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THE CHROMOSOMES OF CATTLE: THEIR ASSOCIATION WITH PHYLOGENIC AND ONTOGENIC PROCESSES WITH AN INTENSIVE STUDY OF THE FREEMARTIN SYNDROME,

The Ohio State University, Ph.D., 1964
Biology–Genetics

University Microfilms, Inc., Ann Arbor, Michigan
THE CHROMOSOMES OF CATTLE:
THEIR ASSOCIATION WITH PHYLOGENIC AND ONTOGENIC
PROCESSES WITH AN INTENSIVE STUDY OF THE
FREEMARTIN SYNDROME

DISSERTATION

Presented in partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the
Graduate School of The Ohio State University

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* * * * * * *

The Ohio State University
1964

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I wish to express my gratitude to Dr. N.S. Fechheimer, Dr. L.O. Gilmore, and Dr. E.F. Paddock, for their advice and criticism during the preparation of this dissertation.

I am especially grateful to Dr. N.S. Fechheimer, for his invaluable counseling through all phases of the problem presented here.

To Dr. D.F. Weseli of the Cattle Blood Typing Laboratory for his antigen admixture analyses and for general advice on technical points pertaining to the work presented, I wish to express my thanks.

I would also like to express my appreciation to the members of the N.C. 2 Dairy Cattle Research project, who aided in securing the animals used in this study and to the many veterinarians, county agents and farmers who cooperated in procuring the research animals.

I would like to extend my thanks to Dr. E.H. Bohl of the Department of Veterinary Medicine for his kind permission to observe the techniques in his laboratory, that were used for kidney cell culture.

To Drs. W. Venzke, B.S. Andriese, and S. Pakes, I would like to extend my thanks for help in
the analysis of the histological preparations.

I would like to thank the members of the slaughtering crew of the Animal Science Department meat laboratory for their cooperation in this project.

To my fellow graduate students, I would like to express my gratitude for their helpful suggestions when questions were posed.

I would like to express my appreciation to the Agricultural Experiment Station and the Dairy Science Department of The Ohio State University for providing the assistantship that made my graduate study possible. I would also like to express my gratitude to the National Institutes of Health for the terminal year predoctoral fellowship which provided the means for finishing the research project.

I would like finally to acknowledge the moral support of my wife, Rhoda, whose understanding has been a great help during my graduate study.
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PUBLICATIONS


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INTRODUCTION

The study of chromosomes in various species as to the diploid number for each, their morphology and aberrations thereof, can lead to a better understanding of phylogenic and ontogenic processes. Various aberrations of chromosome morphology have led to speciation in such genera as Drosophila (White, 1954). In addition, chromosomal aberrations have been associated with many developmental anomalies, which render the individual concerned incapable of propagating the species (Ferguson-Smith, 1961). Of special interest therefore, is the study of sexual differentiation and development and the factors that control it.

Sex is a means whereby genetic variability is maintained among members of a species. The important consideration in this system is the cyclic haploid and diploid phases which, with the synapsis of the chromosomes at meiotic prophase, maintains the variation among genetic constitutions within a species (Witschi, 1960). Male and female differentiation as found in most higher organisms is only a specialization of the hermaphroditic state in which both egg and sperm may be produced by one individual. This specialization prevents self-fertilization and thus aids in
maintaining the variability of the population (Witschi et al., 1957).

Sex may be induced primarily by the environment, the genes, or a combination of both factors. In some lower forms of life, external environment induces the sex. Nutritional factors play a role, with the male sex predominating when the nutritional level is below a certain threshold (Witschi, 1960). In higher vertebrates, sex determination is predominantly genetic, being due to a balance of male and female determining genes. Female determiners are thought to be located on the X chromosome as compared to the male determining genes located on the autosomes and the Y chromosome (Sohval, 1963). The hypothesis that the Y chromosome contains male determining genes (Miller, 1962) is contrary to the situation in insects in which the Y chromosome is almost genetically inert (Bridges, 1916).

Genes act through production, modification, inhibition, or acceleration of enzymes. The enzymes may have either intracellular or intercellular effects. It is through gene action that cellular specialization is thought to take place. Gene action in specialized cells may result in hormones that have an effect on various tissues. The hormones may affect the environment in which the same or other specialized cells are developing into tissue and/or organ systems.
In order to understand the process of sexual development better, research is carried out on various levels of study. At each level, there is an interaction of the effects of genetic forces and environment. It is necessary to consider the findings on all levels. Although the research to be dealt with in this dissertation has been carried out on the chromosomal level, the results must be reconcilable with observations obtained on other levels of study. To this end, much anatomical and endocrinological evidence will be presented in the review of literature that follows.

The objectives of the research undertaken are to develop a technique for cell culture and preparation of metaphase figures of cattle chromosomes, to study the normal morphology, to observe and understand the aberrations present in somatic cells of normal cattle, and to study the chromosomal patterns in an abnormal sexual condition occurring regularly in cattle (the freemartin syndrome).
Cattle chromosomes

The chromosomes of cattle have been studied by various workers since 1892 (Makino, 1944), with Von Bardeleben being the first to estimate the diploid number of chromosomes, reporting 16 chromosomes. Schoenfeld (as cited by Makino, 1944) estimated the diploid number as 20-25 in 1902. Wodsedalek (1920) gave the diploid number as 38. Krallinger (as cited by Makino, 1944) in 1927 was the first to give the diploid number as sixty chromosomes. The early analyses were made from testis biopsies and the counts were variable due to sectioning techniques. Makino (1944) developed the squash technique for cattle chromosomes which made morphological studies more accurate. He confirmed the findings of Krallinger that the diploid number is sixty chromosomes. During the decade of the 1950's, a count of sixty chromosomes for diploid cells and thirty bivalents in the diplotene stage of meiosis was confirmed by Slizynska and Slizynski (1953), Melander and Knudsen (1953), Knudsen (1954, 1956), Leuchtenberger et al. (1956), Grimaldi (1956) and Fechheimer (1957).
The above studies were made with germinal tissue. Beatty and Rowson (1956), using corneal epithelium, concluded that the diploid number of chromosomes of cattle is sixty.

The morphological description of cattle chromosomes as accepted today, was first made in 1959. Melander (1959) observed the chromosomes of cattle in mitotic figures prepared from embryonic lung tissue. The sixty chromosomes were described by him as 58 acrocentric autosomes and two submedially inserted sex chromosomes, the X being among the largest in size and the Y among the smallest. Chiarelli (1960) confirmed the count of sixty chromosomes for cattle somatic tissue, using kidney cells. He described the X as submediocentric, but the Y was not identified, except as one of the smaller chromosomes. Sasaki and Makino (1962) used skin and kidney cells to prepare mitotic figures. Crossley and Clarke (1962), Nichols et al. (1962) and Herschler et al. (1962) used peripheral blood leukocytes in short term culture to prepare mitotic figures. All the analyses confirmed the count and the morphological description of Melander.

Chromosomal aberrations

Heteroploidy. Somatic chromosome aberrations such as variations from the normal diploid number are commonly found. Beatty (1951, 1954, 1957) has
reviewed the types of heteroploidy observed in mammalian somatic tissue. Polyploidy is common in somatic cells, occurring especially frequently in rapidly developing tissue. Aneuploidy may be attributed in some cases, to techniques of preparation of the mitotic figures. It may be found however, any time after the gastrulation stage of development. Heteroploidy may be induced by irradiation, temperature shock, colchicine treatment, or treatment with ether or ethyl alcohol (Beatty, 1951). Cell cultures are prone to increases in heteroploidy.

Endoreduplication. Endoreduplication is a special mode of origin of polyploidy in which the already duplicated chromosomes progress to the next interphase, omitting anaphase and telophase and are reduplicated when mitosis resumes, yielding four-stranded chromosomes. The condition has been reported in tumor cells by Levan et al. (1953) and in irradiated tissue by Bell and Baker (1962), Bell (1964) and Ohnuki et al. (1961). Levan et al. (1953) concluded that endoreduplication is caused by conditions in the culture medium. Bell and Baker (1962) observed that time is a factor in producing endoreduplication in irradiated cultures. Early harvest of the cultures following treatment, led to a higher frequency of endoreduplicated cells and a lower frequency of typical polyploids.
Centric fusion. Centric fusion is a special case of interchange of non-homologous chromosomal pieces following breakage, i.e. a reciprocal translocation. It can be induced by irradiation or chemical treatment according to Burnham (1962). White (pp. 65-67, 1954) has indicated that centric fusions are important in the evolution of animal species. He described the formation of an aneuploid condition as the result of centric fusion between two acrocentric chromosomes.

It is a translocation in which both chromosomes are broken very close to their centromeres. This is a special category of rearrangement as it involves transposition of virtually entire chromosome arms. If two acrocentrics, each consisting of a long limb "distal" to the centromere and a minute second limb "proximal" to it, breakage just occurring proximal to the centromere in one and distal to it in the other and a mutual translocation then takes place, the result is a large metacentric chromosome and a minute element which contains a centromere with a very short region on either side. Both "new" chromosomes will be viable, but the heterochromatic small element may be lost in subsequent generations, especially if its chiasma frequency is less than 1.0 so that it is inherited irregularly at meiosis.

Centric fusions have been observed commonly in species of Drosophila (Painter and Stone, 1935), grasshoppers (McClung, 1917), and reptiles (Matthey, 1945).
Nuclear sex differences

The sex of an individual can be described by the phenotypic sex, or the genotypic sex. In the normal individual, these are the same. However, in cases of an intersexual phenotype, the nuclear sex of individual cells of various tissues may be used to ascertain the genotypic sex.

A karyotype analysis of the chromosomes in various somatic tissues is the direct method of determining genotypic sex. Mitotic figures have been prepared from tissues of the internal organs, the digestive and reproductive tracts, skin, and muscle. Meiotic figures have been prepared from testicular and ovarian tissues. The X and Y chromosomes of most species can be distinguished from one another due to their size differences.

Recent techniques for tissue culture (Madin et al., 1957, Hancock et al., 1959, Moorhead et al., 1960), as well as the older methods of direct preparation from tissue, have aided in the increased use of karyotype analysis as a clinical procedure in cases of intersexuality.

Barr (1959, 1963) observed a chromatin body in the nucleus of 30-70% of the interphase cells of various tissues from human females. It was not observed in cells from males. Although first observed in nerve
tissue, its clinical use has been in smears from buccal mucosa. The presence of the sex chromatin body has been reported also in various other mammalian females (Moore, 1962) and in cattle specifically by Lang and Hansel (1959). The sex chromatin body is considered to be associated with the mammalian XX condition. For each additional X chromosome beyond the basic XX pair per cell, there should be one extra sex chromatin body. Thus in aneuploids such as an XXX cell, two sex chromatin bodies would be expected. In polyploids however, only one sex chromatin body is expected for each 2N number of chromosomes if the sex chromosomes are XX. Thus an XY polyploid cell would be expected to have no sex chromatin body in the interphase nucleus.

Davidson and Robertson-Smith (1954), and Davidson (1961, 1963) described the observation of a sex difference in polymorphonuclear leukocytes. The drumstick shaped lobule, which can be found in 2-10% of such leukocytes in females, is thus a means of determining the nuclear sex of individual cells and is used clinically to diagnose genotypic sex.
Embryology of the reproductive system

It is generally concluded (Arey, 1954; Watzka, 1963) that the embryology of the reproductive system is as described below. Sexual differentiation takes place in three phases (Grumbach and Dorcharme, 1960), first the gonad and then genital ducts and finally external genitalia. Primary sex cells migrate from the epithelium of the hindgut into mesenchyme beneath corresponding segments of the celomic epithelium, the urogenital ridge. The Mullerian duct appears as a groove in the epithelium of the urogenital ridge, whereas the Wolffian duct develops from the mesonephros. Developing gonads of both sexes possess in common a superficial germinal layer, deeper primary germinal cords (medullary cords), rete, and urogenital connections. Gonads differentiate under the influence of the genotypic sex of the embryo. Thus, if destined to become a male, primary sex cords develop into seminiferous tubules and interstitial cells are formed. The Wolffian duct differentiates into the vas deferens and vasa efferentia and accessory sex glands are formed as evaginations of it. Finally external male genitalia are formed from the urogenital sinus. Meanwhile, the Mullerian duct degenerates. If the embryo is destined to become a female, primary sex
cords degenerate and superficial germinal epithelium forms secondary sex cords (cortical cords). The Mullerian duct differentiates, forming the fallopian tubes, uterus and upper portion of the vagina. A corresponding degeneration of Wolffian ducts occur. The urogenital sinus develops into the vestibule and lower part of the vagina.

