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CYTOGENETIC INVESTIGATIONS ON NORMAL AND MALFORMED ANIMALS, WITH SPECIAL REFERENCE TO INTERSEXES*

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CHAPTER I

A. Introduction

1. *General*

In view of the rapid advances made during the last decade in the field of cytogenetics as applied to the elucidation of certain congenital abnormalities and disease syndromes of man (Sohval, 1961) it was deemed advantageous to undertake similar investigations on animals. Pakes & Griesemer (1965), in a recent review have stressed the increasing importance of chromosome aberrations in veterinary medicine. Such studies may in future prove even more significant in view of the increasing use of X-rays and radioactive isotopes, as well as the threat of nuclear weapons. Studies are also under way on the phylogenetic and evolutionary significance of chromosomes in the primate group (Bender & Mettler, 1958; Chu & Bender, 1961).

For cytogenetic studies to be carried out on animals the basic normal morphology and number of chromosomes have to be determined first of all. The variety of species of wild animals found in South Africa offers a ready field for the extension of such studies to animals other than domestic species. The undertaking gains urgency in face of the ever-present danger of extinction of some.

Cytogenetic studies have been facilitated greatly during the last number of years by simplification and adaptation to field conditions of what was formerly a difficult and strictly laboratory procedure. If the simplified technique of today is compared with the cumbersome and difficult procedures of the past it is all the more astounding that so many years had to elapse before heightened interest in another field of biological research, namely biology of radiation, favoured their evolvement.

Thus aceto-orcein squashes and superimposition of camera lucida drawings of serial sections of haploid spermatogenic cells have been replaced mostly by drop preparations of bone marrow cells or tissue cultures, subjected to hypotonic pretreatment and fixation in acetic-alcohol. The standard aceto-orcein stain has been replaced largely by alkaline Giemsa and Feulgen's reaction for DNA. Phytohaemagglutinin and colchicine as agents for procuring larger numbers of metaphase spreads in leucocyte cultures have been useful aids.

The term intersex is used here as a general term for individuals exhibiting intermediate sexual characteristics: part or all of the genital organs of both the sexes—not necessarily both gonads—are represented in one individual. This means that all hermaphrodites are intersexes but all intersexes are not necessarily hermaphrodites. The term intersex as used here has no reference to the ratio of the number of X-chromosomes to sets of autosomes: such an extension in meaning applies to *Drosophila melanogaster* Meigen where, if the ratio of X-chromosomes to number of autosomal sets is intermediate between 0·5 and 1·0, an intersex is indicated.

In studying intersexuality in mammals, it is well to consider its occurrence in other vertebrate classes. A brief comparative review based mainly on that of Armstrong & Marshall (1964) is therefore included.

2. *Comparative review of intersexuality*

Intersexes have always attracted a great deal of attention, even being given a role in certain religious rites (Baker, 1925). The occurrence of intersexes in fowls was considered an evil omen. One of the reasons for such superstitious beliefs was the

infrequent occurrence of intersexes. Nevertheless it has been shown that intersexes can occur in most vertebrate forms and can actually be produced experimentally more particularly in the lower forms.

In an introductory comparative review of intersexuality various terms need some elaboration. *Chimerism* denotes a situation where atypical cells present in an individual were originally derived from another individual, as in freemartins. *Mosaicism* denotes a state where atypical and normal cells in an individual were derived from the same zygote, i.e. the same genetic source. *Hermaphroditism* (ovarian and testicular tissue recognizable in one individual) is divided into *normal hermaphroditism* (functional in many invertebrates and certain fishes) in contrast to *abnormal or teratological hermaphroditism* occurring mostly in more advanced vertebrates especially mammals as an anomaly. *Functional hermaphroditism*, mostly normal, occasionally abnormal, may be *synchronous* (both types of gametes produced simultaneously, e.g. the serranids) or *rudimentary* (normal hermaphroditism, with only one sex functional—the other being rudimentary. It can either be transitory during development or may persist in the adult). Synchronous hermaphroditism may in turn either be *protandrous* (functional, first as male and then as female) or *protogynous* (functional, first as female and then as a male). (In contrast to hermaphroditic forms we have the *gonochoristic* or single sexed individuals or species).

Examples, differing greatly in frequency of occurrence, of all these types can be found amongst fishes, especially the Teleostii, in which group hermaphroditic forms are more numerous than in any other vertebrate group with possible exception of the Cyclostomata (Agnatha). No statistical data exist, however, as to the frequency of these types. Synchronous hermaphroditic types are of special interest and differ surprisingly from mammals in that both eggs and milt may develop to maturity despite the close or intimate contact of the gonads. Such eggs may even be fertilized internally or externally by sperm from the same individual. This forms a serious challenge to the fundamental concept of antagonism between male and female hormonal influences as accepted in the study of vertebrate endocrinology.

Sex determination is considered to be rather primitive in teleosts. This assumption is based principally on morphological grounds. Sex chromosomes, for instance, are cytologically indistinguishable from autosomes. Their presence, however, is revealed by the occurrence of sex-linked inheritance. Sex-linked genes may be localized in the Y as well as X-chromosome and are sometimes exchanged by crossing over (Winge, 1934). From results obtained after crossing two races of guppies *Poecilia reticulata* Winge assumed that autosomes had taken over the function of sex chromosomes. This was, however, later shown to be merely a recognition of the fact that multiple factors affecting sex determination were present on the autosomes. Due to inclusion of teleosts among lower vertebrates the exchange of roles of sex chromosomes and autosomes has been considered primitive but may as a matter of fact actually represent a specialization. Other piscine features considered primitive are: the existence of both male and female heterogamety, sometimes even in the same species; the frequency of genetic sex reversal and the apparent lack of any sex-determining mechanism in some fishes, e.g. *Poecilia vittata.*

Sex chromosomes can only be identified in amphibians by the fact that in male frogs the X and Y-chromosomes conjugate incompletely and therefore at diakinesis form an open tetrad. During the first meiotic division they separate sooner than the autosomal diads (Overzier, 1963). Sufficient evidence exists to indicate that in the Ranidae and Hylidae (Anura) the females are homogametic (XX) and males heterogametic (XY) whereas in more primitive anurans and many Urodela the males are

homogametic (ZZ) and the females heterogametic (ZW). Heteroploids can be produced experimentally by lowering or raising the temperature. Viability in such cases is low and they are either sterile or have a very low fertility. Temperature, various chemicals, androgens and oestrogens can variously alter the sex. Numerous studies have been undertaken on amphibian intersexes, due to the ease with which complete phenotypic sex reversal can be obtained. In parabiosis it has been determined that the masculinizing influence of the testis on the ovary is inversely proportional to the distance between them.

A condition, referred to as transitory juvenile hermaphroditism, in which gonads of male frogs pass through developmental stages where they appear to be ovaries but later develop into testes is encountered in some undifferentiated races.

Among the reptiles persisting intersexuality is encountered in Testudinata (Chelonia) and Lacertilia, is rare in *Sphenodon* and Serpentes (Ophidia) and as far as is known practically non-existent in Crocodilia. Abnormal hermaphroditism has also been encountered in turtles and lizards. Varying degrees of persistence of heterosexual structures can be produced by experimental injection of masculinizing and feminizing hormones in developing embryos. It is still uncertain whether complete sex reversal can occur in reptiles. Abnormal sex ratios have therefore been reported as being due to either sex reversal or differential mortality. No references to sex chromosomes were found.

In birds the male is homogametic (ZZ) and the female heterogametic (ZW). Kosin & Ishizaki (1959) nevertheless demonstrated that Barr bodies are also found in females, the heterogametic sex and not in the homogametic sex as in mammals.

