplasma infections among the animals used in the study and the herd was

free from any agent such as enzootic bovine leucosis virus which might cause transformation of leucocytes. In addition, it is unlikely that the chromosome aberrations were induced to form by the action of theilerial schizonts since there is no indication in the literature to suggest that protozoal organisms interfere with chromosome structure and, furthermore, chromosome aberrations develop during the long-term culture of cells from cattle in the absence of theilerial schizonts (e.g. Nelson-Rees et al. 1964).

Chromosome aberrations can also be induced to form by exposure to various types of radiation (Sax, 1941). However, it is highly unlikely that the aberrations seen in the present study were produced by X-irradiation or radioactive isotopes since no radiographic equipment or radioactive material was used anywhere near the vicinity of the laboratory. Nevertheless, ultra-violet radiation is present in natural daylight and produced by fluorescent light tubes used in the laboratory. However, ultra-violet radiation is unlikely to have had any effect on the cells since it is too weak to pass through the wall of the tissue culture flasks (Uchida, Lee and Byrnes, 1975) and, in any case, the levels required to produce chromosome aberrations are considerably higher than normal background levels (McKenzie, 1941).

Certain chemicals may also induce chromosome aberrations (Sharma and Sharma, 1960). However, the laboratory glassware used in this study was thoroughly rinsed with deionised water prior to sterilisation in order to remove any trace of chemicals used as cleaning agents. Furthermore, all the plasticware was

specifically manufactured for tissue culture purposes and, therefore, it is unlikely that it contained any substance detrimental to normal cell growth. Consequently, there is no evidence to suggest that the chromosome aberrations were induced to form by the action of any exogenous chemical agent.

Although the antibiotic mitomycin-C is known to cause chromosome breaks which subsequently join to form aberrations (Hsu, Patak, Basen and Stark, 1978) the only antibiotics used in the present study were penicillin and streptomycin and both of these are routinely used in tissue culture medium without any apparent effect on chromosome structure. Consequently, it is unlikely that the aberrations were caused by the antibiotics.

In the absence of any external agent which may have induced the formation of chromosome aberrations, it seems highly probable that the karyotypic abnormalities were the direct result of prolonged culture in the unphysiological _in vitro_ environment. This is supported by the fact that the incidence of chromosome aberrations was considerably higher than that which normally occurs _in vivo_ (Moorhead _et al_, 1960). However, whereas various homeostatic mechanisms remove toxic metabolic biproducts and ensure an adequate supply of nutrients to cells _in vivo_ (Cowdry, 1950) these mechanisms do not exist _in vitro_. Consequently, it is possible that the aberrations were caused by either the build up of toxic biproducts or deficiencies of certain nutrients which developed in the culture medium. Nevertheless, any increase in toxic biproducts usually causes a reduction in cell proliferation together with a marked decrease in cell viability (Freshney,

1983) and, since neither of these occurred during the present study, it is probably more likely that the aberrations were caused by deficiencies which developed in the medium. Unfortunately, however, biochemical analyses were not carried out during this study and therefore it is not possible to determine which particular nutrients might be deficient. Nevertheless, the nutrients which are most likely to become deficient are those for which there is an exceptionally high demand and, although the molecular structure of DNA differs in each chromosome (Watson and Crick, 1953), the centromeric region of all cattle chromsomes contains highly repetitive sequences of DNA rich in guanine and cytosine specifically (Kurnit, Shafit and Maio, 1973). Consequently, there will be an extraordinarily high demand for the particular components required to replicate this region and it is conceivable that deficiencies may develop which lead to errors in the DNA replication process. This idea is further supported by the fact that the centromere is the last part of the chromosome to undergo duplication (Schnedl, 1972) and, therefore, many components will already have been used up during the replication of DNA belonging to chromatid arms. Furthermore, chromosome breaks are more likely to develop where irregularities occur in the DNA structure (Evans, 1967) and, therefore, any error in DNA replication would probably predispose that region to breaking which may explain the relatively high frequency of aberrations involving the centromere in this and other studies (e.g. Nielsen and Granzow, 1983). Alternatively, instead of producing breaks, it is conceivable that errors in DNA replication may, in some cases, result in the formation of

chemical bonds between the two duplicate chromosomes and, since this can interfere with chromosome separation (Lawley and Brookes, 1974), the formation of chemical bonds may explain the origin of aneuploid chromosomes found in this and other studies (e.g. Nielsen and Granzow, 1983).