**Time sequences in development**

Early development of human and bovine gonads are very similar (Greenstein and Foley, 1958). The time sequence of bovine development is nearly identical to that of man, although the bovine embryo is progressively larger in size at corresponding stages of development (Winter, 1942). At forty days, the bovine embryo, 22.8 mm. in length, has undifferentiated gonads and duct system present. The corresponding human embryo of almost six weeks, though only 13 mm. in length, also has undifferentiated gonads and duct system.

In human embryos, sexual differentiation starts at 13 mm. in males, but at 18-20 mm. in females. Actual identification of sexes from observation of external genitalia cannot be made until 23 mm. (18 weeks). Lillie (1917) estimated that sexual differentiation starts at 25 mm. (45 days) in bovines.
At ten weeks, genital ducts of the non-genotypic sex begin degenerating. Interstitial cells are present in developing male gonads at the end of the second month and increase in number until the sixth month. After birth, they are not much in evidence, according to Watzka (1963) until puberty, when they increase in numbers. Seminal vesicles evaginate from Wolffian ducts at thirteen weeks and this is followed by development of the external genitalia.

**Intersexuality in man**

Stevenson (1961) estimated the frequency of stillbirths as 2.3% and of congenitally malformed livebirths as 2.5% in man, for a total of 4.8% as compared with 6.25% in cattle (Herschler et al., 1962). Harnden and Jacobs (1961) estimated the frequency of sexual malformations as 0.3% of all human births.

Abnormal sexual development in man has been classified cytogenetically by Harnden and Jacobs (1961). The five categories are: phenotypic males with normal sex chromosome constitutions, phenotypic males with aberrant sex chromosome constitutions, phenotypic females with normal sex chromosome constitutions, phenotypic females with aberrant sex chromosome constitutions, and true hermaphrodites.
Among the conditions described under the categories with aberrant sex chromosome constitutions are gonadal dysgenesis, Turner's syndrome and Klinefelter's syndrome.

Klinefelter's syndrome (Sohval, 1961, 1963, Harnden and Jacobs, 1961), observed in phenotypic males, is characterized by small atrophic testes and therefore eunuchoid proportions. Other somatic effects are also observed. The syndrome is associated with an aneuploid karyotype, having additional X chromosomes present in conjunction with the normal XY. This condition has been observed in mosaics with normal XY cells.

Turner's syndrome (Polani, 1961, Sohval, 1961, 1963) is characterized by maldevelopment of ovaries of phenotypic females and associated with an XO karyotype, a monosomic condition. No sex chromatin body is observed in patients with this syndrome, unless it is accompanied by a mosaic pattern, with XX cells present.

Both Turner's syndrome and Klinefelter's syndrome are considered to be examples of the result of non-disjunction. If all the cells are of the abnormal karyotype, the non-disjunction has probably taken place during meiosis. If a mosaic pattern has been observed, the non-disjunction occurred during mitosis. If the non-disjunction occurred after gastrulation, only certain tissues of the body will have the aberrant karyotype.
Gonadal dysgenesis is a catch-all phrase used to explain various intersexes which have streak gonads. In some cases aberrant karyotypes are observed, but in others, normal karyotypes have been found in all tissues studied. These latter cases may be explained as due to incomplete chromosomal analysis, or small aberrations such as minor deletions, duplications or translocations, or endocrine environment imbalances (Sohval, 1963).

True hermaphrodites, individuals with both testicular and ovarian tissue, also have been observed to be of varying types. Sohval (1963) cites 25 cases of true hermaphroditism reported in the literature, 19 of which were found to have the normal female (XX) karyotype. These cases may be explained by any one of several theories. One is (Ford, 1961) that a disturbance in the balance of sex genes may be quantitatively equal rather than predominantly male or female, hence local changes in the environment during gonadogenesis would govern the differentiation of the gonad. Other theories cited by Sohval (1963) are that of mutant gene effects similar to that shown to fit a case in the goat, and hormonal imbalances.

True hermaphrodites associated with karyotypes other than XX are known as follows: Sandberg et al. (1960) reported true hermaphroditism associated with XY
cells in marrow. Buccal mucosa was chromatin negative. Grumbach et al. (1960) also reported an XY karyotype (skin) associated with true hermaphroditism. Miller et al. (1961) reported true hermaphroditism associated with an XY/X0 mosaic leukocyte karyotype. Buccal smears were chromatin negative. Cells cultured from the left gonadal area where no gonad was found, yielded an X0 karyotype. The right gonadal mass contained seminiferous tubules, Leydig cells and ovarian stroma. Hirschhorn et al. (1960a,b) reported a true hermaphrodite infant with XY karyotype in skin cells and XY/X0 mosaicism in marrow. Fraccaro et al. (1962) observed a true hermaphrodite with a left ovotestis and right primitive testis. Cultures from skin, gonad and blood yielded XX, XXY, and XXYYY karyotypes, respectively. Schuster and Motulsky (1962a,b) observed an individual with a vestigial structure at the site of the left ovary and an histologically identified testis at the site of the right ovary. Both fallopian tube and ductus deferens were present on each side. Tissue was cultured from blood, skin of the arm, and skin of the clitoris. Resultant karyotypes were X0/XY in blood and no drumsticks, X0/XX in skin of the arm, buccal smears chromatin negative, and X0/XX from skin of the clitoris. The explanation offered was that of two somatic non-disjunctions,
the second occurring in a clone of X0 cells in the portion of ectoderm derived from the initial non-disjunction.

Gartler et al. (1962) and Waxman et al. (1962) have reported a true hermaphrodite with XX/XY mosaicism in many tissues. It is interesting to note the proportions of cell types in the various tissues, which appear to be associated with local sexual differentiation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>XX cells</th>
<th>XY cells</th>
<th>XY%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin-left abdomen</td>
<td>8</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Ovary-left side</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Skin-right abdomen</td>
<td>3</td>
<td>10</td>
<td>77</td>
</tr>
<tr>
<td>Ovotestis-ovarian</td>
<td>9</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Ovotestis-testicular</td>
<td>2</td>
<td>12</td>
<td>86</td>
</tr>
<tr>
<td>Clitoris</td>
<td>4</td>
<td>7</td>
<td>64</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>7</td>
<td>6</td>
<td>46</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

When a blood typing analysis indicated that the father had contributed both his blood group alleles, it was suggested that this case was the result of a double fertilization. A better explanation might be fusion of two zygotes, as the propositus was not 3N which would be the expectation from a double fertilization.
The freemartin
Anatomy. John Hunter (1786) first defined the freemartin as follows:

It is a fact known and I believe almost universally understood that when a cow brings forth two calves and one of them a bull-calf and the other to appearance a cow, that the cow-calf is unfit for propagation, but the bull-calf grows up into a very proper bull. Such a cow-calf is called in this country a Free-Martin and is commonly as well known among the farmers as either cow or bull.

The name freemartin, is thought to be derived from the idea that these animals are free from reproductive ability, Saint Martin being the patron saint of fecundity (Gilmore, p. 166, 1952). The Romans were familiar with barren cows, calling them "Taura", i.e. female bull (Hunter, 1786). Hart (1910) reported that in France the animal is called "Taur" and in Brabant, "Bouquetin".

Hunter (1786) described three freemartins and mentioned that a heifer calf born twin to a bull did conceive. Case 1 was five years of age when slaughtered. The vagina was blind, but the uterus was present and divided into horns, at the end of which were found testicular gonads described as abnormal. Case 2 upon slaughter was found to have both an ovary and a testis at the end of each uterine horn and seminal vesicles as well. Case 3 was four years of age when
slaughtered. It had a short vagina, blind uterus with horns and ovaries, but no testicular tissue. An interrupted vas deferens was observed. Hunter could thus report that the hermaphrodite condition was variable.

Sir John Y. Simpson (1872) observed a freemartin and found its anatomy comparable to that of Hunter's case number 2. The second gonad was interpreted as a Wolffian body.

Tandler and Keller (1911) in Germany, and Lillie (1916) in the United States described the condition more thoroughly and associated with bovine placental development. Lillie (1916, 1917a,b, 1922) observed bovine embryos with their placentae. Age varied from pregonadal to fetal stages. The condition itself was found to be variable, as Hunter had observed. Generally however, the freemartin was observed to have a normal vulva, short vagina, poorly developed non-patent uterus and a corresponding partial development of Wolffian derivatives including accessory sex glands. Gonads were described as ovaries, testes or ovotestes, but an histological survey by Chapin (1917) of eleven cases, did not indicate any but medullary sex cords. In some cases these primary cords resembled seminiferous tubules.
The important contribution to etiology of the freemartin syndrome was the observation (Lillie, 1916, Tandler and Keller, 1911) that the condition was associated with an anastomosis of placental blood vessels of the twin pair. (Between the 10 mm. and 20 mm. stages of development, developing embryos elongate and meet in the body of the uterus. Fusion of placentae follows and blood vessels from each side anastomose in the connecting part of the chorion.) A particularly wide anastomosis was usually observed hence each fetus could be both donor and recipient. Lillie (1916) concluded that a constant interchange of blood could therefore take place. In cases where the heifer calf had a normal reproductive tract, Lillie (1923) observed no vascular anastomosis; once scar tissue only was observed at the point of fusion of the placentae and another time no fusion at all was observed. Tandler and Keller were reported by Lillie (1919) to have found among 17 cases, only one in which no vascular anastomosis could be observed. That heifer had a normal reproductive tract.

Cases of severely masculinized freemartins have been reported. Rothe et al. (1961) and Rothe (1952) reported a pair of identical heifer freemartins triplet
to a bull. The heifers had an extremely masculine appearance, small vulva with enlarged clitoris, coarse vulval hair and palpable testes. Upon laparotomy, an undeveloped uterus was found with dangling fallopian tubes and no cervix or ovaries. The gonads were described as histologically normal testes.

Fraser-Roberts and Greenwood (1928) reported an extreme freemartin. The animal had a urethra containing penis projecting from the vulval area. The vasa deferentia were normal with seminal vesicles and other accessory sex glands present. The gonads, with epididymus and spermatic cord, were found to lie in peritoneal evaginations between the abdominal wall muscle and the skin. Histologically, the gonads were composed of small seminiferous tubules and many interstitial elements.

Intersexes resembling the freemartin condition have been reported in other domestic animals, but in very low frequency. Fraser-Roberts and Greenwood (1928), Ewen and Hummason (1947), Stormont et al. (1953), and Moore and Rowson (1958) have reported the condition in sheep. Davies (1913) observed goat hermaphrodites and concluded that certain types resembled the bovine freemartin. Hughes (1927, 1929) extensively
studied swine embryos and observed four cases of placental anastomosis accompanied by intersexuality.

**Hormone hypothesis.** Lillie (1917b) presented evidence that the freemartin, contrary to the hypothesis of Hart (1910), is a sterile genotypic female and one of dizygotic twins. Hart (1910) had postulated that the freemartin is a sterile male, one of monozygotic twins, the syndrome being explained by a Mendelian scheme of potent and non-potent genital determinants, the balance of which is upset when the zygote divides. Lillie (1917b) observed 55 cases of twins, only two of which were not monochorial. In 22 cases in which both ovaries were present, a corpus luteum was observed on each side. The conclusion was reached that monozygotic twins are rare in cattle. Sex ratios observed among twins when the freemartin was considered a genotypic female approximated expected values.