Spontaneous intersexuality has been known in fowls ever since the days of Aristotle. Masculinization of hens occurs quite frequently. A case of complete sex reversal from fertile hen to fertile cock has been described by Crew (1923). The inherent bipotentiality of the gonads of the hen is responsible for the fact that pathological suppression or ovariectomy of the (left) ovary results in testicular development on both sides. This may often instead, lead to the formation of an ovotestis on one or both sides. Hermaphrodites tend to be more numerous in inbred strains of fowl and pigeons. In the latter case they are considered to be genetic males. Gonadal dysgenesis and gynandromorphism also occur. Freemartinism has been seen in cases of heterosexual individuals from double-yolked eggs. Allantoic anastomoses are present in such cases, the male revealing the greater effect. Hermaphrodites have also been reported to be more numerous in hybrid pigeons and hybrid pheasants. In such hybrids males are predominant. Whether this is due to sex reversal or differential mortality is still an open question.

Varying degrees of intersexuality can be produced experimentally by intracoelomic gonadal grafts, by effecting high oestrogen levels prior to sexual differentiation or by irradiation. It is most unlikely that androgens play a role in producing spontaneous intersexes. Intersexual characteristics, developed after hatching, are almost invariably produced in genetic females, as females possess bipotential gonadal primordia.

In mammals intersexes have been reported in a large variety of species. Undoubtedly they may occur in all—it is merely a case of searching for them. As some of the principal forms of intersexes occurring in the sheep, pig and bovine will be dealt with in the succeeding chapters further elaboration at this stage is considered superfluous.

Unfortunately chromosome studies and cytological sexing have up to now been confined mainly to man. Such studies are being extended to include all vertebrates and much information is to be expected therefrom.

3. *Cytological sexing*

This procedure has been an important aid in the furtherance of chromosome studies especially in humans. It offers an easy method of screening suspected individuals for sex chromosome aberrations. It has two aspects:

(*a*) *Nuclear sexing*—It involves the identification of Barr bodies in interphase somatic nuclei. The Barr body was first associated with a sex difference by Barr & Bertram (1949) in neuronal nuclei of the cat and has since become regarded as an extremely important cytological characteristic. The bodies had already, according to Lüers (1958), been noticed by Levi in 1880 and later described by O. Vogt as " Randkörperchen " without their true significance becoming apparent. In later years they were often referred to as chromocentres or as membrane-chromocentres. The Barr body represents the one X-chromosome of the female in a heteropycnotic state—it has undergone " lyonisation " (Lyon, 1962) and is best identified in vesicular nuclei such as those of neurocytons. It varies in size from 0·8 μ to 1·1 μ (Overzier, 1963).

(*b*) *Polymorphic sexing*—This depends upon identification of drumsticks (1·5 to 2 μ in diam.) in polymorphs in blood smears of females, first demonstrated by Davidson & Smith in 1954. As variously shaped appendages are discernible in polymorphs, the position often may become confusing. Hence Kosenow & Scupin (1956) deemed it essential to classify them into four groups.

A. Solid nodules connected by a thin filament to the nucleus, viz. drumsticks, typical for females and occurring very rarely in males.
B. Nodules or tear-drop forms, attached to the nucleus without a thin filament.
C. Small clubs or filaments (< 1 μ), more numerous in males but of no sexual differential importance, as their occurrence is extremely variable.
D. Racket forms, very rare, more numerous in males but of no sexual differential importance.

They devised a formula for differential evaluation of sex in the human. If the numbers of nuclear appendages counted for the first three types are represented by the symbols A, B and C respectively, then the value for the formula $\frac{A + B}{C}$ can be calculated. If the value obtained for this formula is bigger than 0·4 then the individual is a female and if smaller than 0·4 then it is a male. With the help of this formula Schuchardt (1960) and Böhme (1962) could establish a sexual difference in sheep and bovines upon examination of blood smears. Some authors prefer the formula A + B and consider C of no importance.

4. *Chromosome structure and replication*

For convenience a brief introductory review of recent advances concerning chromosome structure and replication is given here.

Contrary to all expectations the electron microscope has not yet completely bridged the gap in our knowledge between the morphological appearance of chromosomes and the proposed chemical structure of DNA as elaborated by Watson & Crick (1953).

Chromosomes (or coloured bodies, socalled because of their great affinity for dyes) are best visualized in metaphase spreads. They consist of two chromatids held together by the kinetochore (centromere). The kinetochore is seemingly responsible for the segregation of the chromatids as it divides just before anaphase commences. The mitotic spindle fibres, originating from the centrioles, are attached to it. Each chromatid consists of two chromonemata spirally coiled. Where the spirals cross, denser staining regions can be seen. These are the chromomeres (socalled " genes " of earlier observers). According to Gimenez-Martin & López-Sáez (1964) each chromatid consists of two subchromatids (i.e. chromonemata) while each of these in turn consists of two middle-sub-chromatids (as seen in preparations of *Scilla nonscripta*). These alternative names indicate some of the differences of opinion regarding ultrastructure of chromosomes. Moses (1964) however, defines the term chromatid as half a replicated chromosome which in turn consists of one or several chromonemata. The latter terminology will be used in this work.

The position of the centromere, as the primary constriction, is largely responsible for defining chromosome morphology: chromosomes therefore can be metacentric (median centromere), acrocentric (or telocentric—centromere at one end), submetacentric (centromere near the middle, submedian) or subtelocentric (subterminal) (Levan, Fredga & Sandberg, 1964). Chromosomes cannot exist for long without centromeres. If absent, chromosomes fail to orientate themselves or move properly during mitosis. Some human chromosomes possess satellites—a phenomenon which may be extended to some other primates and even some plants. Each chromosome consists of varying amounts of euchromatin (genetically active), and heterochromatin (generally genetically inactive) seen as granules in a stained interphasic nucleus. Sohval (1961) gives an excellent glossary of cytogenetical terms.

Although the morphological concept of a polynemic structure is upheld by most authors it has not yet been possible to evolve a single, generally accepted structural pattern for mammalian chromosomes due to difficulties encountered in explaining detailed structural and uncoiling mechanisms. Kaufmann (1960) regarding the fibrillar structure of chromosomes states: " . . . . pairs of pairs form ascending orders in a structural hierarchy, as is strikingly demonstrated in the polytene chromosomes of salivary-gland cells of Diptera ".

Chromosomes, therefore, are built up of many microfibrils, the exact number being still a subject of some controversy. These may range from 16 to 128 (average of 64). Such estimates are based on microfibril counts in ultramicrographs: yet no certainty exists as to whether the fibrils seen each represent a single twisted strand or a bunch, twisted and coiled spirally (Moses, 1964). He mentions the following proposed chromosome models:—

(*a*) The side-chain model of Taylor (1958) according to which short, helical DNA strands are attached to both sides of each half of a hypothetical chromosomal backbone consisting presumably of protein. The strands form complementary pairs, each attached to corresponding sites on the respective halves of the hypothetical backbone. On replication the halves of the backbone peel apart, simultaneously unwinding the complementary DNA strands. Each half with its complementary strands can then be fully replicated according to the template hypothesis. This model lacks only one mechanical specification namely that of representing a fixed linear arrangement of the genes: the DNA strands with one end waving freely are not so arranged. To meet this objection the following model was proposed.