In common with the results of other research involving the long-term culture of cells (e.g. Hsu, 1961) chromosome aberrations were not recognised in the present study until cells were in culture for at least six weeks. Nevertheless, eukaryote cells contain endoplasmic reticulum which stores various components necessary for DNA replication (Palade, 1975) and therefore it is unlikely that any aberration caused by the deficiency of nutrients would occur until after this reservoir is depleted. Furthermore, it is unlikely that minor errors in DNA replication would result in a major karyotypic abnormality and, therefore, several lesions probably accumulate before any aberration is produced. This idea is supported by the results of earlier research which shows that, whereas chromosomes can tolerate small irregularities in DNA structure, a break develops if several molecular aberrations occur in the same region (Lawley and Brooks, 1974). However, although chromosome aberrations developed in cells from each animal during this study, the passage at which they were first recorded was different in each case. Variations in the onset of chromosome aberrations also occurs among animals of other species including the human, mouse (Hsu, 1961), sheep and goat (Nelson-Rees et al, 1967). However, to date, there is no suitable explanation for this.

Nevertheless, the genetic composition of each individual is unique (McKusick and Ruddle, 1977) and, therefore, it is conceivable that some animals contain chromosomes with a molecular structure which is more susceptible to deficiencies which develop in vitro than others. Alternatively, however, it may be that the endoplasmic reticulum of some animals is less efficient at producing and storing the components necessary for DNA replication and, therefore, deficiencies will develop sooner in these animals. Nevertheless, in order to discuss these two possibilities further it would be necessary to compare the DNA molecular structure and endoplasmic reticulum of cells from different animals and, to date, this has not been carried out. However, there is some evidence which supports the possibility that the molecular structure of chromosomes may have some effect on their susceptibility to forming aberrations since certain human chromosomes are more susceptible to radiation-induced aberrations than others (Lee and Kamra, 1981).

Although the occurrence of chromosome aberrations which subsequently disappeared during the earlier part of this study is consistent with the results of some research workers (e.g. Hsu, 1961), others (e.g. Levan, 1956) did not observe this early peak. Nevertheless, the latter group of research workers examined the karyotype of cells only occasionally during the earlier part of the study and therefore it is possible that aberrations did occur during this period but remained undetected. Furthermore, the formation of chromosome aberrations which subsequently disappear during the earlier period of long-term culture seems to be a relatively common phenomenon since it is recognised in a large

variety of cell types cultured from various different species (Hsu, 1961). Nevertheless, in spite of this, there is no clear explanation for the apparent disappearance of these aberrations. However, it is currently believed that chromosome aberrations which develop during long-term culture may provide the cell with a growth advantage over others *in vitro* (Freshney, 1983) and, since many chromosome aberrations such as inversions and reciprocal translocations may not be recognisable in cattle using conventionally stained giemsa preparations (Gustavsson, 1980), it is conceivable that the earlier aberrations in this study were followed by the formation of certain structural rearrangements which were not recognisable in the giemsa preparations but provided the cells with a growth advantage over those containing the earlier aberrations. Consequently, these cells would survive at the expense of all others and the karyotype of the cell population would apparently return to normal. However, in order to investigate this possibility further, it would be necessary to differentially stain the chromosomes during the earlier period of long-term culture using various banding techniques in an attempt to identify aberrations which are not recognised by conventional staining and, to date, this has not been attempted. Furthermore, not all structural rearrangements can even be detected by banding (Gustavsson, 1980) and, therefore, more intricate methods such as high resolution banding (Yunis *et al*, 1978) or chromosome mapping (McKusick and Ruddle, 1977) may be necessary.

An alternative explanation for the disappearance of aberrations which developed during the earlier part of the study

might be that they caused degeneration of the cells. However, this is unlikely since, although cell viability was measured only once during this part of the study, less than 1% of the cells were dead and, furthermore, there was no reduction in the rate of cell proliferation.

During the second half of the study, the proportion of cells with karyotypic aberrations progressively increased in each animal which demonstrates that not only were these cells viable but also that they increased in number at the expense of normal cells. Consequently, these results are in accordance with the currently held theory that certain chromosome aberrations provide cells with a growth advantage over normal cells (Freshney, 1983). However, in animal 1, the proportion of cells with karyotypic abnormalities subsequently fell by 30% towards the end of the study. It is possible that this decline was caused by degeneration of the karyotypically abnormal cells although this seems unlikely since 88.5% of the cells were viable at the end of the study. Nevertheless, viability was not measured during the immediately preceding passages and, therefore, it may be that cell death occurred undetected during this period. However, again, this is unlikely since such a large loss of cells would probably have been apparent during microscopic examination of the culture medium. It is also unlikely that the karyotypically abnormal cells were in the process of ageing during this period since this phenomenon is accompanied by a reduced rate of cell proliferation (Hayflick and Moorhead, 1961) which did not occur in the present study. The most probable explanation for the reduction in the proportion of karyotypically abnormal cells is