Lillie (1916) proposed an explanation of masculinization of the genotypic female based on hormonal exchange. The earlier developing male gonad produces a hormone which, entering the female via the placental anastomosis, causes a modification of ovaries and reproductive tract in a masculine direction. A precedent for his hypothesis was the conclusion of
Bouin and Ancel (1903) that interstitial cells have a trophic role locally vis-a-vis the sexual elements in the genital primordium.

Bissonette (1928), using three sets of triplets, tested the theory that degree of masculinization is correlated with the amount of male hormone present as measured by the number of males and females in triplet sets. No such correlation was observed.

Lillie (1923) reported a case in which only very fine capillaries were anastomosed, and concluded that size of the anastomosis and degree of masculinization were uncorrelated.

Willier (1921) and Bissonette (1924) categorized the different degrees of intersexuality using the gonads and tracts respectively. They both concluded that the variability was a function of time of introduction, and intensity or duration of action of male sex hormones.

**Endocrine experiments**

Hormone injections in laboratory mammals. Attempts were made to test the endocrine hypothesis by reproducing the freemartin condition in laboratory mammals. Moore and Price (1930) observed that there was no antagonism when male and female hormones were simultaneously injected into castrated rats. Testis hormone caused
accessory sex glands to develop to normal size in the presence of estrogen as well as alone. Green and Ivy (1937) observed the results of injecting testosterone and testosterone propionate in varying dosages into pregnant rats. Although many stillbirths and resorptions occurred, 27 normal males and 19 females with varying degrees of intersexuality were obtained. In a later report based on additional data, Green, Burrill and Ivy (1938a) presented observations of Wolffian derivatives present in association with fallopian tubes, uterus and vagina, which were normal. Hamilton and Wolfe (1938) obtained the same results and noted that the reproductive system was functional. Green, Burrill and Ivy (1938b) injected androsterone in varying dosages and again produced females with Wolffian duct derivatives next to the uterus, which developed normally. The degree of masculine development was correlated with the stage of pregnancy at which injections were made and with amount of hormone injected.

Moore (1941, 1944a,b), using the marsupial, opossum, after duplicating the above mentioned results with pregnant rats, attempted to alter the reproductive tract by treating new-born. Opossum was thought an ideal animal for embryological experimentation as it is born sexually undifferentiated, the gonad not being
recognizable as to sex until the third day postpartum. Androgens were applied, first as an ointment and later as injections. Results were similar to those obtained with indirect hormone treatments of rat embryos. In males, androgens stimulated the external genitalia and the accessory sex glands. In females, both Wolffian and Mullerian derivatives were stimulated. Estrogen injections in males also resulted in stimulation of both Wolffian and Mullerian derivatives. Moore (1941) could not stimulate production of sex hormones in opossum until day 70 postpartum in the male and day 100 in the female. This was much later than duct differentiation which occurred at day 30. Moore (1944a,b) concluded that sexual differentiation was under control of genotypic sex determining factors and not controlled by gonad secreted hormones. Moore (1950) hypothesized that certain stimulating humoral agents issue from the entire somatic complex of the organism and favor homologous duct development, and that the genotypic constitution may be overridden in cases such as that of the freemartin.

Gonadectomy. Moore (1941) had attempted gonadectomy in opossum and obtained normal duct development. Using rabbit, Jost (1947a) had first duplicated the injection experiments outlined above. Jost (1947b) however, did not obtain the same results as Moore
when he performed gonadectomy in embryonic rabbits. Rabbit embryonic gonad is sexually differentiated on day 15 post conception, and somatic parts, which are alike on day 19, are differentiated on day 26. By gonadectomy of rabbit embryos from day 19 through day 24 post conception, Jost was able to produce a series of demasculinized male tracts, the most severely demasculinized being 19 day castrates. This inhibition of Wolffian duct development was not matched by Mullerian duct inhibition in castrated females, the only variation from normal being a smaller diameter uterus. Mullerian ducts of early male castrates were not inhibited and as a result early male and female castrates developed similarly. Unilateral castration of males in early stages of duct development had no effect on Wolffian duct development, although some persistence of Mullerian duct on the castrated side was noted. Parabiosis experiments led to normal development in all cases. Jost (1947b, 1953, 1954) explained Moore’s gonadectomy results as being due to late castration. He concluded that fetal testis stimulates male duct development and causes degeneration of Mullerian ducts. As testosterone injections do not have this effect, fetal testis hormone is different in action from that of the mature gonad’s hormonal secretions. Burns (1949) however, noted that effects
similar to that of the bovine freemartin had not been demonstrated with laboratory mammals.

Gonad transplants and culture. Holyoke (1949), using rat, grafted embryonic ovaries into animals with testes. The testes grew normally, but ovaries developed an abnormal medullary portion, although cortex was normal. He concluded that ovary is more easily influenced by environment than is testis. Holyoke (1957), using embryonic rabbit gonad, made grafts to adult ovaries and testes. No abnormal differentiation was observed in either ovarian or testicular tissue. MacIntyre (1956) transplanted embryonic rat gonads, both ovary and testis, to a position side by side on the kidney of castrated adults. In the 25 pairs that grew well, the testis differentiated normally. All ovaries were adversely affected when compared with ovarian transplant controls. In nine cases, the ovarian portion (cortex) was inhibited almost completely, leaving only a few isolated sex cords and some poorly developed follicles. The 14 remaining cases had evidence in addition, of tubular structures similar to seminiferous tubules. Ovaries transplanted into non-castrated males developed normally as did embryonic gonads of rabbits as reported by Holyoke (1957). Turner and Asakawa (1964) obtained similar results with mice, and reported evidence of primary spermatogenesis in the ovotestis so produced.
Holyoke and Beber (1958) repeated the pairing experiment of MacIntyre with rabbit and rat embryonic gonads in tissue culture, after reproducing the effect obtained by MacIntyre with gonads transplanted into castrated rabbit hosts (Holyoke, 1956). In 17 cases of male-female pairs grown in vitro, embryonic ovaries developed abnormally, having no cell nests, but medullary cords. When ovaries were grown alone or in pairs, cortical differentiation proceeded normally. The conclusion was reached therefore, that gonadal inductors and adult sex hormones are not the same.

**Hormone injection in bovines.** Mason et al. (1957) and Bogart et al. (1958) reported an intersex heifer calf born in the course of a hormone treatment study of beef cattle. Two pregnant cows were treated with 1 mg. testosterone per kg. of body weight beginning the 35th day post conception. One animal gave birth to a normal male calf, the other produced an intersex heifer. This heifer had ovaries with pinhead follicles, short and thickened fallopian tubes, hypoplastic uterine horns, lacking normal coiling. The body of the uterus was missing, as was the cervix. The urethra was bifurcated with one duct emptying into the vagina and the other following the penis pathway to a point midway between the four teats. The heifer was considered
to be an example of the effect of sex hormones in production of the freemartin syndrome, contrary to results observed in experiments with laboratory mammals.

**Extent of the freemartin syndrome and identification**

The freemartin syndrome has been observed to extend further than possible hormonal exchanges via anastomosed chorionic blood vessels. These considerations as well as external symptoms of intersexuality have been used in early identification of freelmartins. Swett et al. (1940) recommended close observation of external anatomy of heifer calves of bisexed twin pairs. An enlarged clitoris and coarse vulval hair are indications of masculinization of the reproductive tract. Gilmore (p. 169, 1952) suggested inserting a test tube into the vagina of day old heifers to test for a shortened vagina, which is characteristic of the syndrome. Swett et al. (1940) suggested that after one month, mammary gland development is retarded and may be so designated by a practiced observer.

Owen (1945), and Owen et al. (1946), having noted Lillie's reports of an anastomosis of fetal blood vessels between twin embryos, observed that fraternal
bovine twins, as a consequence of anastomosis, had erythrocyte antigenic types that were identical. In addition, antigenic specificities could be delegated to two distinct populations of cells; a mosaic existed. Lazear et al. (1953) reported the frequency of vascular anastomosis in cattle twins as measured by erythrocyte antigen specificity, to be 91% of dizygotic twin pairs (101 of 111 pairs).

Billingham et al. (1952), and Anderson et al. (1951) observed that bovine fraternal twins, unlike their human counterparts, whether of like sex or unlike sex, are in some manner mutually tolerant to grafts of each other's skin. This effect corresponds to the anomalous conformity of erythrocyte agglutinin types. Of 42 fraternal twin pairs tested, 86% did not reject reciprocal skin grafts. The frequency is not unlike that reported by Lillie (1923) for chorionic anastomosis in twins, or Lazear et al. (1953).

Moore et al. (1957), using sex chromatin body to identify cell types, found no evidence of mosaicism in nerve cells of central nervous system in bisexed bovine twins. Ohuma and Nishikawa (1963) confirmed the above report with their findings in nerve tissue from three anatomically intersex heifer twins.
Fechheimer et al. (1963) observed a mosaic pattern of cell types among mitotic figures derived from cultured leukocytes of freemartins. Ohno et al. (1962) also reported mosaicism, in marrow of animals of bisexed bovine twin pairs. Makino et al. (1962) did not observe mosaicism in bovine bisexed twins in either leukocytes or lung tissue, but their study was based on a small number of observations.

Ohno et al. (1962) have reported possible mosaicism in testicular tissue of males twin to freemartins. It was concluded that the mitotic figures observed were not from leukocytes.

**Fraternal twin mosaicism in other mammals with vascular anastomosis**

Mosaics that result from placental blood vessel anastomosis, have been reported in various species. Stormont et al. (1953) and Moore and Rowson (1958) reported such in sheep. In both instances, the females were intersexes. Hughes (1929) observed placental fusions in swine, which were associated with intersex females. The frequency of anastomosis however, is very low for these two species. In marmoset, in which twinning occurs frequently, fetal vascular anastomosis is the rule rather than the exception (Wislocki, 1939).
No effect on the reproductive tract was observed however. Benirschke et al. (1962a,b) reported marrow mosaicism in marmoset bisexed twins, but no evidence of mosaicism in lung or kidney tissue.

Human mosaics have been reported occasionally. Dunsford et al. (1953) reported that a fertile woman had 60% type 0 cells and 40% type A cells. Later, Dunsford and Stacey (1956) observed that there was a partial breakdown of the acquired tolerance to A antigen when the woman became pregnant for the fourth time. Booth et al. (1957) reported a set of fraternal twins, each member with both A and 0 blood types. The ratios reported were 86% A, 14% 0 for the male twin and 1% A, 99% 0 for the female twin. W.M. Davidson was asked to study the polymorphonuclear leukocytes of the male twin. He observed drumsticks in a proportion compatible with the mixture of cell types as shown by blood group analysis. Nicholas et al. (1957) reported a set of fraternal twins with mosaicism of cell types. Using antigenic methods, they demonstrated mosaicism involving the ABO, MN, and rhesus systems. In addition, drumsticks were observed in 6 of 318 polymorphonuclear leukocytes from the female, and 6 of 338 from the male. Davidson et al. (1958) studied blood films of women previously shown to have mixed populations of erythrocyte antigen
types. Neutrophils were counted until six drumsticks were found. These counts were compared with normal women's counts and the proportion of female cells estimated. The results correlated well with the proportions expected from antigen admixture studies. In two cases where both members of a twin pair were available, the proportions of cells of each type were not the same for both of the twins. Woodruff et al. (1962) also presented data which indicate that the proportion of cell types is not the same in members of a twin pair. Lewis et al. (1963) however, reported a pair of human fraternal twins with similar proportions of erythrocyte antigen types. In addition, the percent of leukocytes and erythrocytes of the two types were of the same magnitude. Fechheimer et al. (1963) reported that in cattle twins, proportions of cells of the two types of leukocytes were of the same magnitude in both animals.