(*b*) An end-to-end chain model originally suggested by Freese (1958) in which the free ends of the short DNA helices are joined by protein " linkers " thereby forming long chains in which " linkers " joining like strands alternate with those connecting complementary strands. Thus instead of one backbone there are two (although these are intermittent protein blocks joined by flexible bonds involving Ca-ions) with DNA helices crossing between them somewhat like the steps of a ladder. The two spines may meet forming a tube which may then become coiled into a tight helix. The Ca-bonds are supposed to give them the necessary flexibility, which allows for folding and coiling. In this model lamp-brush chromosomes could be produced by alignment of " linkers " along the chromosome axis. On replication stretching of the chain causes unwinding of the helices and duplication can take place.

(*c*) A multistranded model proposed by Steffensen (1959) in which five or six hierarchies of pairs of DNA molecules are twisted together as the strands of a rope. Replication and untwisting present problems in this case.

It has been established by autoradiographic methods that DNA replication takes place only during interphase (vegetative phase). It has been subdivided (Bullough, 1963) into (*a*) an early interphase (also referred to as G1); (*b*) a dichophase in which the cell " decides " between synthesis leading to differentiation and synthesis leading to division and (*c*) prosphase during which preparation for mitosis takes place. The prosphase is subdivided into: (i) a phase of DNA synthesis, the duration of which can be exactly determined by the use of tritiated thymidine, and (ii) an antephase (or G2) as a short predivision stage. This is followed by mitosis (prophase, metaphase, anaphase and telophase). During prophase the chromosomes become visible and are progressively contracted by differential coiling of the chromonemata to reach the maximum state of contraction during metaphase. It is understandable therefore, that the metaphase stage is the most suitable stage for numerical determinations and morphological observations of the chromosomes.

In the numerous preparations investigated during the course of this investigation it has been noticed that chromosomes in metaphase spreads of young individuals are smaller than those of adults. This must presumably be due to an increase in protein content as no evidence exists that DNA is increased. In spreads where heterochromosomes are very distinct, e.g. bovine, it is often seen that the one X-chromosome tends to be more contracted and darker stained than the other one. It is assumed that the darker stained one had undergone " lyonisation ".

B. Material and Methods

The animals used in this investigation were obtained as miscellaneous specimens sent in or offered to the departments of Genesiology and Anatomy respectively as freaks or as result of the general appeal by the Department of Agricultural Technical Services for twins. In case of the sheep the Afrikaner × Persian crossbred animal was obtained in the Soekmekaar district. Unfortunately the male twin brother had been sold to a butcher while correspondence to obtain the pair was being conducted. The other intersex animal, a Karakul, was presented by the State Veterinarian, Omaruru, South West Africa.

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The technique used in this investigation was originally based on that of Sandberg, Crosswhite & Gordy (1960). As the work progressed numerous adaptations have been made. As presently used, its prescription is as follows:—

1. Add one or more millilitres of bone marrow aspirate (from sternum or ribs), obtained by means of a two-inch long, gauge 16 Salah sternal puncture needle to either one of the following solutions.
   (*a*) Ten ml of 0·6 per cent dextrose in 0·7 per cent sodium chloride in a conical graduated centrifuge tube, with addition of 0·2 ml of heparin (1000 IU/ml) as anticoagulant, or
   (*b*) nine ml of 0·6 per cent dextrose in 0·7 per cent sodium chloride in a conical graduated centrifuge tube with addition of 1 ml of 10 per cent potassium oxalate solution as anticoagulant. The latter anticoagulant was found to be more suitable than heparin for domestic animals as a stock solution could be prepared and used whenever required. With heparin very diffuse fibrin threads still appeared in goat and pig marrow after hypotonic treatment; these often prevented cells from being spun down.
2. Centrifuge at 350 rpm for 3 to 5 min, discard the supernatant fluid and add 10 ml of 0·44 per cent sodium citrate. If cell concentrate exceeds 0·5 ml progressively more hypotonic sodium citrate must be added. Mix by gentle inversion.
3. Incubate in a waterbath at 37° C for 15 minutes. Centrifuge for 3 to 5 min as before. Discard the supernatant fluid.
4. Add ± 5 ml of freshly prepared fixative (acetic acid: 1 part, methyl alcohol: 3 parts) to the cell sediment without disturbing it. If sediment is rather voluminous (more than 0·25 ml) gently raise it by pipetting some fixative gently along the side of the tube underneath the sediment without breaking it up thus ensuring rapid fixation. Allow to fix for at least 30 minutes.
5. Resuspend the cells by breaking up the sediment with gentle pipetting. If cell suspension is moderately turbid drop preparations can be made directly, otherwise it can be spun down again and resuspended in acetic-alcohol to give the correct density. If it is too concentrated, metaphases do not spread out sufficiently; if too dilute, metaphases are too far apart.

   (Exposure of the cell suspension to 45 per cent acetic acid in the refrigerator for one hour or more is optional. In tissue cultures it was found to be unnecessary and often actually detrimental to staining and spreading of chromosomes. It is recommended in cases of insufficient spreading).
6. Coat cold glass slides on one side only with a very thin layer of a dilute (2 drops to 5 ml of distilled water) solution of Mayer's albumin-glycerine. It reduces surface tension and allows even spreading of the cold water film. It should not be allowed to dry on the slide as it then takes up the stain used later. Rinse in iced water, drain, hold the slide at a slight angle and drop 3 to 4 drops of cell suspension separately on the coated surface starting at the upper end. The drops should not be allowed to overlap each other. After each drop has fallen, aid its rapid spreading by blowing hard but evenly. After excess fluid has been drained evaporate quickly by gently heating over a gas flame. Suitable spreads can be obtained from such cell suspensions even after they have been kept in the cold for three weeks.

7. When the slides are dry, fix for 3 to 5 min in pure methyl alcohol, then rinse in a 50 per cent solution and stain for 3 to 6 hours in a 10 per cent Giemsa solution in distilled water to which a few drops of ammonia have been added. Rinse in tap water, wipe the slides underneath and allow to dry.
8. Remove any stain deposit by briefly dipping individual slides in a jar of oil of cloves, blot dry and rinse in two successive jars of xylol. Scan directly for mitotic spreads or after mounting in cedar wood oil or Permount.

Where chromosomes from somatic tissues other than haemopoietic tissues were required these organs were collected in sterile containers immediately after slaughtering the animal and tissue cultures prepared. To actively growing cultures colchicine was added (to a strength of 2 μ gm/ml of culture) at 8 a.m. and spreads made three hours later. The cells were loosened from the test tubes in which they were cultured either by scraping with a polythene spatula or by the use of Versene. The cell suspensions in culture medium thus obtained were spun down and treated exactly as above, starting from step 2.

CHAPTER II

CYTOGENETICS OF THE PIG—*SUS SCROFA DOMESTICA* LINNAEUS

A. THE NORMAL PICTURE

1. *The karyotype*

This term as used here signifies " . . . . a systematised array of the chromosomes of a single cell prepared either by drawing or by photography, with the extension in meaning that the chromosomes of a single cell can typify the chromosomes of an individual or even a species " (Böök, *et al.* 1960).

(*a*) *Review of literature*

Wodsedalek (1913), who according to Makino (1951) was the first to investigate pig chromosomes, found the diploid number to be 18 in males and 20 in females. Hance (1917), Makino (1944) and Sachs (1954) establised it as 40. Krallinger (1931), Bryden (1933), Hillebrand (1936), Crew & Koller (1939), Melander (1951), Gimenez-Martin, López-Sáez & Monge (1962), Stone (1963), McConnell, Fechheimer & Gilmore (1963), Di Antonio (1964) have all determined it as $2n = 38$. With the exception of Wodsedalek, who could find no Y-chromosome, no one experienced difficulty in establishing the X-Y mechanism in pigs.