that a chromosome aberration which is not discernable by conventional staining formed in a cell with the normal karyotype and that the daughters of this cell were better suited to the *in vitro* environment than the other cells. Consequently, the proportion of apparently normal cells would increase at the expense of cells with abnormal karyotypes. If the period of long-term culture in this study had been longer then it is possible that the proportion of cells with an apparently normal karyotype would continue to increase. However, various research workers (e.g. Nielsen and Granzow, 1983) demonstrated that different chromosome aberrations are continually developing during prolonged *in vitro* culture. Consequently, other aberrations would probably occur in cells from animal 1 and if one of these involved a change in karyotype and provided the cell with a growth advantage over the apparently normal cells then the proportion of cells with an abnormal karyotype would rise again.

Polyploidy also occurred in the present study during passages when chromosome aberrations were detected which tends to suggest that the two events are, in some way, related to each other. This observation was also made by other research workers (e.g. Hsu, 1961) although the precise mechanism involved in the formation of the polyploid cells is unknown. Nevertheless, it is probably associated with an increased frequency of endomitosis or endoreduplication since both these processes occur occasionally in mammalian cells (Nagl, 1978) and it may be that these processes are more likely to occur in cells with chromosome aberrations than in normal cells. It is also possible that

polyploid cells were caused to form by the action of colcemid since this substance inhibits spindle formation (Inoue, 1952). However, colcemid is unlikely to be the sole cause since it was used only at a level which produces the occasional polyploid cell (Herreros et al, 1966). Another possibility is that polyploidy was the result of cell fusion although this is probably unlikely since there was no evidence of binucleate or multinucleate giant cells in either this or other long-term culture studies (e.g. Nielsen and Granzow, 1983).

Although it would be an almost impossible task to identify each individual chromosome aberration which developed during this study, G-banded karyotypes were prepared at passage 90 from a random selection of mitotic spreads and, therefore, it seems reasonable to assume that the most commonly occurring aberrations were probably present in these karyotypes. Consequently, it is interesting to note that two of the 26 karyotypes contained a chromosome inversion which would not be apparent in conventionally stained spreads and, therefore, chromosome aberrations were probably present in a higher proportion of cells than that which was recorded by conventional staining. Chromosome inversions have previously been reported in cattle (e.g. Popescu, 1976) although the one identified in the present study is of particular interest since, unlike previous reports, the centromere is divided in two by the inversion. However, relatively few G-banded karyotypes have been prepared from cattle and it may be that this inversion also exists in vivo but, to date, is undetected. On the other hand, there is no report of inversions which split the centromere in other species including

the mouse and human (Kaiser, 1980) and, therefore, it is possible that the aberration developed as a consequence of the unnatural in vitro environment. This is supported by the facts that not only does the centromere of cattle contain highly repetitive sequences of DNA rich in guanine and cytosine (Kurnit, Shafit and Maio, 1973) but also that this area is the last to undergo replication (Schnedl, 1972) and, therefore, since nutrients in the culture medium were rapidly metabolised, it is possible that the medium was unable to provide an adequate supply of all the components necessary for replication and that this interferred with chromosome duplication to such an extent that a break was produced in the centromere. Furthermore, an inversion would result if a break also occurred in the chromatid arm and the broken ends fused with each other.

An abnormal C-banding pattern was obtained on one of the largest chromosomes in mitotic spreads belonging to animal 21 in which the inversion was identified. However, since different mitotic spreads were used for each of the banding techniques it is not possible to be entirely certain that both abnormal banding patterns belonged to the same chromosome aberration. Nevertheless, this seems highly likely for several reasons. Firstly, the abnormal C-banding pattern occurred only in mitotic spreads belonging to the animal with the inversion involving chromosome number 2. Secondly, the C-banded karyotypes demonstrated that the abnormal chromosome not only corresponded in size to number 2 but also that the position of the dark C-bands was exactly the same as the unstained G-band and thirdly,

the G- and C-banded centromeric regions were both correspondingly smaller than usual. Consequently, it seems reasonable to assume that the abnormal C- and G-banding pattern both belonged to the same chromosome aberration.