Rendel et al. (1962) in a study of cattle quintuplets, four females and one male, observed differences in proportions of various erythrocyte antigen types. Two females had the same proportions of cell types as the male twin and were greatly masculinized as compared with the other two females which had a different erythrocyte antigen ratio. Such a difference in
proportions of erythrocyte antigen types had not been observed by Owen et al. (1946) who reported a set of quintuplet calves with all five members of the set having lysis reactions to A antigen for 83.8 - 85.3% of cells. Rendel explained his different admixture ratios as due to later anastomosis of the two more normal twins to the circulation of the male than that of the more masculinized females to the male. Stone et al. (1963) have indicated that the proportions found in a mosaic of cell types has a tendency to shift over time.

Ryan et al. (1961) gave an explanation for the intersexual nature of bovine mosaic females, as compared with reproductively normal humans and marmosets. They demonstrated the presence of an enzyme that converts androgen to estrogen in the placenta of marmosets. The enzyme could not be demonstrated in bovine placental tissue.

Zeitschmann and Krolling, as cited by Koch (1963), suggested tentatively, that intersexual anomalies such as the freemartin, are blastogenically or chromosomally determined at a time when the chorion is avascular. In that case, vascular anastomosis would be either an effect of or co-incident with the condition rather than a cause.
Animals involved

The animals involved in this study were cattle from institutional and private herds in Ohio. Singles were obtained mainly from The Ohio State University dairy herd; three singles were procured from private individuals. Bisexed multiple birth animals were located in The Ohio State University herd, The Ohio Agricultural Experiment Station herd, and through the cooperation of the Ohio North Central Dairy Cattle Breeding Project, in the State of Ohio institutional herds. In addition, animals on private farms were located through cooperation with the cattle blood typing laboratory at The Ohio State University, which receives requests periodically for blood typing analysis of twin pairs, and through the efforts of various individuals.

Blood samples were taken from the animals involved in this study and leukocyte cultures were set up. The remainder of each sample was then used for blood typing analysis by the cattle blood typing laboratory. If the two analyses indicated placental anastomosis, the heifer was considered a freemartin and an attempt was made to obtain her reproductive tract.
Purchases were made of privately owned animals and these were sold to and slaughtered by The Ohio State University, Department of Animal Science, Meats Division. Animals belonging to The Ohio Agricultural Experiment Station that were considered freemartins were also slaughtered by the Department of Animal Science, Meats Division. From these animals, the reproductive tracts were obtained and photographed. In some cases the gonads were preserved, sectioned, and analyzed histologically. In addition, kidney tissue was obtained and cultures of kidney cells attempted.

Animals belonging to The State of Ohio institutional herds that were considered freemartins, were slaughtered at the London Correctional Institution, London, Ohio. From these animals, the reproductive tracts were obtained and photographed and the gonads in a few cases were preserved, sectioned and analyzed histologically. As it was not possible for me to be present when the slaughter took place at the London Correctional Institution, the tracts were removed by the attending veterinarian.

**Leukocyte cultures**

**Preparation of the culture.** The technique to be described here is an adaptation for cattle leukocytes of the technique described by Moorhead *et al.* (1960)
for the culture of human peripheral blood leukocytes. This culture technique was made possible by the discovery of Nowell (1960) that phytohemagglutinin, a plant extract, is an initiator of mitosis in cultures of human leukocytes.

Thirty ml. blood samples are drawn into sterile Vacutainer tubes by inserting a 20 gauge needle in the jugular vein. Each tube contains 0.1 - 0.2 ml. sodium heparin (1000 units/ml.). The tubes are then stored in a refrigerator or a bucket of ice for at least 15 minutes. (For samples obtained at a great distance from the laboratory, the storage time is up to five hours.)

The refrigerated tubes are placed in a centrifuge for five minutes at 1500 rpm. (350 x G), five degrees C. The plasma and buffy-coat, which contains the leukocytes, with some erythrocytes is removed with a sterile pasteur pipette, placed in sterile tubes, and left at room temperature for 1½ hours. The tubes are then centrifuged at room temperature for two minutes at 300 rpm. (9 X G). With a pasteur pipette, a three ml. aliquot of the supernatant plasma and leukocyte mixture is placed in a sterile 100 ml. screw top culture bottle containing seven ml. TC 199 (Difco), a synthetic culture medium prepared according to the formula of Morgan et al. (1950), and containing in addition one ml. antibiotic
mixture per 100 ml. culture medium. The antibiotic mixture consists of 10,000 units aqueous penicillin, 10,000 micrograms dihydrostreptomycin and 5,000 units nystatin per 100 ml. Bactophytohemagglutinin P (Difco) in the amount of 0.1 ml. is added to each culture bottle. The cultures are placed upright in an incubator oven for 72 hours at 37 degrees C.

Harvest of cell cultures and preparation of mitotic figures. At the end of 68 - 70 hours, 0.1 ml. 0.05% colchicine in triple distilled water is added to each culture bottle. The cultures are reincubated for 2 - 4 hours. The culture medium is then decanted into centrifuge tubes (non-sterile) and centrifuged for five minutes at room temperature, 800 rpm. (142 X G). The supernatant liquid is removed with a pasteur pipette and cells are washed in prewarmed (37 degrees C.) Hanks' balanced salt solution (Hanks and Wallace, 1949). The resulting cell suspension is again centrifuged at 800 rpm. (142 X G) for five minutes at room temperature. Following removal of all but 0.5 ml. of the supernatant liquid, 1.5 ml. prewarmed (37 degrees C.) distilled water is added. The cell suspension is incubated at 37 degrees C. for four minutes and then centrifuged at room temperature for four minutes, 600 rpm. (84 X G). The supernatant liquid is removed and 4 - 5 ml. of fresh cold fixative (three parts methanol to one part
glacial acetic acid) is carefully added so as not to disturb the cells at the bottom of the tube. The tubes are then refrigerated for thirty minutes. After resuspending the cells, the suspension is centrifuged at five degrees C. for five minutes, 600 rpm. (84 x G). The supernatant liquid is removed and fresh fixative added in an amount necessary to obtain a concentrated cell suspension.

Two drops of cell suspension are placed on a clean slide that has been filmed with iced distilled water. The slides are dried either by waving horizontally in front of an electric fan (Rothfels and Siminovitch, 1958) or by burning off the fixative (Scherz, 1962).

The slides are stained with a 1% solution of aceto-orcein, prepared according to the formula of Darlington and LaCour (1950) and mounted in piccolyte.

**Kidney cell cultures**

The technique used for preparation of bovine kidney cells for culture was an adaptation of the technique of Hancock (1957), and Hancock et al. (1959) for culture of swine kidney cells. The monolayer technique has been used in the culture of many tissues of domestic and laboratory animals (Madin et al. (1957).

Bovine kidney was obtained immediately upon slaughter and returned to the laboratory in a sterile
covered pan. Working under a sanitized hood, the kidney was removed to a second sterile pan with sterile forceps. The membrane covering the kidney was cut away and slices made in the cortical portion with a sterile scalpel. The slices were cut away and removed to a sterile petri dish containing prewarmed (37 degrees C.) sterile Hanks' balanced salt solution (Hanks and Wallace, 1949). Remainder of the kidney was discarded. The slices were transferred to a sterile 50 ml. centrifuge tube and minced with a double pointed pair of sterile scissors. Fresh prewarmed Hanks' solution was added and the mixture decanted into a sterile 125 ml. Erlenmeyer flask. The mixture was swirled after addition of more sterile Hanks' solution to the 50 ml. mark, and the supernatant liquid decanted. Prewarmed (37 degrees C.) sterile trypsin in Hanks' solution (0.25%) was added to the 75 ml. mark and swirled, decanted and the process repeated. (The trypsin (Difco 1:250) was prepared as a 1% solution in Hanks' solution, kept frozen and diluted before use. The trypsin solution was kept at pH 7.6 - 7.8 by addition of 4.4% sodium bicarbonate.)

After the second trypsin rinse, the flask of minced kidney was placed on a magnetic stirrer at 27 degrees C. Slow agitation in the trypsin solution dissolves the intercellular cementum of kidney tissue. After 30 - 40
minutes, the kidney pieces had a fuzzy appearance. The supernatant liquid was decanted and the rinses with Hanks' solution and 0.25% trypsin solution repeated. The final trypsin rinse was again agitated slowly on the magnetic stirrer until the kidney appeared fuzzy. (If room temperature is lower than 27 degrees C., agitation time must be increased.)

The supernatant liquid was decanted into a sterile beaker through sterile gauze, placed in sterile centrifuge tubes and centrifuged at room temperature for eight minutes, 600 rpm. (84 x G). The supernatant liquid was removed and the cells suspended in prewarmed (37 degrees C.) sterile TC MK medium (Difco, Melnick, 1955), a lactalbumin hydrolysate medium containing in addition, one ml. antibiotic stock solution and five ml. bovine serum per 100 ml. The concentration of cells (¹/₄ ml. in five ml. of medium) was 5%. A ten ml. aliquot of cell suspension was incubated in a 100 ml. sterile screw top culture bottle placed on its side in a 37 degrees C. incubator oven. After three days, the medium was changed by decanting the supernatant liquid and adding 10 ml. of prewarmed TC MK medium. The face of the culture bottle was covered with kidney cells by this time. Then 0.1 ml. colchicine (0.05% in triple distilled water) was added to the medium and the culture reincubated overnight.
Harvesting procedures were similar to that employed for leukocytes, after the cells had been scraped from the glass surface with a rubber tipped glass rod. Swelling of cells was accomplished by either addition of distilled water (Moorhead et al. 1960) or incubation with prewarmed 0.7% sodium citrate solution for ten minutes (Fraccaro et al., 1960). Slide preparation and staining techniques were the same as for leukocytes.

Cytological analysis

Slides were scanned under a low power objective (10X) and mitotic figures studied under an oil immersion objective (98X). When available, phase contrast objectives were used to add to the clarity of the morphological delineation of the mitotic figures.

Polyploid cell frequency was determined while scanning slides in regular order under a low power objective. Chromosome counts, morphological descriptions of the autosomes and sex chromosomes, identification of aneuploidy, centric fusion and mosaicism, and the photographs of mitotic figures were made with an oil immersion objective. Karyograms were prepared from enlarged photographs.

Erythrocyte antigen analysis

Personnel of the cattle blood typing laboratory, under Dr. D.F. Weseli, made the analysis for evidence of erythrocyte antigen admixture, an indication of
placental anastomosis in twins. If partial lysis occurred with any reagent that was expected to yield an all or none reaction, the sample was considered to give evidence of erythrocyte antigen admixture. In addition, an identical blood type for members of a dizygotic twin pair was considered to be evidence of erythrocyte antigen admixture.

Reproductive tract analysis

Reproductive tracts of freemartins were obtained at slaughter, extraneous tissue cleaned away, and photographed. A value of masculine development was given to each tract based on gross and histological observations. Categories were determined by grouping the similar reproductive tracts. Four groupings were made. The categories are given values 1 - 4 according to degree of deviation from the normal female reproductive tract. It is assumed that a highly masculinized tract shows no evidence of Mullerian duct development and has a high degree of Wolffian duct development. The gonads are testicular or ovarian. (Category 4). An intermediate tract is one with both Mullerian duct and Wolffian duct derivatives partially developed. A tract with testicular gonadal tissue is considered Category 3. A tract with ovarian gonadal tissue alone is considered Category 2. A reproductive tract with inadequate development of
the Mullerian duct derivatives, but with no evidence of Wolffian duct development is considered to be masculinized to a slight degree (Category 1).

**Statistical analysis**

Statistical tests for possible correlations of percentage of cells of male origin between paired members of multiple birth sets showing sex chromosome mosaicism, and of degree of masculinization of the freemartin's reproductive tract with the proportion of leukocytes of male origin observed in the multiple birth set, were taken from the text by Snedecor (pp. 173-177, 1955).

The sample correlation coefficient \( r \) was calculated and a test of the hypothesis, \( \rho = 0 \) was made using the test of Fisher as described by Snedecor. Confidence limits, based on the sample from which \( r \) was calculated, were set on the value of \( r \).