This investigation was undertaken due to the discrepancy which existed in the literature on chromosome counts in this species and as one of the necessary preliminaries to cytogenetic studies on abnormal individuals.

(*b*) *Results*

Observations were made on bone marrow spreads from two boars and two sows of the Large White breed. Only well-spread metaphase spreads were selected for counting. These are listed inTable 1 below and represented in Plates 1 to 3:—

TABLE 1.—*Distribution of chromosome number per number of metaphases counted*

Chromosome Number	37	38	39	41	76
No. of metaphases (2 males)	1	40	0	2	2
No. of metaphases (2 females)	0	30	1	0	1

Preparations from bone marrow collected between 9.30 and 10.30 a.m. (winter months only) contained most mitoses, whereas those collected between 8 and 9 a.m. and between 11 and 12 a.m. had mostly mature erythrocytes and granulocytes with few metaphases. More data are being collected to confirm or disprove the suspicion of a timelinked developmental cycle in bone marrow in this species.

Bone marrow collected at 10 a.m. from the manubrium of a pig immediately after routine slaughtering, i.e. after immersion of the carcass in hot water at 62° C for 5 min to soften its hair, contained no metaphases. It is believed that the high temperature stimulated metaphases to complete division, no new ones being formed.

(*c*) *Discussion and conclusion*

It is evident that present observations (Table 1) confirm the chromosome number of domestic pigs as $2n = 38$. Melander's report (1951) on the old Swedish variety as having $2n = 30$ is, therefore, of considerable interest and should be confirmed by means of more modern techniques.

It is difficult to apply the Denver nomenclature of human chromosomes (Böök, *et al.*, 1960) strictly to those of the pig. In the pig we have long and short, metacentric, submetacentric, acrocentric and subtelocentric chromosomes. To arrange them simply in descending order of length would cause confusion. It is advisable to group similar types of more or less corresponding lengths together and to arrange such groups in descending order of length.

This complies with the necessary simplicity and freedom from ambiguity of the Denver system without too great a deviation therefrom. Six groups of autosomes can thus be formed (Plate 3). Group 1 contains a single largest pair of submetacentrics. Group 2 to 4 contains three pairs of acrocentrics of different lengths. Only in groups 5 to 8 and 9 to 13 are difficulties encountered.

Group 5 to 8, according to the author's classification, begins and ends with a typical subtelocentric pair [differing in this respect from that of Gimenez-Martin, *et al.* (1962) who, instead also begin their next group with a subtelocentric pair]. These are relatively easy to pick out in spreads. In between are two pairs of submetacentrics.

Group 9 to 13 also consists of submetacentrics, the second pair (10) being almost metacentric and resembling the X-chromosomes very closely. Chromosome 13 often, and 9 occasionally show a lighter zone around the centromere, making their identification relatively easy. In spreads, chromosome 13 has often been seen to break transversely at the centromere, giving the appearance of two extra chromosomes. This may be the reason for the difference in chromosome counts (38 or 40) obtained in the past. Chromosomes 11 and 12 are submetacentrics.

Group 14 to 15 contains two pairs of small metacentrics.

Group 16 to 18 contains three pairs of acrocentrics, the first pair being comparatively large and the last one very small. The latter occasionally appears as single rodlike chromosomes without distinct evidence of a centromere. This is probably an artefact.

The sex chromosomes are both metacentric, the Y being the smallest chromosome present.

The chromosome length progressively decreases during prophase and metaphase due to greater coiling of the chromonemata. This length further varies according to the cell type, being greater in myelocytes and proerythroblasts but smaller and more condensed in polychromatophilic erythroblasts. As homoplastic haemopoiesis normally takes place, metaphases of the latter cells are relatively frequent. The individual length of homologous chromosomes is also further influenced by varying degrees of spreading (Plates 1, 2 & 3). This effect is impossible to control with present methods. It may often cause great variation in length of chromosome pairs and militates against successful pairing off. From these observations it is obvious that in karyotypes the actual length of chromosomes is of relatively little importance but their relative length of vast significance.

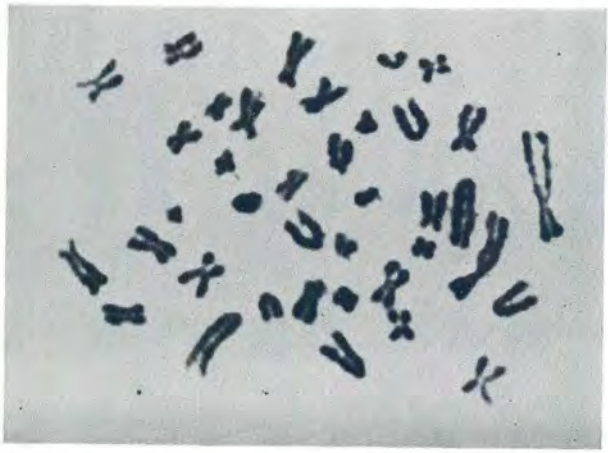

PLATE 1.—Chromosomes of an adult boar

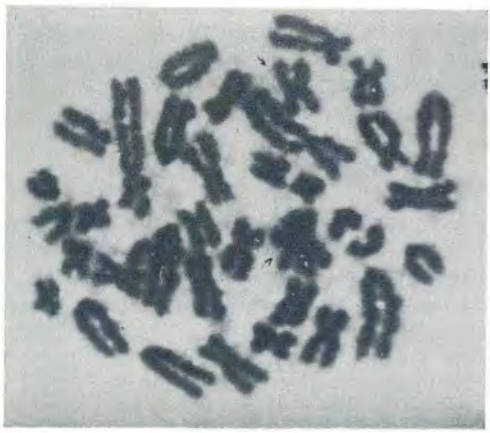

PLATE 2.—Chromosomes of an adult sow. X-chromosomes at arrows

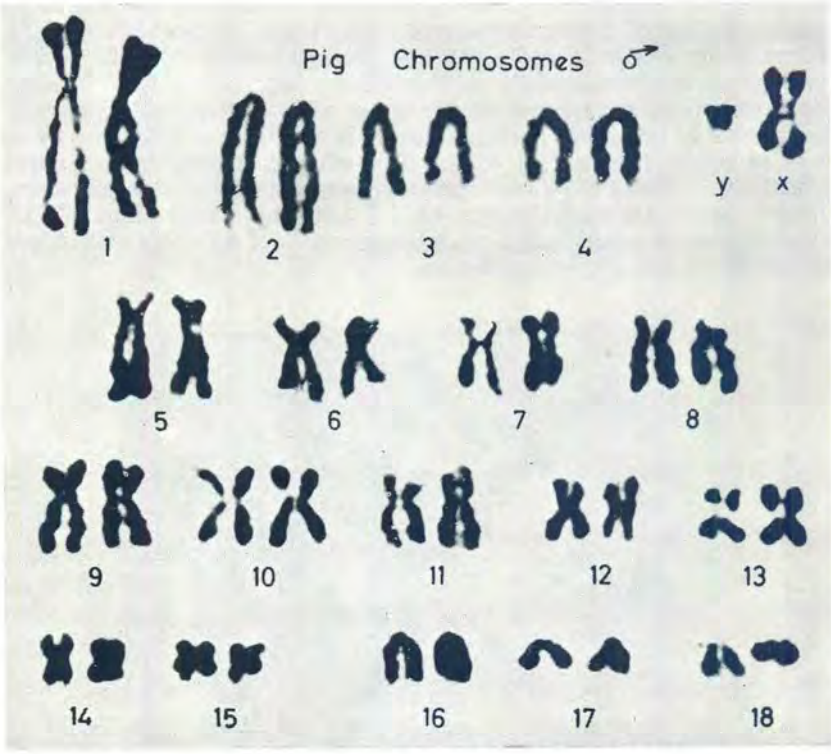

PLATE 3.—Karyogram of pig chromosomes

An alternative method of arranging the chromosomes of a pig is that advocated by Stone (1963) and Di Anionio (1964) in which non-acrocentrics are arranged in one group according to length while the acrocentrics and heterochromosomes form two other groups. Although the method given in this paper is preferred, much can be said in favour of a three-group karyogram.