It is interesting to consider whether division of the centromere into two separate parts by the inversion would upset the normal process of chromosome separation during anaphase since the centromere acts as the point of spindle fible attachment (Inoue, 1952). However, the spindle fibres only attach to one specific part of the centromere called the kinetochore (Hoskins, 1969) and, therefore, the normal separation of duplicate chromosomes is unlikely to be affected unless this part of the centromere is also involved in the aberration. Fortunately, the position of the kinetochore is easily located on the centromere since it forms its narrowest part (Hoskins, 1969) and, since the narrowest part remained in its usual position at the centromeric tip of the chromosome inversion and since there was no evidence of any other constriction elsewhere, it seems reasonable to assume that the kinetochore was not involved in the inversion and the the process of chromosome separation was probably unaffected. If the kinetochore had been transferred to the chromatid arm along with the large portion of centromere then this would probably have been evident by a constriction in this region. Alternatively, if the kinetochore had been split in two by the inversion, then two constrictions would probably be visible and, if both sections of the kinetochore remained active, then a chromatin bridge would form between them during anaphase with the subsequent fragmentation of the chromosome (McClintock, 1942).

However, since this process usually results in death of the cell, it is unlikely that any aberration which splits the kinetochore would be found in viable cells during long-term culture.

By using the G-banding technique, it was possible to identify each individual chromosome, with the exception of the group 22-23, from the banding patterns described in Section 2.31. However, as in Chapter 2, it was impossible to distinguish between chromosomes number 22 and 23 since they had the same banding pattern. Consequently, there were five G-banded karyotypes in which the aneuploid chromosomes could not be identified other than that they belonged to the group 22-23. Nevertheless, four of these karyotypes contained six chromosomes belonging to this group and, therefore, each member of the group was probably trisomic. This is supported by the finding of two other karyotypes with trisomic chromosomes in this study and, furthermore, by the fact that trisomy is reported to be the most frequently encountered aberration to develop during long-term cell culture (Nielsen and Granzow, 1983). Alternatively, however, it may be that two of the chromosomes were members of the same homologous pair which would make the other chromosome tetrasomic. This is supported by the finding of one other tetrasomic karyotype in this study, although tetrasomy is not commonly recognised in cells during long-term culture (Nielsen and Granzow, 1983). Nevertheless, there are two conceivable ways in which tetrasomy may occur. Firstly, it could be produced by non-disjunction involving a chromosome which was already trisomic, in which case one daughter cell would be tetrasomic and

the other would be normal and, secondly, by non-disjunction involving both chromosomes of a pair in which case one daughter cell would be tetrasomic and the other nullisomic. However, both of these processes are relatively complex, which probably explains why tetrasomic cells are rarely found. Consequently, it is probably more likely that the unbalanced karyotypes were trisomic for both members of group 22-23.

The most commonly cited cause of trisomy is non-disjunction during mitosis whereby one daughter cell is trisomic and the other monosomic (Uchida et al, 1975) and, therefore, assuming that both chromosome number 22 and 23 are trisomic in the karyotypes containing six of these chromosomes, it seems reasonable to assume that the karyotype with only two of these chromosomes probably represented a cell which was correspondingly monosomic for both chromosomes. This is supported by the finding of two other monosomic cells in this study and, although the specific trisomic counterparts of these cells were not identified, they probably belonged to the large group which was not karyotyped. Alternatively, it may be that the chromosomes missing from group 22-23 were lost during the fixation process in which case aneuploidy would be a technical artefact. Nevertheless, this is probably unlikely since great care was taken to ensure that only the best preparations with the most evenly-spread chromosomes were selected for karyotyping. It is also unlikely that both chromosomes belonged to the same homologous pair since this would make the cell nullisomic for the other chromosome and, although the loss of one chromosome can be compatible with life (Uchida et al, 1975), the loss of both

members of a pair usually results in death of the cell (Uchida et al, 1975).

The results of cytogenetic research in the 1960's and 70's showed that supernumerary chromosomes can also be produced by an error in DNA replication which leads to the duplication of a chromosome twice (Battaglia, 1964; Ford, 1971). However, these observations were made in meiotic preparations only and since there is no report of it occurring in somatic cells, it is perhaps unlikely to have been responsible for trisomy in the present study. Nevertheless, the in vitro environment of cells is not necessarily ideal and, therefore, it is conceivable that deficiencies might occur which interfere with normal DNA replication. Furthermore, there were twice as many trisomic as monosomic karyotypes prepared during this study and, therefore, it may be that whereas those with a corresponding monosomic karyotype were the result of non-disjunction, the others were produced by extra chromosome duplication. It is also possible, however, that the reason fewer monosomic karyotypes were prepared is that these cells were less viable than their trisomic counterparts. This is supported by research in the mouse which shows clearly that monosomic embryos tend to die earlier than trisomic ones (Gropp et al, 1974) and, consequently, further research is required in order to determine whether or not extra chromosome duplication occurs during the culture of somatic cells.