The chi square test of heterogeneity (Snedecor p. 214, 1955) polyploidy frequency among individuals, was used to determine if the variation observed was due to factors other than chance.
RESULTS

Chromosome number and morphology

Mitotic figures prepared from cultured leukocytes of a bovine male and female and karyograms prepared from the mitotic figures, are illustrated in Figures 1, 2, 3, and 4. Sixty was decided upon as the normal diploid number of chromosomes because it was found in 70% of the cells studied (861 of 1230). The 58 autosomes are acrocentric, the short arms, well illustrated in Figure 1, appearing as "knobs" on the otherwise U-shaped chromosomes. Only the sex chromosomes have submedially inserted centromeres. In metaphase figures from a normal male, the X and Y chromosomes are easily identified as the X is among the largest of the chromosomes and the Y among the smallest.

Aneuploidy was estimated at 12.3% (122 of 994 cells) by assuming an equal number of 60+ and 60- cells. The rationale was that broken cells, i.e. aneuploidy caused by treatment of cells during harvest of cultures, would be eliminated from the analysis. Aneuploidy is caused by mitotic non-disjunction which should yield a monosomic cell for each trisomic cell. An aneuploid cell (66 chromosomes) and its karyogram are illustrated in Figures 5 and 6.
Figure 1

Metaphase figure of leukocyte of male origin
Figure 2
Karyogram of the previous metaphase figure
Figure 3

Metaphase figure of leukocyte of female origin
Figure 4

Karyogram of the previous metaphase figure
Figure 5

Aneuploid metaphase figure of leukocyte of male origin with 66 chromosomes
Figure 6

Karyogram of previous aneuploid metaphase figure
Polyploidy was calculated at 3.45% by counting 500 dividing cells in each of ten animals and 1000 in another. The range of the eleven values was from 0.4% to 5.6%. Table 1 contains the data. A test of heterogeneity was significant at the 0.01 level. Thus the variation in polyploidy from one animal to another was due to factors other than chance. No analysis of the variation was made. Figure 7 is a mitotic figure of a tetraploid cell of male origin.

Figure 8 is a mitotic figure of a polyploid cell of the type thought to arise by endoreduplication. Note that each chromosome has a replicated mirror image. This single example was the only such polyploid observed.

Centric fusion

Figures 9, 10, and 11 are mitotic figures and a karyogram, of cells of female origin, from a freemartin triplet with a presumed centric fusion of two acrocentric autosomes. The "new" submedially inserted chromosome is larger than the X chromosome. Presumably, the minute element, formed from the fusion of the short arms of the acrocentric chromosomes has been lost as all the 25 cells of female origin observed had this aberration and only 59 chromosomes. This presumed centric fusion was also observed in the three cells
Table 1

Frequency of polyploid cells among cultured leukocytes from eleven animals, arranged in order of increasing percentage of polyploid cells.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Sex</th>
<th>Number Polyploid Cells</th>
<th>Number Diploid Cells</th>
<th>Total Number Cells</th>
<th>Percent Polyploid Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-101</td>
<td>F</td>
<td>2</td>
<td>498</td>
<td>500</td>
<td>0.4</td>
</tr>
<tr>
<td>C-109</td>
<td>F</td>
<td>11</td>
<td>489</td>
<td>500</td>
<td>2.2</td>
</tr>
<tr>
<td>C-100</td>
<td>M</td>
<td>12</td>
<td>488</td>
<td>500</td>
<td>2.4</td>
</tr>
<tr>
<td>C-102</td>
<td>M</td>
<td>15</td>
<td>485</td>
<td>500</td>
<td>3.0</td>
</tr>
<tr>
<td>C-85</td>
<td>F</td>
<td>34</td>
<td>966</td>
<td>1000</td>
<td>3.4</td>
</tr>
<tr>
<td>C-70</td>
<td>F</td>
<td>17</td>
<td>483</td>
<td>500</td>
<td>3.4</td>
</tr>
<tr>
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<td>F</td>
<td>19</td>
<td>481</td>
<td>500</td>
<td>3.8</td>
</tr>
<tr>
<td>C-93</td>
<td>F</td>
<td>22</td>
<td>478</td>
<td>500</td>
<td>4.4</td>
</tr>
<tr>
<td>C-91</td>
<td>F</td>
<td>23</td>
<td>477</td>
<td>500</td>
<td>4.6</td>
</tr>
<tr>
<td>C-96</td>
<td>F</td>
<td>24</td>
<td>476</td>
<td>500</td>
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</tr>
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<td>C-72</td>
<td>M</td>
<td>28</td>
<td>472</td>
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<td>5.6</td>
</tr>
</tbody>
</table>

\[ X^2 = 31.4772, \text{ d.f. } = 10, \text{ p}<.0005 \]
Figure 7

Tetraploid metaphase figure of leukocyte of male origin
Figure 8

Metaphase figure of leukocyte of male origin with 60 endoreduplicated chromosomes
Figure 9

Metaphase figure of leukocyte of female origin with presumed centric fusion of two autosomes
Figure 10

Karyogram of previous metaphase figure with "new" submediocentric autosome
Figure 11

Early metaphase figure of leukocyte of female origin with presumed centric fusion of two autosomes
of female origin found in leukocyte cultures from the two male triplets of the set. No such aberration was observed in the cells of male origin found in leukocyte cultures from the triplets. The dam was observed to have only normal cells of female origin.

**Sex chromosome mosaicism**

A mosaic pattern of cells of male origin (XY) and cells of female origin (XX) was observed in the mitotic figures prepared from cultured leukocytes of animals from bisexed multiple birth sets. No such mosaic pattern was observed in mitotic figures prepared from cultured leukocytes of single born animals. The animals observed for sex chromosome mosaicism are listed in Table 2, with the numbers of leukocytes of male origin and female origin, the percentage of leukocytes of male origin, and the results of an analysis for erythrocyte antigen admixture. Among the thirty bisexed multiple birth sets from which animals were tested, only three gave no evidence of either sex chromosome mosaicism or erythrocyte antigen admixture. Thus 90% of heifers from bisexed multiple birth sets were considered freemartins. Two thirds of 21 freemartins, for which at least eleven metaphase figures were analyzed, were found to have a preponderance of leukocytes of male origin. A male triplet, for
Table 2

Animals tested for mosaicism of male and female origin cells.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Sex</th>
<th>Erythrocyte Antigen</th>
<th>Cytogenetic Analysis</th>
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<tr>
<td></td>
<td></td>
<td>Admixture</td>
<td>Number of Cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>XX</td>
</tr>
</tbody>
</table>

Multiple birth sets with more than eleven cells per set.

- **C-21**  F  Untested  12  3  15  20.0
- **C-29**  F  Untested  5  9  14  64.3
- **C-55**  M  Positive  7  30  37  81.1
- **C-58**  F  0  5  5  100.0
- **C-56**  F  Positive  6  33  39  84.6
- **C-57**  F  Positive  6  16  22  66.7
- **C-59**  F  Positive  31  32  63  50.8
- **C-60**  F  Positive  8  24  32  75.0
- **C-61**  F  Positive  0  21  21  100.0
- **C-68**  F  Positive  16  4  20  20.0
- **C-69**  M  17  3  20  15.0
- **C-70**  F  Positive  8  22  30  73.3
- **C-71**  M  2  28  30  93.3
- **C-72**  M  1  31  32  96.9
- **C-80**  F  Negative  30  0  30  0.0
- **C-81**  M  0  30  30  100.0
- **C-82**  F  Positive  12  18  30  60.0
- **C-83**  M  2  1  3  33.3
- **C-84**  F  Negative  24  6  30  20.0
- **C-89**  M  Positive  9  21  30  70.0
- **C-90**  F  11  19  30  63.3
- **C-77**  M  Positive  24  6  30  20.0
- **C-91**  F  103  3  106  2.8
Table 2 (contd.)

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<th>Value1</th>
<th>Value2</th>
<th>Value3</th>
<th>Value4</th>
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<td>30</td>
<td>100.0</td>
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<tr>
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<td>30</td>
<td>60.0</td>
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</tbody>
</table>

Multiple birth sets with less than twelve cells per set.

<table>
<thead>
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<th>Value1</th>
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<th>Value3</th>
<th>Value4</th>
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<tbody>
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<td>7</td>
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<td>C-28</td>
<td>F</td>
<td>Untested</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>62.5</td>
</tr>
<tr>
<td>C-31</td>
<td>M</td>
<td>Positive</td>
<td>3</td>
<td>8</td>
<td>11</td>
<td>72.7</td>
</tr>
<tr>
<td>C-40</td>
<td>M</td>
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<td>5</td>
<td>5</td>
<td>100.0</td>
</tr>
<tr>
<td>C-53</td>
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<td>0</td>
<td>4</td>
<td>0.0</td>
</tr>
<tr>
<td>C-73</td>
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<td>Negative</td>
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<td>0</td>
<td>10</td>
<td>0.0</td>
</tr>
<tr>
<td>C-75</td>
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<td>Positive</td>
<td>4</td>
<td>7</td>
<td>11</td>
<td>63.6</td>
</tr>
</tbody>
</table>
Table 2 (contd)

**Single-born animals with more than ten cells per animal.**

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<th></th>
<th></th>
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<th></th>
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<td>11</td>
</tr>
<tr>
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<td>23</td>
</tr>
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<td>C-26</td>
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<td>Untested</td>
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<td>0</td>
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<td>C-85</td>
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<td>Negative</td>
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</tbody>
</table>

**Single-born animals with less than eleven cells per animal.**

<p>| | | | | |</p>
<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
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<tr>
<td>C-8</td>
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</tr>
<tr>
<td>C-12</td>
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<td>Untested</td>
<td>4</td>
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<td>C-16</td>
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<td>0</td>
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<tr>
<td>C-17</td>
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<td>Untested</td>
<td>2</td>
<td>0</td>
</tr>
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</table>
which five metaphase figures were analyzed, was found to have no leukocytes of female origin, contrary to expectation, as the erythrocyte antigen admixture analysis had been positive. Unfortunately, no growth was obtained in cell cultures from the two heifer triplets. In another case, a female twin did not have evidence of erythrocyte antigen admixture, but did have a mosaic pattern of leukocytes of male and female origin. In this case, the male twin was not available for study so that admixture could not be documented on the basis of identical blood types for the two animals of the pair.

It was noted that within each multiple birth set for which the heifer was considered a freemartin, that the percentages of cells of male origin were of the same magnitude for all animals. Table 3 contains a statistical test of the correlation of percentage of cells of male origin between paired members of multiple birth sets with sex chromosome mosaicism. The arcsine transformation was used to adjust for the difference in variance among percentages. For the triplet and quintuplet sets, each animal was paired with each of its sibs. As these sets had been considered three-zygote and five-zygote sets by color pattern analysis, the growth of cells of male and female origin would be compared among
Table 3

Correlation of percentage of cells of male origin between paired members of multiple birth sets.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>% XY cells</th>
<th>Arcsine transf.</th>
<th>Animal No.</th>
<th>% XY cells</th>
<th>Arcsine transf.</th>
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<td>9.63</td>
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<td>26.56</td>
</tr>
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<td>C-68</td>
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</tr>
<tr>
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<td>C-107</td>
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</tr>
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<td>58.89</td>
<td>C-71</td>
<td>93.3</td>
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<td>96.9</td>
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$\epsilon_{xy}=6286.52; \epsilon_{x^2}=8376.35; \epsilon_{y^2}=5913.08; r=0.893$

$H:0, \epsilon=0; t=8.403, \text{d.f.}=18, p<.01; 95\% \text{conf. int.}=0.745-.957$
animals with 50% of their genes in common, just as with the dizygotic twin pairs. The correlation of percentage of cells of male origin between the paired members of multiple birth sets with mosaicism is 0.893. This sample correlation is significantly different from zero, so that a positive correlation is assumed for the population as a whole (t=8.403, d.f.=18, p<.01). The 95% confidence interval around the sample correlation coefficient is 0.745 - 0.957.