2. *Cytological Sexing*

(*a*) *Review of literature*

(1) *Nuclear sexing.*—Cantwell, Johnston & Zeller (1958) investigated sex chromatin from eight males, ten females and seven intersexes in liver, heart, kidney, thyroid, pancreas, adrenal, cerebrum, dorsal root ganglia, sympathetic ganglia, cerebellum and spinal cord. They found positive results in nervous tissue only, the spinal and sympathetic ganglia being the most successful. They established the average size of the Barr body as $0{\cdot}84 \times 1{\cdot}12\ \mu$; it was present in 95·2 per cent of neuronal nuclei in normal females, in 93·5 per cent in intersexes and in 7·2 per cent in normal males.

Schmidtke (1957), investigating sex chromatin from four males and four females, found that chromocentres next to the nucleolus of the various neuronal cells (similar to the bovine) were of no differential significance but only those in the karyoplasm

or next to the nuclear membrane. Chromocentres were found to be present in 3·1 per cent of neuronal nuclei (apart from those next to the nucleolus) in males and in 73·4 per cent of female nuclei. They were totally absent in 82·9 per cent of male neuronal nuclei and 12·3 per cent of female nuclei. Sexual differentiation based on neuronal sex chromatin is, therefore, possible.

Struck (1961) found the epithelial cells of the buccal mucosa to be classified into three types:

I. Fine chromatin granules with 2 per cent containing a distinct sex chromatin body next to the nuclear membrane. This is not present in males.

II. Coarse chromatin granules, no sex chromatin.

III. Very fine chromatin. No sex chromatin.

Sexual dimorphism could be established in buccal smears of pigs and intersexes (four positives per 100 nuclei of Type I).

Hard & Eisen (1966) found buccal smear preparations unsuitable for revealing the presence of sex chromatin bodies. Buccal smears therefore have a limited application.

(2) *Polymorphic sexing*.—Due to the ease with which polymorphic sexing can be done, authors have applied this method to the pig more than nuclear sexing.

Lüers & Struck (1960) found no drop forms (drumsticks without handles) or drumsticks in neutrophils in blood smears from male pigs and 0 to 1·6 per cent drumsticks and as many drop forms in blood smears from females (total counted 500 neutrophils). Rentsch, Bruschke & Schulz (1960) found 1 to 3 drumsticks in 200 neutrophils, Kraft (1960) found 3 to 7 per cent drumsticks in castrated animals. Wollrab & Lichtner (1963) found an average of 0·4 drumsticks in 500 neutrophils in male pigs and 12 drumsticks per 500 neutrophils in females. They found A + B >10 always indicated a female. Sessile nodules were also found to be more numerous in females, 6 to 24 (average 10·6), than in males, 0 to 8 (average 2·5). Eighteen out of 25 boars had no drumsticks whereas four had one and the other three, two drumsticks. Small clubs and rackets in general are more numerous in males but great individual differences exist.

(*b*) *Results*

(1) Nuclear sexing was found applicable only to neuronal nuclei. The peripheral ganglia were most suitable in this respect.

(2) Blood smears from three Large White boars and three Large White sows were examined for nuclear appendages of neutrophils. The results are given in Table 2.

TABLE 2.—*Nuclear attachments as found per* 1000 *neutrophils. Figures given in each case are averages of* 5000 *neutrophils counted*

Sex	Drum-sticks	Sessile nodules	Small clubs	Rackets
2955 ♀	10	19	10	0
2878 ♀	3	16	9	0
2954 ♀	1	13	4	0
2951 ♂	0	13	6	0
2876 ♂	1	18	10	1
2952 ♂	1	12	7	0

(*c*) *Discussion and conclusion*

It is disappointing that drumsticks in neutrophils of females are so scarce. It is even more frustrating to note that they also occur in males. This limits their usefulness in nuclear sexing. Most authors have observed this paucity in sows and the presence of occasional drumsticks in boars. Lüers & Struck (1960), however, reported drumsticks to be absent in boars. Wollrab & Lichtner (1963) noted a differential frequency in favour of sows by means of which their sex could be determined. Although this is possible, a large number of doubtful cases will always be encountered. For critical evaluation the method should therefore be discarded: the drumstick count in females is too low to be seud as a criterion in routine sexing.

Drumsticks are rare in human males (Overzier, 1963) but appear to be relatively more frequent in boars. In the light of the investigations of Hughes (1929) and Ohno, Trujillo, Stenius & Christian (1962) the question arises whether boars in which drumsticks occur are not perhaps chorionic dizygotic twins of females from which early transfusion of blood cells took place. In the light of further investigations on intersexes, this possibility can be excluded completely: to date not the slightest indication of chimerism has ever been encountered in the pig. On the other hand it may mean that the single X-chromosome in the boar behaves differently from that of human males, appearing in the resting phase occasionally as heterochromatin as Ohno (1963) has suggested.

B. The Intersex condition in Pigs

1. *Introduction*

In the past it has always been a problem whether these animals were genotypically male or female. Goldschmidt (cit. Freudenburg & Widmaier, 1959) was of opinion that in general no zygotic male intersexes existed in mammals, whereas Dantschakof (1941) came to the opposite conclusion in her experiments. Crew (1923) considered sexual intergrades to be males with insufficient male hormone produced. This hypothesis was repudiated by Baker (1925). Arthur as late as 1959 still considered the porcine hermaphrodite to be a behavioural male.

The consensus of opinion even among medical men tended towards considering them genotypic males (Freudenburg & Widmaier, 1959). In connection with nuclear sexing they make the following significant statement: " . . . . und liesze sich auf diesem Wege das zygotische Geschlecht der Intersex mit Sicherheit bestimmen so käme die Lehre von der Intersexualität bei Säugetieren ein gutes Stück voran ".

Freudenburg (1958) states that intersexuality occurs in 0·2 per cent of the total German pig population. The Senior State Veterinarian, Pietermaritzburg, reported that out of a total of 80 piglets on a farm in the Estcourt district 22 were intersexes; this indicates how high the incidence of intersexes can be. Johnston, Zeller & Cantwell (1958) found in the litters in which intersexes appeared that there were 88 normal males, 1 cryptorchid, 26 intersexes and 94 normal (?) females, i.e. 26 intersexes out of 209 piglets. True hermaphrodites (Hermaphroditismus ambiglandularis) were usually not suspected until post-mortem examination. Male pseudohermaphrodites (Hermaphroditismus testicularis) could be detected from behaviour and external appearance.

Pond, Roberts & Simmons (1961) reported true hermaphrodites to be about one-tenth as common as pseudohermaphrodites whereas in isolated herds as many as 20 per cent of the progeny could be affected. They found too, that the sex ratio was heavily in favour of males (68 males, 22 females and 16 intersexes). This was not the case in Johnston and co-workers' experiments.