Although relatively few G-banded karyotypes were prepared in this study, these belonged to cells which were selected at random

and, therefore, it seems reasonable to assume that the most commonly occurring aneuploid chromosomes were present in these karyotypes. Consequently, it is interesting to consider whether the same chromosomes are involved in aneuploidy *in vivo*. To date, the only aneuploid chromosomes which have been positively recognised in cattle are number 17 (Herzog, Hohn and Rieck, 1977) and the sex chromosomes (e.g. Logue, Harvey, Munro and Lennox, 1979) which tends to suggest that the chromosomes involved in aneuploidy *in vivo* differ from those *in vitro*. Nevertheless, relatively few cytogenetic studies have been carried out in cattle and, therefore, it is possible that other chromosomes are involved in aneuploidy *in vivo* but, to date, are undetected. This is supported by the fact that cases of aneuploidy are reported in which the aneuploid chromosome is unidentified and may be different from previously reported cases involving chromosome 17 (e.g. Mori, Sasaki, Makino, Ishikawa and Kawata, 1969). Consequently, it is possible that some of these aneuploid chromosomes are the same as those identified in the present study. This is further supported by the fact that virtually every chromosome in other species including the human (Warburton *et al*, 1980) is involved in aneuploidy *in vivo* and there is no evidence to suggest that cattle chromosomes should be an exception. However, the relative incidence of particular chromosomes involved in aneuploidy varies considerably (Warburton *et al*, 1980) and many cases of aneuploidy die during gestation (Warburton *et al*, 1980). Consequently, further research involving cytogenetic examination of embryos is required in order to determine whether the chromosomes which are most frequently

aneuploid *in vivo* are the same as those which occur most frequently *in vitro*.

The presence of a Robertsonian translocation usually increases the incidence of non-disjunction during meiosis due to the adjacent separation of chromosomes during cell division (Tettenborn and Gropp, 1970). However, none of the G-banded chromosomes was aneuploid for either chromosome number 1 or 29 which tends to suggest that the 1/29 Robertsonian translocation was not involved in non-disjunction during the present study. Furthermore, each of the 300 or so mitotic spreads which were examined from the carrier animals in this study were heterozygous for the 1/29 Robertsonian translocation whereas non-disjunction would result in the formation of monosomic cells without the translocation (Tettenborn and Gropp, 1970). Consequently, the results of this study provide clear evidence that the behaviour of the 1/29 Robertsonian translocation during mitosis is different from that during meiosis where non-disjunction occurs in approximately 6% of cells (Logue and Harvey, 1978[a]). However, meiosis is a much more complicated chain of events than mitosis and, consequently, the chances of failure during some part of the process are more numerous which may explain the relatively high incidence of non-disjunction during meiosis. Furthermore, the incidence of meiotic non-disjunction increases with age (Hassold, Jacobs, Kline, Stein and Warburton, 1980) which tends to suggest that the aberration is also influenced by hormonal and other physiological factors which may not affect somatic cells to the same degree.

Although each of the G-banded metacentric chromosomes was the same size and consisted of a dicentric fusion between both members of the homologous pair number 15, metacentric chromosomes of various sizes developed during this study and, therefore, other chromosomes were also involved in the formation of these aberrations. Nevertheless, each metacentric chromosome was evidently also the result of a dicentric fusion between a homologous pair since the C-banded preparations show that, without exception, all metacentric chromosomes consisted of two distinct centrally positioned dark centromeric bands separating two lightly stained chromosome arms of equal length. If non-homologous chromosomes had been involved in the fusion then the chromosome arms would be of different lengths. These findings are, therefore, in contrast with the results of Nelson-Rees et al (1964) which show that submetacentric chromosomes formed during long-term cell culture and that these possibly represented Robertsonian translocations involving non-homologous chromosomes. However, there are several important differences between the protocol of these two studies which may explain the different results. For example, instead of using fresh cells, Nelson-Rees et al (1964) used cells which had been frozen. Nevertheless, this probably did not adversely affect the chromosomes since the cells were frozen and thawed without any significant loss in viability. Furthermore, it is unlikely that freezing would be responsible for the formation of chromosome aberrations since these also developed in cells which were never frozen (Nelson-Rees et al, 1967). Another difference is that the cells in the Nelson-Rees study were cultured in a different type of medium