Table 4 is a list of the multiple birth sets from which the freemartin's reproductive tract was available. The sets are categorized according to the degree of masculinization of the freemartin's reproductive tract. The categories are as described in the Materials and Methods (p. 42). Examples of the various categories, with a normal female tract for comparison, are shown in Figures 12, 13, 14, 15, and 16. The individual cases cited in Table 4 are described in the appendix.

Table 5 contains a statistical test of the correlation of percentage of cells of male origin in the multiple birth set with the degree of masculinization of the freemartin's reproductive tract. The percentage of cells of male origin for the multiple birth set, because of larger numbers, was used as a more accurate estimate of the true percentage of cells of male origin. The arcsine transformation was then used to adjust for
Table 4

Multiple birth sets categorized by degree of masculinization of the freemartin's reproductive tract.

<table>
<thead>
<tr>
<th>Category</th>
<th>Animal No.</th>
<th>No. of cells per set</th>
<th>Percent XY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>XX</td>
<td>XY</td>
</tr>
<tr>
<td>1</td>
<td>C-68,69</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>C-77,91</td>
<td>127</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>C-123</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>C-115,116</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>C-100,101</td>
<td>36</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>C-124</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>C-29</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>C-60</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>C-106</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>C-89,90</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>C-70,71,72</td>
<td>11</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>C-55,58</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>C-61</td>
<td>0</td>
<td>21</td>
</tr>
</tbody>
</table>
Figure 12
Normal female reproductive tract
(18 mo. old single born heifer)
Figure 13

Category 1. Inadequate development of the Mullerian duct derivatives; no evidence of Wolffian duct development.
Figure 14

Category 2. Both Mullerian duct and Wolffian duct derivatives partially developed; ovarian gonadal tissue only.
Category 3. Both Mullerian duct and Wolffian duct derivatives well developed; Testicular and ovarian gonadal tissue present.
Figure 16

Category 4. No evidence of Mullerian duct derivatives; High degree of Wolffian duct development; Gonadal tissue testicular and/or ovarian.
Table 5

Correlation of percentage of cells of male origin of the multiple birth set, with the degree of masculinization of the freemartin's reproductive tract.

<table>
<thead>
<tr>
<th>Animal Nos.</th>
<th>% XY cells</th>
<th>Arcsine transf.</th>
<th>Masc. Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-68,69</td>
<td>17.5</td>
<td>24.7</td>
<td>1</td>
</tr>
<tr>
<td>C-77,91</td>
<td>6.6</td>
<td>14.9</td>
<td>2</td>
</tr>
<tr>
<td>C-123</td>
<td>26.7</td>
<td>31.8</td>
<td>2</td>
</tr>
<tr>
<td>C-115,116</td>
<td>50.0</td>
<td>45.0</td>
<td>2</td>
</tr>
<tr>
<td>C-100,101</td>
<td>55.6</td>
<td>48.2</td>
<td>2</td>
</tr>
<tr>
<td>C-124</td>
<td>60.0</td>
<td>50.8</td>
<td>2</td>
</tr>
<tr>
<td>C-29</td>
<td>64.3</td>
<td>53.3</td>
<td>2</td>
</tr>
<tr>
<td>C-60</td>
<td>75.0</td>
<td>60.0</td>
<td>3</td>
</tr>
<tr>
<td>C-106</td>
<td>56.7</td>
<td>48.9</td>
<td>4</td>
</tr>
<tr>
<td>C-89,90</td>
<td>60.0</td>
<td>50.8</td>
<td>4</td>
</tr>
<tr>
<td>C-55,58</td>
<td>83.3</td>
<td>65.9</td>
<td>4</td>
</tr>
<tr>
<td>C-70,71,72</td>
<td>88.0</td>
<td>69.7</td>
<td>4</td>
</tr>
<tr>
<td>C-61</td>
<td>100.0</td>
<td>90.0</td>
<td>4</td>
</tr>
</tbody>
</table>

n=13; ∑ xy=182.82; ∑x²=4584.63; ∑y²=14.31; r=0.714

H:0, ρ=0; t=3.3844, d.f.=11, p<.01

95% conf. int. = 0.269-0.907
the difference in variance among percentages. The correlation of 0.714 was found to be significantly different from zero, so that a positive correlation is assumed for the population as a whole \((t=3.384, \text{ d.f.}=11, p<.01)\). The 95% confidence interval around the sample correlation is 0.269 - 0.907. This large interval is attributable to small sample size.

**Kidney cell cultures**

Kidney cell cultures, attempted after slaughter, were successful in two cases, both from females of bisexed twin pairs. As was expected, the number and morphology of the chromosomes were the same as that obtained with the leukocyte cultures. Figure 17 is a mitotic figure prepared from a kidney cell culture of a bovine freemartin. In neither culture was sex chromosome mosaicism observed (see appendix, cases C-91 and C-123) in six and 27 cells respectively. Although mosaicism was such that 26.7% of leukocyte cells of C-123 were of male origin, no kidney cells of male origin were observed, an indication that the kidney is not affected by the mosaic condition.

**Artificially produced mosaicism**

Two cultures of leukocytes were set up with cells from unrelated animals of the opposite sex in each culture. Normal controls for each animal were also
Figure 17

Metaphase figure of kidney cell of female origin
prepared. In both of the mixed cell cultures, a mosaic pattern of cells of male and female origin was observed. Both cell types grew well. As an equal amount of cell suspension from each animal had been added to the culture medium, it was concluded that variation in percentages of cell types observed in freemartin cell cultures was not due to culture environment.

**Hypothesis**

The results reported here, lead to the postulation of an hypothesis that the freemartin is not the result of a hormone from the early developing male gonad, which masculinizes the gonad and reproductive tract of the female to which it is connected by a vascular anastomosis, but that the presence of the Y chromosome in hematopoietic tissue is an indication of a direct effect of the Y chromosome on freemartin sexual development.
DISCUSSION

Chromosome number and morphology

The diploid number of chromosomes in somatic cells of cattle as arrived at from the observations presented here, agrees with many previous reports in the literature (Beatty and Rowson, 1956; Melander, 1959; Chiarelli et al., 1960; Nichols et al., 1962; Crossley and Clarke, 1962; Sasaki and Makino, 1962; Ohno et al., 1962). The morphological description given here is similar to recent descriptions of cattle chromosomes (Melander, 1959; Sasaki and Makino, 1962; Ohno et al., 1962; Crossley and Clarke, 1962; Nichols et al., 1962). The short arms of the acrocentric autosomes have been well delineated by use of the flame drying technique of Scherz (1962). This technique however, causes a great deal of cell breakage. Broken cells account in part for the percentage of normal diploid cells reported. Broken cells were counted when the sex chromosomes were both present, so that the number of cells used in the mosaicism analysis might be increased. Other factors contributing to a lowering of the percentage of normal diploid cells observed are the frequency of aneuploid and polyploid cells.
The aneuploidy estimate of 12.3% in somatic cells can be explained by various factors that cause abnormal mitosis. Beatty (1951) stated that cells in culture are prone to heteroploidy. Temperature shock, colchicine treatment and other abnormal environmental conditions have been the most likely contributors to the aneuploidy reported.

The incidence of polyploidy in somatic cells as reported here, varied significantly among the eleven animals surveyed (p<.01). The differences may be attributed to culture technique variations among samples (slight temperature differences or colchicine treatment effects) as well as to genetic considerations. Any analysis of variation must necessarily assume strictly controlled environmental conditions or repeated cultures of cells from each animal.

The endoreduplicated polyploid condition is associated with abnormal environments. This type of anomaly has been produced in significant numbers in tumors (Levan and Hauschka, 1954) and irradiated cultures (Bell and Baker, 1962; Ohnuki et al., 1961). That endoreduplicated cells "revert" to the usual tetraploid form of polyploidy has been indicated by Bell and Baker (1962).
High frequencies of aneuploidy and polyploidy in somatic tissue are associated with abnormal conditions in the individual and with normally rapidly growing tissue (Beatty, 1951). The frequencies reported here, when allowances are made for technical factors, are considered not to be of a large magnitude.

**Centric fusion**

The third metacentric chromosome, larger than the X, and repeatedly observed in leukocytes of female origin of a set of placentally anastomosed triplets, is interpreted as having evolved by centric fusion which is rare in mammals. Although the third metacentric chromosome was observed only in leukocytes, no other tissue being available for study, it is assumed that it may be found in tissues throughout the soma and in the germ line. That the third metacentric chromosome was not observed in leukocytes of the dam, eliminates the prospect of inheritance through the maternal side of the pedigree. Four theoretical models for the origin of such a metacentric chromosome by the process of centric fusion, are as follows: 1. Formation during meiotic division of oogenesis in the dam or spermatogenesis in the sire. 2. Inheritance through the paternal side of the pedigree. 3. Formation during early mitotic divisions
in the zygote. Formation during mitotic divisions later in development, but necessarily before formation of primordial hematopoietic tissue of female origin, which produced leukocytes of the anomalous type only. The fact that the cells of male origin of the triplets did not contain the third metacentric chromosome indicates that the sire could not be homozygous for it.

In this case, although the number of chromosomes and of centromeres has been reduced by one, the number of chromosome arms (numero fundamentale, Matthey, 1945) remains the same, 62. Thus the total genetic constitution is still present, although position effects of the gene loci may be different than in the normal condition. The effect of this presumed centric fusion on the phenotype of the individuals in question appeared to be nil, but this may be due to the preponderance of cells of male origin in all three individuals, the presence of which may have overcome the effects of the cytogenic anomaly in the cells of female origin.

Sohval (1963) discusses major translocations as possible causes of abnormal human karyotypes. Reciprocal "translocations" of arms between the X chromosomes may lead to the formation of a large and a small medio-centric chromosome. Transverse division of the centromere at mitosis, leading to isochromosome formation,
may also produce a large and a small mediocentric chromosome. In the human karyotype however, only five of the 23 pairs of chromosomes are acrocentric so that the probability of a centric fusion occurring between two non-homologous chromosomes with subsequent loss of the small element, is reduced as compared with mammals such as cattle, which have many pairs of acrocentrics. Ford (1961) has cited cases associated with mongolism in which a small acrocentric chromosome had fused with the X chromosome. The total chromosome number in the mongoloids was still 46, but the 21st pair was in reality trisomic. Lejune and Turpin (1961) reported a case of human polydyspondylism associated with what may be a centric fusion similar to that described here for cattle. The diploid number of chromosomes was given as 45 with an extra submediocentric chromosome and two missing acrocentrics. The condition was postulated as fusion of two acrocentrics of the 13-15 and 21-22 groups. This case is different from that reported here in that it is associated with a clinical anomaly, which leads one to assume that the small element, lost during subsequent divisions, contained important genetic material.

The centric fusion type of reciprocal translocation is considered to have great evolutionary significance (White, 1954; Matthey, 1945). It has been shown by
McClung (1917), Painter and Stone (1935), and Matthey (1945), that among species of grasshoppers, Drosophila, and lizards respectively, differences in phenotype are associated with differences in chromosomal number, although the "numbre fundamentale" is the same for each of the various species. The differences in chromosome number were explained by White (1954), Matthey (1945) and many others, to be due to centric fusions between autosomes. The evolutionary significance of centric fusion may be extended to mammals. The bovine, for example, has 60 chromosomes (nf = 62), whereas the ovine has 54 chromosomes (nf = 62). The cytogenetic anomaly described in this report presumably increased the extent of the variability possible in the bovine, but appeared to have no grossly detrimental effects on any of the triplets, which is the expectation for the classic centric fusion. If the hypothesis that the presence of normal cells of male origin has overcome the effects of any chromatin loss, is rejected, these individuals might be cited as evidence of the feasibility of the explanation of species formation in mammals by means of centric fusion. It would seem obvious that the probability of such centric fusions would be greater in genera with a large number of acrocentric pairs of chromosomes, such as the bovidae, than in those such as the primates. The comparative rarity of examples of centric fusion in human species may be explained thusly.
Sex chromosome mosaicism

The frequency of sex chromosome mosaicism among fraternal twins in cattle as reported here (90%) is of the same magnitude as that reported for erythrocyte antigen admixture, 91% (Lazear et al., 1953), for acceptance of reciprocal skin grafts, 86% (Billingham et al., 1952) and for vascular anastomosis of the placental blood vessels, 96.4% (Lillie, 1923), 94.1% (Tandler and Keller as cited by Lillie, 1919). The fact that no sex chromosome mosaicism is found in single-born animals and that erythrocyte antigen admixture tests were in agreement with the observations of sex chromosome mosaicism in bisexed multiple birth sets, is an indication that sex chromosome mosaicism is associated with the freemartin syndrome. Ohno et al. (1962) have observed the reproductive tracts of three freemartins and found that abnormal tracts are associated with marrow sex chromosome mosaicism. That report is in accordance with the observations reported here on thirteen animals.