Pig intersexes can assume practically all intermediate stages: those with ovaries only are usually referred to as *female pseudohermaphrodites*; those with both testes and ovaries on one or both sides as *hermaphrodites*; those with only testes as *male pseudohermaphrodites* (Plate 6) and those with testes and ovary only on one side as *lateral hermaphrodites*. The term intersex is used for any of these types. The nature of the accessory organs is not taken into account for these definitions.

Cohrs (1962, p. 809) considers the abovementioned nomenclature outdated. He states: " Nach dem Ergebnissen der neueren experimentell-genetischen Forschung ist diese Einteilung des Zwittertums in einen echten und falschen Hermaphroditismus nicht mehr haltbar. Auch der sogenannten Pseudohermaphroditismus ist in gleicher Weise wie der Hermaphroditismus verus echtes Zwittertum, das nur unter einer besonderen Erscheinungsform verläuft ". He prefers to classify hermaphrodites as follows:—

1. Hermaphroditismus ambiglandularis (Zweidrüsenzwitter). It can appear in three forms:
   (*a*) Hermaphroditismus glandularis bilateralis (on both sides either a testis and an ovary or an ovariotestis).
   (*b*) H. glandularis unilateralis [on the one side either a testis, an ovary or ovariotestis and only ducts (♂ or ♀) on the other side].
   (*c*) H. glandularis alternans (on one side a testis and on the other an ovary).
2. Hermaphroditismus testicularis (Hodenzwitter).
3. Hermaphroditismus ovarialis (Eierstockzwitter).

Moszkowicz (cit. Cohrs, 1962) compiled a scheme of principal types of testicular intersexes in man. Such a scheme is still lacking in animals.

Cohrs states that two types of sexuality exist: a chromosomal and a hormonal. The former is responsible for hermaphroditic states while the latter exerts an augmenting effect (" Hilfswirkung ") on the already formed individual. Intersexes, therefore, do not develop by chance but as a morphological expression of a biological " Gesetzmäszigkeit " (p. 812).

In the pig there appears to be a general rule as to the nature of the accessory genital structures in intersexes. They depend to a certain extent on the type of gonad present. Asdell (1942) found that there is a general tendency for the presence of a testis to suppress the oviduct and uterus to some extent but not completely, whereas the presence of an ovary may suppress the male genital ducts either completely or partially. Rare exceptions to this general tendency do occur, however. Both male and female ducts and associated gland systems develop in the presence of an ovotestis. A uterus is, therefore, invariably present in all pig intersexes. [It may, however be severely suppressed and represented only by an enlarged uterus masculinus as in case VII (see later)]. Asdell ascribed the development of ducts and associated glands to the initial stimulus provided by the gonadal hormones produced by the type of gonad present, but gave no idea as to why the one or the other gonad develops. In view of the foregoing he advanced the view that development of the accessory reproductive organs is influenced by the nature of the gonadic tissue on the same side.

Krediet (1934) in castration studies on intersexes came to the contrary conclusion: " Een intersexentestis bevordert even goed den groei van een intersexenbaarmoeder als een intersexenovarium of een ovariotestis dat doet ".

This morphological fluctuation which is encountered in the ducts and gland systems of the genital tract of intersexes is the result of variation in the development of a potentially bisexual genital system, an initial characteristic of all mammalian embryos.

According to Schuchardt (1960) and Benoit (1964), five different criteria are used in circumscribing sexual development:

(*a*) The genetic or chromosomal criterion, according to which sex is determined at fertilization by the chromosomal constitution.

(*b*) The germinal criterion, whereby the sex of the germ cells produced is used as standard.

(*c*) The gonadal criterion according to which sex is identified by the histological appearance of the gonads. This need not correspond to the morphological sex.

(*d*) The gonopherous criterion, based on the morphology of the genital pathways.

(*e*) The somatic criterion according to which sex is identified by the secondary sexual features and the psychic characters.

These standards are ideally suited to closer circumscription of varying degrees of human intersexuality. Lack of information renders their application to animal intersexes premature.

Intersexes are mostly sterile. Nevertheless, a few rare cases of pregnancy in hermaphroditic sows (Folger, 1932; Smidt, 1962; Hulland, 1964; Benoit, 1964) have been reported. Folger's pig was an intersex but was pregnant. On the right side a uterus, oviduct, infundibulum and an ovary with fifteen corpora lutea were present. On the left side the uterus tapered to a point, with testis, epididymis and ductus deferens in that vicinity. No spermatogenesis was present. Each horn of the uterus had four foetuses.

Hulland's (1964) case is of considerable interest as it had an apparently normal right ovary, containing 12 corpora lutea, whereas the left gonad consisted of an irregular mass of epididymal and sterile testicular tissue. This sow had given birth to four live pigs, that died after birth; three dead ones were extracted from the uterus while a further two were found partially autolysed in the peritoneal cavity as a result of a ruptured uterus. Benoit (1964) also often found ovulation, as determined by the presence of ova and corpora lutea, to be normal in intersexes but spermatogenesis was consistently absent. This finding was also supported by Brambell (1929) and Pond, Roberts & Simmons (1961). Baker (1925), however, observed early spermatocytogenesis in one case but no actual spermiogenesis. Krediet (1939) stated that in testes of newly born intersexes spermatogonia may be encountered between the living cells or in the lumina of the spermatogenic tubules. They degenerate during the puberal period.

The first definite determination of the zygotic sex of intersexes was done by Cantwell, Johnston & Zeller (1958) who found the nuclear sex (determined in nerve cells) of seven intersexual pigs to be female: four were male pseudohermaphrodites, two were hermaphrodites and one had gonadal agenesis. Lüers & Struck (1960) found the neutrophils of a pig intersex to possess drumsticks—thus being regarded by them as female. Pond, *et al.* (1961) found their intersexes all to be genetic females on determination of Barr bodies in sections of liver and spinal cord.

Although Makino, Sasaki, Sofuni & Ishikawa (1962) had been the first to report on chromosome determinations done on pig intersexes, Gerneke (1964), at the time of publication, had been unaware of the former paper. In all cases studied the nuclear sex was found to be female.

2. *Results*

A description of eight intersexes studied is given:

Case I (2905)

The first opportunity for cytogenetic studies on pig intersexes arose when a gonadal male pig intersex (male pseudohermaphrodite) of the Large White breed, about two months old, was referred to this department.

The chromosome number was determined on bone marrow preparations, made according to the previously outlined technique. It was found to contain the normal number of $2n = 38$ (see Table 3). Two X-chromosomes were present (Plate 5), constituting the normal female configuration. The few aneuploid metaphases listed are of no significance, as they could have occurred during preparation or as a result of non-disjunction of chromosomes. The two tetraploids listed are spreads of early anaphases. No visible morphological aberrations of the chromosomes were found (Plates 4 & 5).