and, since the results of this study suggest that the development of chromosome aberrations may be a consequence of the unphysiological in vitro environment, it is possible that the medium used by Nelson-Rees et al (1964) was more conducive to the development of Robertsonian translocations than that which was used in the present study. This possibility is supported by the observation that chromosome aberrations develop sooner in cells grown in one type of medium than another (Sasaki and Sasaki, 1962) although the significance of this observation is complicated by the fact that cells from different tissues were used in the experiment. Consequently, the development of chromosome aberrations may also be dependent on the type of tissue cultured. This possibility is of particular relevance to the present discussion since Nelson-Rees et al (1964) cultured kidney cells whereas lymphocytes were used in the present study. Furthermore, the kidney cells probably consisted of a mixture of epithelial and interstitial cell types and, since the submetacentric chromosomes developed in only a proportion of the cells, it is possible that different chromosome aberrations develop in different cell types. Another important difference between the studies is that, whereas the lymphocytes used in the present study were passaged every 2-3 days, the kidney cells were passaged every 7-14 days. Consequently, although submetacentric chromosomes were first observed in the kidney cells during passage 53, these cells had already been cultured for at least twice as long as those in the present study and, therefore, it is possible that the development of certain chromosome aberrations

is influenced by the age of the cell line. In contrast to this, however, other research workers identified Robertsonian translocations which formed in cells after only relatively few weeks of culture (e.g. Nielsen and Granzow, 1983), although these cells were from the mouse which introduces species as yet another possible variable.

In view of the many factors involved, therefore, it is not possible to determine which were responsible for the development of submetacentric chromosomes in the Nelson-Rees study yet not in the present study. Nevertheless, it would be wrong to conclude that the absence of any submetacentric chromosomes in this study is conclusive evidence that Robertsonian translocations will not form in bovine lymphocytes during long-term culture. For example, it is possible that the aberration would develop in lymphocytes belonging to other animals, or if the cells were cultured for a longer period, or if a different culture medium was used or if cells from a different tissue were used. Consequently, in order to determine whether the 1/29 Robertsonian translocation will develop during prolonged in vitro culture, it would be necessary to culture various types of cells from several different animals in different types of culture medium. Cytogenetic examination could then be carried out at regular intervals and if, for example, the 1/29 Robertsonian translocation developed in cells growing in a particular medium, then the constituents and deficiencies which develop in that medium could be compared with others in order to identify any difference which may have been responsible for the formation of the aberration. If any difference was detected then the

possibility that this could result in the formation of the translocation *in vivo* could be considered. Alternatively, if the 1/29 Robertsonian translocation did not develop in any of the cells undergoing *in vitro* culture, then it would be reasonable to assume that the environmental conditions were not conducive to its formation. Consequently, it would then be possible to alter various aspects of the environment in attempt to induce formation of the translocation. If the translocation formed when certain essential nutrients were deficient, then the possibility that these nutrients might become deficient during an *in vivo* situation such as starvation or dietary imbalance could be considered. Alternatively, if a rise in temperature resulted in the translocation, then it is conceivable that it is more likely to form *in vivo* during pyrexic conditions. It is also possible that the aberration might be induced to form in cells from only certain animals which would tend to suggest a genetic predisposition.

Since the chromosomes in this study which were most frequently involved in aneuploidy belonged to group 22-23, this tends to suggest that these chromosomes are particularly predisposed to non-disjunction *in vitro*. This is further supported by the observation that aneuploidy of these chromosomes was recognised in cells from three of the six animals investigated. However, neither chromosome 22 nor 23 has been identified in aberrations which occur *in vivo*. It may be that these aberrations exist but are undetected. However, the finding of aneuploidy involving chromosome 17 in different animals by

several independent groups of research workers (e.g. Herzog et al, 1977) tends to suggest that aneuploidy in vivo involves this particular chromosome more frequently than any other. Furthermore, a large proportion of the G-banded karyotypes (33.3%) prepared during this study contained a dicentric fusion between the homologous pair number 15, whereas the most common centric fusion in vivo is the 1/29 Robertsonian translocation (Long, 1985). Consequently, the aberrations which developed in vitro are not the same as those which occur in vivo.

This is also the situation in other species including the human and the mouse (Chen and Ruddle, 1971). However, whereas research workers use somatic cells to study the formation of aberrations in vitro, aberrations which occur in vivo may be the result of errors in meiotic division. This is supported by the results of meiotic studies in cattle (Logue and Harvey, 1978[a]) and other species including the human (Martin, Balkan, Burns, Rademaker, Lin and Rudd, 1983) which show that approximately 10.6% and 4.2%, respectively, of secondary spermatocytes produced by normal animals are unbalanced. Consequently, cases of aneuploidy in vivo are probably the result of fertilisation between unbalanced products of meiosis and normal gametes and this has been demonstrated in cattle (Popescu, 1980; King et al, 1981; present study), humans (Hamerton, 1970), and mice (Gropp et al, 1974) heterozygous for various different Robertsonian translocations. Furthermore, there is clear evidence that structural rearrangements can result from errors in meiotic divisions since the de novo formation of a 13/14 Robertsonian translocation in the human occurred in the germ cell of a mother

with the normal karyotype (Gosden et al, 1979), and an estimated 3.3% of spermatozoa from normal men contain chromosomes with various structural abnormalities (Martin et al, 1983).