Correlation of percentage of cells of male origin between members of multiple birth sets was found to be high in the data analysed from the eight sets of multiple births, 0.893. Reports in the literature on this point however, indicate conflicting observations.
In cattle, Ohno et al. (1962) have reported data on two sets of twins. One set had exactly equal proportions of cells of male origin (marrow) based on counts of 50 and 100 cells, while the other set had opposite proportions of cells of male origin based on 22 and 23 cells. Owen et al. (1946) had observed that erythrocytes with alternative antigenic specificities were contained in the same proportions in members of a set of quintuplets. Rendel et al. (1962) had observed different proportions of erythrocytes with alternative antigenic specificities in two groups of a set of cattle quintuplets, but explained these results as being due to differences in time of vascular anastomosis with the male. Lewis et al. (1963) found that the proportions of erythrocytes with alternative specificities were similar in a set of human twins, but Booth et al. (1957) reported twins with opposite proportions of erythrocyte types as measured by antigenic specificities. Davidson et al. (1958), using drumstick identification in two sets of twins, observed that the proportions of leukocytes of male and female origin were different for the male and female twin. The response of twins to anastomosis of the chorionic blood vessels is thus different in human and cattle twins. Anastomosis occurs frequently in cattle and is accompanied by
intersexuality in the female. Anastomosis occurs rarely in humans, but often in marmosets, another primate. In primates the female twin is normal. In cattle, proportions of cell types are similar in twins, whereas in man, the host's cell type usually prevails. The differences may be due to either time of anastomosis, a function perhaps of the type of placenta, or to the environment of the host animal. In cattle, the cell type of the host does not necessarily have a preferential environment. The two artificially mosaic cultures produced by mixing male and female leukocytes from unrelated animals grew well. Two thirds of the freemartins had a preponderance of cells of male origin. Perhaps cells of male origin have a selective advantage in cattle.

The thirteen freemartins slaughtered in this study were found to have varying degrees of masculine development. Such variation has been reported in the literature by Hunter (1786), Tandler and Keller (1911), and Lillie (1917). From detailed descriptions of reproductive tracts of the freemartins observed by Hunter (1786), Simpson (1872), Lillie (1917), Willier (1921), Fraser-Roberts and Greenwood (1928), and Rothe et al. (1961), twelve of the tracts described and pictured in the appendix, can be deemed typical
for freemartins. The other case, C-68 however, is of a lower degree of masculinization than any of the animals previously reported as freemartins. No animals with as severe a degree of masculinization as that reported by Fraser-Roberts and Greenwood (1928), or Rothe et al. (1961) were observed. The categorization of reproductive tracts was similar in some respects to the categorizations of Willier (1921).

Correlation of degree of masculinization of freemartin reproductive tract with percentage of cells of male origin for the thirteen multiple birth sets was 0.714 with a 95% confidence interval of 0.269 - 0.907. The correlation was significantly different from zero, (p<.01), indicating that a positive correlation does exist, based on this sample. The establishment of such a correlation necessarily leads to a reexamination of the hypothesis of etiology of the freemartin syndrome and therefore of sexual development in mammals.

Witschi et al. (1957, 1960) have shown that in lower vertebrates, the most important role in sexual differentiation is played by external environment. In higher vertebrates, the genes which condition cellular environment are considered to be of prime importance in sexual differentiation. These genes are located mainly
on the sex chromosomes, especially the Y which contains strongly masculinizing factors (Miller, 1962). This last has been learned by observation of mammalian sexual abnormalities which have been associated with abnormal sex chromosome numbers and morphology (Sohval, 1963). In cases of sexual abnormalities for which no abnormal karyotypes are observed, intersexuality is described as the result of hormonal imbalances or gene mutations (Sohval, 1963). In true hermaphrodites, the above have been tentative explanations in 19 of 25 cases in which a normal XX karyotype was found.

The most interesting case of sex chromosome mosaicism associated with intersexuality in a true hermaphrodite from my point of view, is that reported by Gartler et al. (1962) and by Waxman et al. (1962). In that case, the proportion of cells of male and female origin within the gonads and reproductive tract was associated with type of local sexual differentiation. From these observations, one might postulate that intersexuality in mammals is the result of an imbalance of genetic factors, as observed by aberrant sex chromosome constitution, acting through gene-enzyme relationships and producing tissues of variant types either directly or via hormone production. Willier (1921) associated groin position of the gonad of the freemartin with testicular
development. Rothe et al. (1961) observed freemartins with palpable gonads in the udder area that were described as histologically normal testes.

The experiments of Jost (1947a,b), MacIntyre (1956), Holyoke and Beber (1958), and others, have indicated that developing reproductive tracts and gonads can be modified in laboratory animals by means of gonadectomy or adjacent male-female gonad cultures and transplants. Jost (1947b) however, was unsuccessful in modifying the reproductive tracts of developing female embryos with parabiosis experiments. Ryan et al. (1961) demonstrated an aromatization reaction by the marmoset placenta. The enzyme so demonstrated, typically changes androgens to estrogens. Ryan et al. (1961) therefore postulated that presence of this enzyme prevented the marmoset female twin from becoming an intersex. Such an enzyme could not be demonstrated in the bovine placenta. The conclusions of Moore (1941), MacIntyre (1956), and Holyoke and Beber (1958) that the gonadal inductor substance produced by differentiating male gonad was not the same as adult male hormones, may affect the explanation of Ryan et al. Gonadal inductors have not yet been recovered and analyzed and therefore it cannot be assumed that they are androgens.
Benirschke et al. (1962a, b) have concluded that hematopoietic tissue is exchanged via placental anastomosis, from observing mosaicism in marrow of marmoset twins. Presence of erythrocytes of different antigenic specificities, demonstrated by Owen et al. (1945) in cattle twins with placental anastomoses, also indicates that hematopoietic tissue is exchanged. Ohno et al. (1962) have indicated possible mosaicism in gonadal tissue of males which are twin to freemartins. The above observations, when linked with the positive correlation of percentage of cells of male origin with the degree of masculinization of reproductive tract, lead to the postulation of the hypothesis that the freemartin is not the result of a hormone from the early developing male gonad which masculinizes the gonad and reproductive tract of the female to which it is connected by a vascular anastomosis, but that the presence of the Y chromosome in hematopoietic tissue is an indication of a direct effect of the Y chromosome on freemartin sexual development.

One question remains, an explanation of normal sexual development in the male twin. One possible explanation is that male gonads which develop somewhat earlier than those of the female, are not affected by the exchange of tissue due to prior onset of the
developmental process. The early development of interstitial cells leads to the maintenance of the normal cellular environment for masculine development despite the presence of cells of female origin. A second possible explanation is that developing ovarian tissue is more easily modified by cellular environment than developing testicular tissue. A third explanation is that cells of male origin have a strongly masculinizing effect so that cells of female origin only cause feminine development in the absence of cells of male origin.

The association of the Y chromosome with inter-sexuality in the freemartin bears out the importance of genetic balance in sexual differentiation in mammals.
SUMMARY AND CONCLUSIONS

The normal diploid number of chromosomes in cattle is sixty, being composed of 29 pairs of acrocentric autosomes and one pair of submedially inserted sex chromosomes. The X is one of the largest chromosomes and the Y is one of the smallest.

Frequencies of aneuploidy and polyploidy in cattle leukocyte cultures were 12.3% and 3.45%, respectively. The variation in incidence of polyploid cells from one individual to another was significantly different, but variations in culture techniques may explain many of the differences obtained.

A third metacentric autosome was reported in a set of triplets which also had sex chromosome mosaicism and only 59 chromosomes in the cells of female origin. It is interpreted as having evolved by centric fusion. As no apparent detrimental effects of the fusion were observed in the animals, it may be cited as an example which indicates the feasibility of the theory of speciation via cytogenetic anomalies such as centric fusion, if one rejects the hypothesis that the presence of normal cells of male origin can overcome the effects of a cytogenetic anomaly in the cells of female origin.
Sex chromosome mosaicism was reported in 90% of the 30 bisexed multiple birth sets tested. The observation of percentage of cells of male origin of the same magnitude among members of multiple birth sets was analyzed and found to have a correlation of 0.893 with a 95% confidence interval of 0.745-0.957. Reproductive tracts from thirteen slaughtered freemartins and a normal heifer were compared and a categorization of the reproductive tracts based on degree of masculinization was made. The percentage of cells of male origin in the multiple birth sets was found to be significantly correlated (0.714) with the degree of masculinization of the reproductive tract. No sex chromosome mosaicism was observed in mitotic figures from cultures of kidney tissue of two freemartins. It was hypothesized therefore, that the freemartin condition in cattle is caused by the presence of cells of male origin in the female, rather than by an hormonal exchange by means of the anastomosed chorionic blood vessels. On the basis of this hypothesis, the freemartin condition is interpreted as an effect of the strongly masculinizing potential of the Y chromosome in mammals.
APPENDIX

Case Histories

Case C-29

Holstein heifer twin out of cow #2463 from London Correctional Institute, London, Ohio.

Birth date: 16 July, 1962

Blood sample: 7 August, 1962; 5 XX cells, 9 XY cells.

Slaughtered: 16 October, 1962

Description of the reproductive tract:

Grossly, intersex. Category 2.
1. Gonads present; ovarian position.
2. Uterine horns slightly developed.
3. Lower portion of uterus is undeveloped Mullerian duct.
4. Accessory sex glands present.
5. No cervix.
Figure 18

Case C-29; Reproductive tract
Case C-58, 55  Holstein twins out of cow #1258  
from Orient State Hospital,  
Orient, Ohio.

Birth date:  19 January, 1963

Blood samples:

C-55  M.  30 January, 1963;  7 XX cells, 30 XY cells.  
C-58  F.  8 February 1963;  0 XX cells,  5 XY cells.

Heifer slaughtered:  18 September, 1963

Description of the reproductive tract:

1.  Gonads not recovered; assumed due to position in groin area.
2.  Mullerian ducts not developed.
3.  Wolffian ducts developed into elongated tube.
4.  No accessory sex glands present.
5.  Vagina short, non-patent.

Blood group analyses:

<table>
<thead>
<tr>
<th>System</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>F</th>
<th>J</th>
<th>L</th>
<th>S</th>
<th>Z</th>
</tr>
</thead>
</table>
C-55    reaction | A* 01* E1* C1* X2 | F - L* H1* Z |
C-58    reaction | A* 01* E1* C1* X2 | F - L* H1* Z |

* Partial reaction.

This twin pair shows evidence of admixture due to partial reactions. The blood types are identical.
Figure 19

Case C-58; Reproductive tract
Case C-60  Holstein heifer twin #1626 from the Ohio Agricultural Experiment Station, Wooster, Ohio.

Birth date:  August, 1961

Blood samples:  27, February, 1963; 5 XX cells, 23 XY cells.  21, October, 1963; 3 XX cells, 1 XY cell.