TABLE 3.—*Chromosome number as per number metaphases counted*

Chromosome number	28	35	38	39	76	Total
Metaphases counted	1	1	60	2	2	66

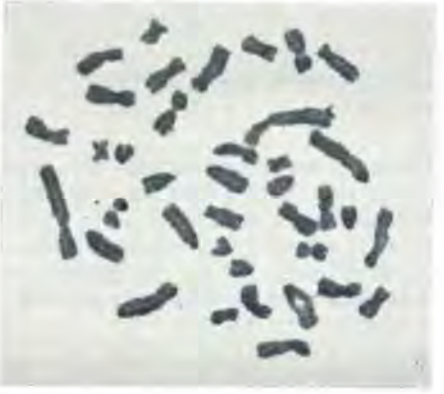

PLATE 4.—The appearance of the chromosomes of a gonadal male pig intersex in a bone marrow cell at metaphase

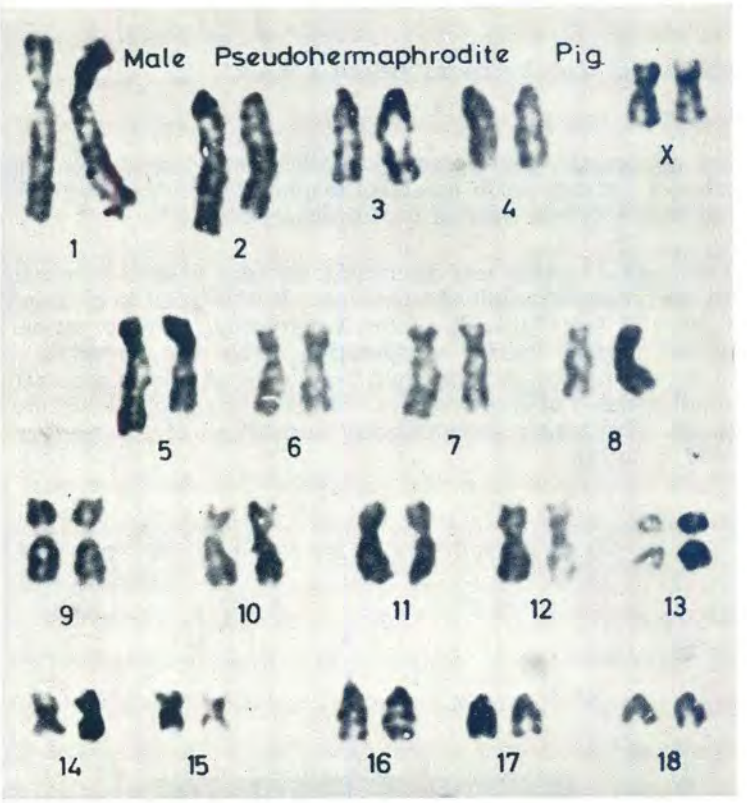

PLATE 5.—A karyogram of the chromosome spread in Plate 4

The genital tract (Plate 6) was characteristic of a gonadal male intersex (H. testicularis) in showing a greater development of male than female features. Two distinct testes with epididymes (right epididymis only partly attached to the testis), ductus deferentes and all male accessory glands were present. The penis was rudimentary and projected caudodorsally, giving the impression of an enlarged clitoris. The crescentic urethral opening—5 mm wide—was situated on the dorsal aspect of the penis approximately 6 mm from its tip.

The preputium was relatively large and resembled the ventral commissure of the rima pudendi of a sow. The opening of a blind preputial fossa was situated caudal to the urethral opening; the tip of the penis was exposed. Ovaries were absent and the body and horns of the uterus were well formed, typical of tubular bisexuality. The

internal spermatic lymphnodes, attached to the pampiniform plexuses, were present. These were probably the structures described by Johnston, *et al.* (1958) in the following terms: "The ovarian bodies were not composed of true ovarian tissue. Sections appeared more similar to the lymph glands". The fallopian tubes could be seen running along the dorsal ridges of the epididymal tails and ending on the medial side of the testes in infundibula (arrow on left testis Plate 6). The organs on the left side were larger than those on the right. The corpus uteri ended in a minute opening on the colliculus seminalis. No openings of the ductus deferentes were found.

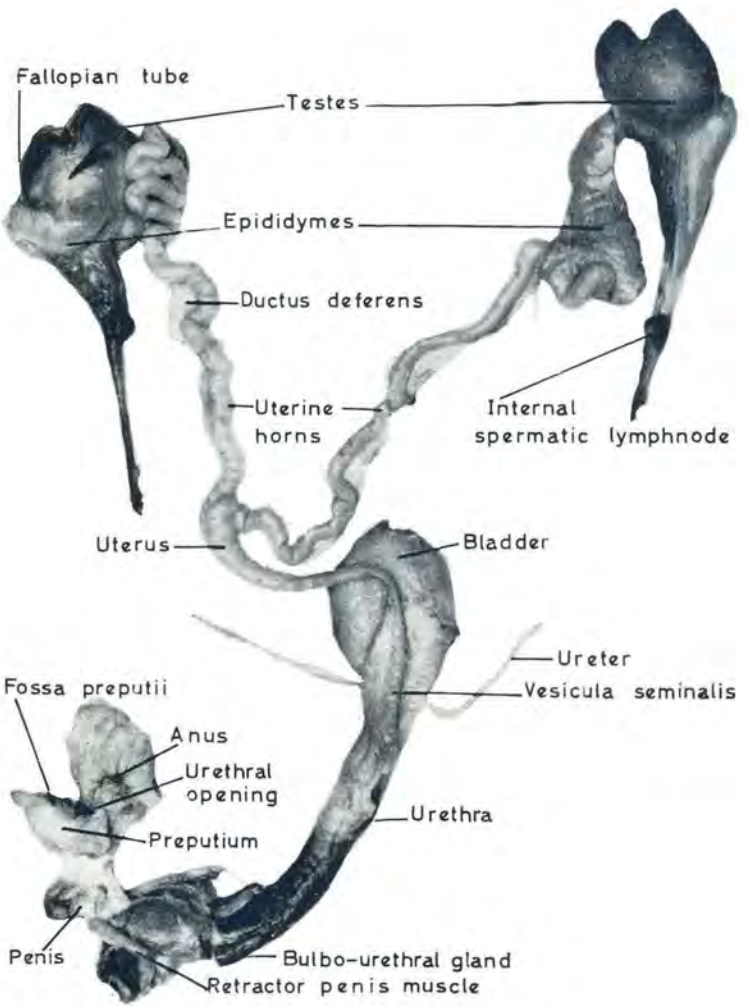

PLATE 6.—The genital organs of a gonadal male pig intersex

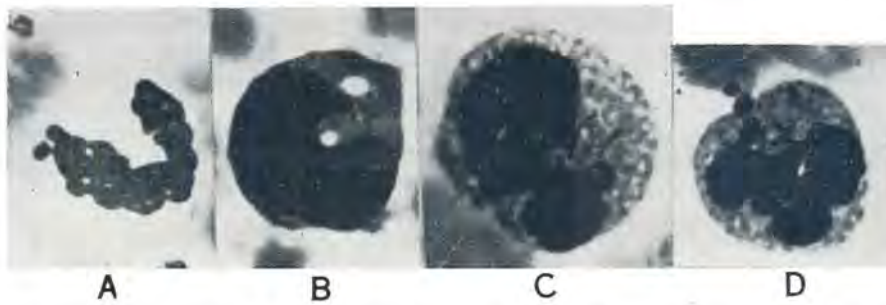

PLATE 7.—The sex chromatin appendages in neutrophils of a gonadal male pig intersex. In A & B (B being a myelocyte) " drumsticks " are seen, in C a sessile nodule and in D a sessile nodule and a structure considered to be a " racket " are shown

The testes had a uniform structure throughout and contained no spermatogonia in the seminiferous tubules, only Sertoli cells, with numerous cells of Leydig between the tubules. These cells are induced by the testis tubules (Krediet, 1939) and develop from the mesenchymal cells. This inducing substance might primarily be formed by the Sertoli cells, as spermatogonia are present only as a rare exception and in any case degenerate during the prepuberal period.

The penis, prostate and epididymis were not examined microscopically; the other organs had a normal histological structure.

" Drumstick " counts on 5,000 neutrophils showed an average of two drumsticks (Plate 7), 30 sessile nodules and three small clubs per 1000 neutrophils.