There are several stages during meiosis at which chromosome aberrations may develop. For example, two chromosomes of a bivalent structure may fail to separate during anaphase of the first meiotic division in which case the subsequent separation of the two homologues during the second meiotic division would lead to the formation of two disomic and two nullisomic gametes. Alternatively, if no chiasma forms between a duplicate pair of chromosomes then the paired homologues may fall apart and appear as univalents (Patau, 1963). These may either drift at random to the two poles during the first meiotic division and divide irregularly in the second or, they may divide mitotically in anaphase I and, being single chromatids in the second division, they cannot divide any more and therefore drift at random to the poles or misdivide. Similar behaviour is also exhibited by univalents if they result from an original failure to pair during prophase I (Patau, 1963). Furthermore, recent electron microscopic observations during meiosis show that complicated structures formed by chiasmata during prophase I often result in breaks (von Wettstein, Rasmussen and Holm, 1984) and, if the wrong broken ends fuse together, then this would result in a structural aberration. Consequently, more relevant results would probably be obtained by using meiotic preparations to study the in vitro formation of aberrations which occur in vivo.

CHAPTER SIX

CONCLUSION

Although the number of breeding records from animals heterozygous for the 1/29 Robertsonian translocation were insufficient to demonstrate the anticipated small reduction in fertility caused by early embryonic death, the finding of three aneuploid embryos sired by an animal heterozygous for the translocation supports the results of earlier studies which showed that the fertility of animals carrying the translocation is reduced due to the fertilisation of unbalanced gametes produced by non-disjunction during meiosis (Logue and Harvey, 1978[a], Popescu, 1978). The subsequent death of these aneuploid embryos causes a reduction in non-return to service rates (Gustavsson, 1969) and, therefore, prolongs the interval between successive calvings. This results in a considerable financial loss to the agricultural community [calculated to be £3.50 per cow per day (Esslemont, Baillie and Cooper, 1985)] which could be avoided by the eradication of carriers from the national breeding herd. However, in spite of removing all carrier bulls from Artificial Insemination Centres, the incidence of the translocation was reduced in Sweden by only 2% over ten years (Gustavsson, 1979). Consequently, in order to effectively eradicate the aberration, it would be necessary to cull all carrier cows also. This would be a major problem in Sweden since the translocation is distributed throughout the country and affects a large number of animals [estimated to be several thousands (Gustavsson, 1969)]. However, the results of the present study tend to suggest that, at the moment, relatively few members of the British Friesian breed carry the translocation.

Furthermore, the translocation appears to have developed de novo in the proband's dam or a recent predecessor and,therefore, is probably restricted to a limited number of herds containing progeny from that animal. Consequently, the 1/29 Robertsonian translocation could be successfully eradicated from the British Friesian breed by tracing these animals and culling those which are carriers.

It is foreseeable that opposition to eradication might be encountered if an animal of superior genetic potential is found to carry the translocation. However, only 50% of offspring inherit the condition (Gustavsson, 1969) and, therefore, in this case it would not be necessary to cull that particular animal providing progeny with the translocation are excluded from breeding. Alternatively, it may be that the translocation chromosome itself contains valuable genetic information which would be lost if all carriers were eradicated. However, this information could be preserved by storing frozen semen and embryos from animals carrying the translocation and, therefore, be available for use at a later date if necessary.

Another objection to eradication might be the expense involved in identifying carrier animals. However, from the results obtained during this study, it seems likely that relatively few British Friesian cattle are effected and, therefore, karyotypic analysis would probably involve only those herds containing animals closely related to the proband. Nevertheless, the longer eradication is delayed, the greater the cost is likely to be since the incidence of carriers will probably rise as successive generations inherit the

translocation.

The precise mechanisms whereby chromosome aberrations, such as the 1/29 Robertsonian translocation develop *in vivo* are unknown. However, with the advancement of science, animals are being increasingly exposed to irradiation and chemicals which produce chromosome breaks and, therefore, it is conceivable that one or more of these agents may be responsible for at least some of the aberrations which occur *in vivo*. Consequently, a logical progression of the cell culture study, would be to expose cells to various forms of radiation and chemicals and determine whether the aberrations which develop include any of those which occur *in vivo*.