Slaughtered:  22 October, 1963

Description of the reproductive tract:

Grossly, intersex.  Category 3.
1.  Gonad well developed; in ovarian position on one side.
2.  Gonad on other side poorly developed, only gonadal tissue; position toward upper back.
3.  Uterine horns present; filled with fluid.
4.  Body of uterus poorly developed.
5.  Wolffian ducts well developed.
6.  Accessory sex glands present.
7.  No cervix.
8.  Vagina short.

Histologically: Possible testicular tissue, spermatogonia and what might be seminiferous tubules in the well developed gonad.

Blood group analyses:

System  A  B  C  F  J  L  S  Z
Reaction  D  G  O₁*  Y₂*  E₁*  C₁  F -  L*  H₁  Z

* Partial reaction

This twin shows evidence of admixture due to partial reactions.

For photograph of reproductive tract see Figure 15, page 69.
Case C-61

Jersey heifer twin #1635 from the Ohio Agricultural Experiment Station, Wooster, Ohio.

Birth date: September, 1961

Reported estrous cycle: February, 1963

Blood sample: 27 February, 1963; 0 XX cells, 21 XY cells.

Slaughtered: 22 October, 1963

Description of the reproductive tract:

1. Gonads well developed; located in groin area.
2. Wolffian ducts well developed.
3. Mullerian ducts degenerated.
4. No accessory sex glands present.
5. No cervix.
6. Vagina short, non-patent.
7. Vulva small.
8. Clitoris enlarged.

Histologically:
1. Embryonic epididymus present.
2. Differentiating vas deferens present.
3. Some juvenile ovarian tissue present in the gonad.

Blood group analyses:

System A B C F
Reaction A A* E G O Y B G Q T Y C W F V
System J L S Z
Reaction J S *

* Partial reaction

This twin shows evidence of admixture due to partial reactions.
Figure 20
Case C-61; Reproductive tract
Case C-68, 69

Holstein twins, #1708 and #1550 respectively, from the Ohio Agricultural Experiment Station, Wooster, Ohio.

Birth date: 12 March, 1963

Blood samples:

C-68 F. 6 June, 1963; 16 XX cells, 4 XY cells.
C-69 M. 6 June, 1963; 17 XX cells, 3 XY cells.


Description of the reproductive tracts:

C-69: Grossly, that of a normal male.

C-68: Grossly, intersex. Category 1.

1. Gonads in ovarian position, well developed, but hard and ridged.
2. Fallopian tubes present.
3. Horns of uterus fairly well developed, but lack elongated coiling of a normal tract.
5. Cervix present.
6. Vagina normal.
7. No Wolffian derivatives observed.

Blood group analyses:

<table>
<thead>
<tr>
<th>System</th>
<th>A</th>
<th>B</th>
<th>F</th>
<th>J</th>
<th>L</th>
<th>S</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-68 reaction</td>
<td>A</td>
<td>G* Y2* E1* Ox A1</td>
<td>F</td>
<td>- -</td>
<td>H'</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C-69 reaction</td>
<td>A</td>
<td>G* Y2* E1* Ox A1</td>
<td>F</td>
<td>- -</td>
<td>H'</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Partial reaction

This twin pair shows evidence of admixture due to partial reactions. The blood types are identical.

For photograph of C-68 reproductive tract, see Figure 13. page 67.
Case C-70, 71, 72, 85  Holstein triplets and dam from Ed J. Brown Farm, Route #3, Norwalk, Ohio.

Date of Birth:  10 October, 1962

Blood samples:

C-70 F.  10 June, 1963;  8 XX cells, 22 XY cells.
15 April, 1964
  Additional XX cells observed for presumed centric fusion anomaly.
C-71 M.  10 June, 1963;  2 XX cells, 28 XY cells.
C-72 M.  10 June, 1963;  1 XX cell,  31 XY cells.
  All cells of female origin have the cytogenetic anomaly, a presumed centric fusion.
C-85  Dam  13 August, 1963;  20 XX cells,  0 XY cells.
  The cells of the dam do not have the presumed centric fusion.

Slaughtered heifer:  16 April, 1964.

Description of the reproductive tract:

  1.  Vagina short, non-patent.
  2.  Accessory sex glands present.
  4.  Wolffian ducts developed.
  5.  Gonad appears testicular.
  6.  Epididymus present.

Blood group analyses:

<table>
<thead>
<tr>
<th>System</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>F</th>
<th>J</th>
<th>L</th>
<th>S</th>
<th>Z</th>
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<tr>
<td>C-70 reaction</td>
<td>A* O₁* A₂ I₂* W* X₂ F V* J* - H¹ Z*</td>
<td></td>
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<tr>
<td>C-71 reaction</td>
<td>A* O₁* A₂ I₂* W* X₂ F V* J* - H¹ Z*</td>
<td></td>
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<tr>
<td>C-72 reaction</td>
<td>A* O₁* A₂ I₂* W* X₂ F V* J* - H¹ Z*</td>
<td></td>
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<tr>
<td>C-85 reaction</td>
<td>A G Y₂ E₁ I₂ WC₁X₂ F J L H¹ Z</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tbody>
</table>

* Partial reaction

The triplets are considered admixed due to partial reactions.  In addition, they have identical blood types.
Figure 21

Case C-70; Reproductive tract
Case C-77, 91
Holstein twins purchased from
Roger Martin Farm, Wooster, Ohio.

Birth date: February, 1963

Blood samples:

C-77 M. 11 June, 1963; 24 XX cells, 6 XY cells.
C-91 F. 6 September, 1963; 33XX cells, 0 XY cells.
19 September, 1963; 70XX cells, 3 XY cells.


Kidney cell cultures:

C-91 6 XX cells, 0 XY cells.

Description of the reproductive tracts:

C-77 Grossly, that of a normal male.
Histologically, spermatogenesis in a young testicle.

C-91 Grossly, intersex. Category 2.
1. Gonad in ovarian position.
2. Mullerian ducts poorly developed.
3. Wolffian ducts intermittent.
4. No accessory sex glands.
5. No cervix.

Histologically:
1. Oviduct present
2. Ovarian stroma in medullary area of gonad.
3. Much connective tissue in gonad.

Blood group analyses:

System A B C F J L S Z
C-77 reaction A* B G* O J* O' W* X2 F - - H' Z
C-91 reaction A* B G* O J* O' W* X2 F - - H' Z

* Partial reaction

This twin pair shows evidence of admixture due to partial reactions. The blood types are identical.
Figure 22

Case C-91; Reproductive tract
Case C-89,90

Holstein twins out of cow #2425
from London Correctional Institute,
London, Ohio.

Birth date: 24 July, 1963

Blood samples:

C-89 M. 4 September, 1963; 9 XX cells, 21 XY cells.
C-90 F. 4 September, 1963; 11 XX cells, 19 XY cells.


Description of the reproductive tract:

1. One gonad recovered; in groin position.
2. Mullerian derivatives not developed.
3. Wolffian ducts developed.
4. Accessory sex glands present.
5. No cervix.

Histologically, ovarian stroma observed in the gonad.

Blood group analysis:

System A B C F J L S Z

C-89 reaction A* B* G O2 Q Y2 A' D'* X1 F - - H' Z
C-90 reaction A* B* G O2 Q Y2 A' D'* X1 F - - H' Z

* Partial reaction

This twin pair shows evidence of admixture due to partial reactions. The blood types are identical.
Figure 23
Case C-90; Reproductive tract
Case C-100,101 Holstein twins out of cow #2551 from London Correctional Institute, London, Ohio.

Birth date: 25 August, 1963

Blood samples:

- C-100 M. 24 September, 1963; 21 XX cells, 20 XY cells.

Heifer slaughtered: 3 February, 1964

Description of the reproductive tract:

Grossly, intersex. Category 2.
1. Two sets of gonads, in the ovarian position.
2. Mullerian ducts intermittent.
3. Wolffian ducts partially developed.
4. Accessory sex glands present.
5. No cervix.

Histologically: each gonad with ovarian stroma.

Blood group analyses:

System A B C F J L S Z
C-100 reaction - B* G* O1* Y2 D' X1 F - S*H'* Z
C-101 reaction - B* G* O1* Y2 D' X1 F - S*H'* Z

* Partial reaction

This twin pair shows evidence of admixture due to partial reactions. The blood types are identical.
Case C-101; Reproductive tract
Case C-106

Holstein-Angus X Hereford crossbred twin purchased from Robert Hodges Farm, Cambridge, Ohio.

Birth date: Spring, 1962


Description of the reproductive tract:

1. Well developed gonads, in groin position.
3. Wolffian derivatives well developed.
4. Accessory sex glands present.
5. Cervix absent.
6. Vagina short, non-patent.

Histologically: fairly normal epididymus present.

Blood group analyses:

System

<table>
<thead>
<tr>
<th>A</th>
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<th>F</th>
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<td>AHYD</td>
<td>I*</td>
<td>E3*</td>
<td>G*</td>
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<td>C1RWX2</td>
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System

<table>
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</thead>
<tbody>
<tr>
<td>LHI*</td>
<td>-</td>
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</tbody>
</table>

* Partial reaction

This twin shows evidence of admixture due to partial reactions.

For photograph of the reproductive tract, see Figure 16, page 70.
Case C-115,116  Holstein twins out of cow #2018
from London Correctional Institute,
London, Ohio.

Birth date:  14 November, 1963

Blood samples:

C-115 M. 18 November, 1963;  3 \(XX\) cells,  3 \(XY\) cells.
C-116 M. 18 November, 1963; 15 \(XX\) cells, 15 \(XY\) cells.


Description of the reproductive tract:

Grossly, intersex. Category 2.
1. Two sets of gonads, in ovarian position.
2. Mullerian derivatives poorly developed.
3. Wolffian derivatives fairly well developed.
4. Accessory sex glands present.
5. No cervix.
6. Vagina short, non-patent.

Histologically: each gonad has ovarian stroma.

Blood group analyses:

<table>
<thead>
<tr>
<th>System</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>T</th>
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<td>(Y_1)</td>
<td>(A_1^f)</td>
<td>(Y')</td>
<td>(X_1)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>C-116 reaction</td>
<td>D</td>
<td>(O_x)</td>
<td>(Y_1)</td>
<td>(A_1^f)</td>
<td>(Y')</td>
<td>(X_1)</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

No partial reactions observed, but both members of the set have identical blood types.
Figure 25

Case C-116; Reproductive tract
Case C-123 Holstein twin purchased from Raymond Starbuck, Plain City, Ohio.

Birth date: 21 May, 1963

Blood sample:

24 January, 1964; 22 XX cells, 8 XY cells.

Slaughtered: 14 February, 1964

Kidney cell culture: 27 XX cells, 0 XY cells.

Description of the reproductive tract:

Grossly, intersex. Category 2.
1. Gonad in ovarian position.
2. Mullerian duct intermittent.
3. Wolffian duct intermittent.
4. Accessory sex glands present.
5. No cervix.
6. Vagina short, non-patent.

Histologically: gonad contained ovarian tissue; one follicle observed.

Blood group analyses:

System A B C F J L S Z
Reaction B₂ Y₂ E¹ G¹ F - - H¹ Z
* Partial reaction

This twin shows evidence of admixture due to partial reactions.
Figure 26

Case C-123; Reproductive tract
Case C-124

Holstein twin out of cow #2301 from London Correctional Institute, London, Ohio.

Birth date: 27 November, 1963

Blood sample:

3 February, 1964; 12 XX cells, 18 XY cells.

Slaughtered: 24 February, 1964

Description of the reproductive tract:

Grossly, intersex. Category 2.
1. Two sets of gonads, in ovarian position.
2. Uterine horns partially developed.
3. Lower portion of Mullerian duct poorly developed.
4. Partial development of Wolffian ducts.
5. Accessory sex glands present.
6. No cervix.
8. Clitoris enlarged.

Histologically: ovarian stroma present in each gonad.

Blood group analyses:

System A B C F J L S Z
Reaction  A B 01 A1 G' C2 X F -- H' * *

* Partial reaction

This twin shows evidence of admixture due to partial reactions.

For photograph of the reproductive tract, see Figure 14, page 67.
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