Alternatively, certain physical features, such as variations in temperature, oxygen tension and hydration, increase the incidence of chromosome breaks in plants (e.g. D'Amato and Gustafsson, 1948) and, since similar conditions occur in animals during pyrexia, hypoxia and dehydration, it is conceivable that some of the aberrations which occur *in vivo* developed in animals suffering from a pathological condition which upsets the tissues' normal environment. This idea could be explored further by exposing cells to different physical conditions *in vitro* and identifying the aberrations which develop. However, chromosomes behave differently during meiosis and mitosis and, since the majority of heritable chromosome aberrations probably developed during meiosis (Griffen and Bunker, 1967), more relevant results would probably be obtained by using meiotic preparations.

Appendix

The Preparation of Sterile Ground Up Tick Supernate (GUTS) containing Theileria parva sporozoites

The sterile GUTS which contained the Theileria sporozoites used in this study to stimulate rapid cell proliferation was prepared by C.G.D. Brown of the Centre for Tropical Veterinary Medicine, Edinburgh using the following method based on the technique described by Brown (1983).

Twenty adult ticks (Rhipicephalus appendiculatus) infected with Theileria parva were fed on the ears of a rabbit for four days to stimulate multiplication of the sporozoites. After this period, the ticks were decontaminated by washing in a universal bottle containing 1% benzalkonium chloride (Roccal, Winthrop Laboratories) and sterilised by rinsing three times in 70% ethanol. Next, the ticks were washed three times in Eagles Minimum Essential Medium (MEM) with Hank's Salts (Gibco) which contained antibiotics at double strength (200 iu/ml penicillin, 200 µg/ml streptomycin, 100 µg/ml nystatin) ensuring that the ticks were completely immersed for ten minutes during the last wash. The old medium was then replaced with fresh medium supplemented with 3.5% bovine plasma albumin (Armour Fraction V, Sigma) and the ticks were poured into a sterile mortar where they were ground thoroughly with a sterile pestle. This produced a suspension, which was collected in a sterile universal bottle, and sediment, which was ground again with 5 ml of the supplemented medium. After repeating this process five times, the accumulated suspension was centrifuged for five minutes at

100 x g to produce a supernatant which was drawn into a sterile syringe and filtered (8 μ with a prefilter, Millipore). The filtrate (GUTS) was collected in a sterile universal bottle and the presence of sporozoites was confirmed by centrifuging a 100 μl drop onto a microscope slide at 150 x g for ten minutes and staining with 10% buffered giemsa solution (Gurr's R66, pH 6.5, B.D.H. Chemicals).

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Addendum

A degree of ambiguity exists over the precise definition of the term centromere. Early research workers (e.g. Inoue, 1952) used the terms kinetochore and centromere synonomously. However, more recently, the term kinetochore is used to describe a specific part of the centromere to which the spindle fibres attach during cell division (King and Standsfield, 1985) and contemporary research workers (e.g. Ford et al., 1980) now use the term centromere to describe the region of the chromosome containing chromatin with highly repetitive sequences of DNA which become tightly condensed during cell division and cause this area to stain differently from the rest. This definition is used in the present study and, due to its effect on staining, the type of chromatin in the centromere is called heterochromatin (King and Standsfield, 1985). In the present study, centromeric heterochromatin is situated in the unstained region of the G-banded autosomes which have a corresponding dark stained C-band and therefore centromeric heterochromatin is involved in the inversion identified by G- and C-banding in Chapter Five.

In addition to G- and C-banding, cytogeneticists are increasingly using R-banding techniques which produce a series of light and dark bands on each chromosome such that the areas which are stained dark by G-banding stain lightly with R-banding and vice versa (e.g. Yunis, 1981). Furthermore, in cells which are cultured in the presence of the base analogue 5-bromodeoxyuridine (BrdU), the chromosomes incorporate the base analogue instead of thymidine into their structure and the R-banding pattern can be

produced by subsequently staining the chromosomes with either Giemsa or fluorescent dyes (Yunis, 1981). Research workers in Italy recently adapted this technique for use with cattle chromosomes and demonstrated that the number of bands is greater in prophase than metaphase chromosomes (Iannuzzi and Di Berardino, 1985). Furthermore, certain bands form landmarks which divide the prophase chromosomes into sections and can be used to establish the identity of individual chromosomes. However, since only metaphase chromosomes were used in the present study, it was not possible to investigate the high resolution banding patterns produced by modifications of either the G- or R-banding techniques.

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