According to its karyotype, the present case is a gonadal male intersex (H. testicularis) with a female chromosomal constitution. Although drumstick sexing showed it to be chromatin positive, one hesitates to draw specific conclusions from so small a number, the more so because this method is unreliable in the pig (Gerneke, 1964). None the less, it supports the observations on the chromosome picture.

Case II (3198)

This was a gonadal male pseudohermaphrodite (H. testicularis) being one of two intersex siblings received from a farmer in the Brits district, case III being the other.

It had two well-formed scrotal sacs, with both testicles completely descended and well-formed. The testicular tissue was dark brown in colour. Before slaughtering, the scrotal sacs had reached a considerable size partly due to fluid distension of the uterine horns which originated close to the head of the epididymis as blindly ending but enlarged tubes (Plate 8). This fluid filled the bladder and uterus and caused considerable distension of the latter. It was yellowish-brown in colour. The presence of fallopian tubes could not be ascertained due to excessive distension of all tubes.

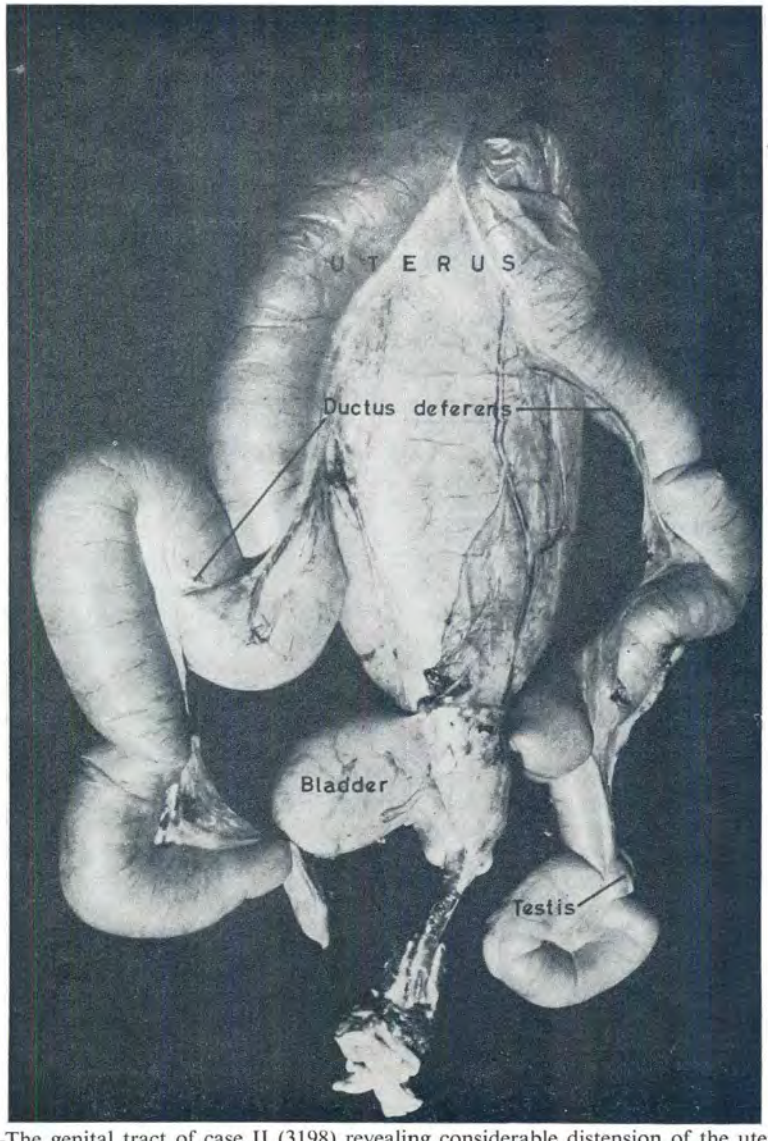

PLATE 8.—The genital tract of case II (3198) revealing considerable distension of the uterine body and horns. The left testis was removed for culture preparations

The ductus deferens was continued from the tail of the epididymis along the enlarged uterine horns and lateral sides of the body of the uterus to end blindly at the utero-vaginal junction: a small utero-vaginal opening was present. The epididymis, although small, was completely formed. None of its tubules extended into the uterine cavity as described in a case by Johnston, *et al.* (1958). The last portion of the ductus deferens was enclosed by tree-like extensions of the vesicula seminalis. A small prostate and two laterally situated bulbo-urethral glands were present. All male accessory ducts and glands were excessively hypoplastic.

The clitoris was enlarged to represent an immature penis with the urethral opening situated dorsally. Urination through this dorsal opening was naturally directed upwards and backwards and took place in spurts.

Histologically the atrophic seminiferous tubules were lined only by Sertoli cells. Spermatogonia were completely absent. The excessive numbers of Leydig cells, which were responsible for the dark brown appearance of the testes, were comparatively more numerous than in the normal adult testes. This was also found to be the case by Johnston, *et al.* (1958).

A single paranucleolar chromatin body was present in most Sertoli cell nuclei after formalin fixation but mostly away from the nucleolus in Zenker-fixed sections. Although originally considered as the one X-chromosome of the female complement in hetero-pycnosis, careful comparison with the normal male testis indicated such a concept most likely to be erroneous. Apart from this body one or mostly two other, but smaller non-specific chromocentres were also consistently seen between the nucleolus and the nuclear membrane, i.e. in the karyoplasm.

The ductus deferens was lined by pseudostratified columnar epithelium with short stereocilia. Slight epithelial invaginations, simulating initial glandular formations, possessed rather vesicular nuclei, many of which had a distinct plano-convex Barr body situated against the nuclear membrane. They were identical to those seen in the nuclei of the fallopian tube of the pig.

The uterine wall, which was very thin and fibrous due to the degree of distension presented the picture of a typical endometritis. The endometrium consisted of granulation tissue which was composed of numerous vertically directed arterioles, no glands, numerous plasma cells, neutrophils and active fibroblasts. The myometrium was mostly replaced by fibrous tissue. No epithelial lining was present, but this could have become desquamated due to autolysis. Numerous haemorrhages were responsible for the blackspotted macroscopic appearance of the endometrium.

The adrenal glands were of special interest as examination of the cortex of each revealed extensive focal cellular hypertrophy (Plate 9) but very little lipid storage. The smaller groups of hypertrophied cells were mostly seen in the zona reticularis or tending to develop from similar cells often seen in the periphery of the cortex. The hypertrophied cells very much resembled those of the corpus luteum possessing a more eosinophilic endoplasm and a less eosinophilic ectoplasm. Fine lipoid droplets could be identified throughout the hypertrophied cells. Mitotic figures were present though very rare, thereby indicating a certain degree of hyperplasia. The focal cellular hypertrophy represented adenomatous changes of the cortex. Otherwise the adrenal glands appeared to be quite normal, judged histologically. Biochemical assays were not undertaken.

Metaphase spreads made from cultures of one testis and the left kidney revealed the following:—

(*a*) The testis culture yielded 92 excellent spreads, all of which had 38 chromosomes with an XX complement. No indication of any male chromosome was seen.

(*b*) The kidney culture yielded 25 excellent spreads, all of which also had 38 chromosomes with an XX complement. Again no indication of any male chromosome was found.

Metaphase spreads of bone marrow cells obtained by sternal puncture from the living animal yielded a total of 167 spreads of which only four cells had 39 chromosomes, the extra one being a minute dot-like structure resembling a Y-chromosome, i.e. about 2 per cent of the cells contained this small structure. It was definitely not part of chromosome 13 as these were both present.

No polymorphic sexing was considered necessary as chromosomal evidence was sufficiently indicative of the sex.

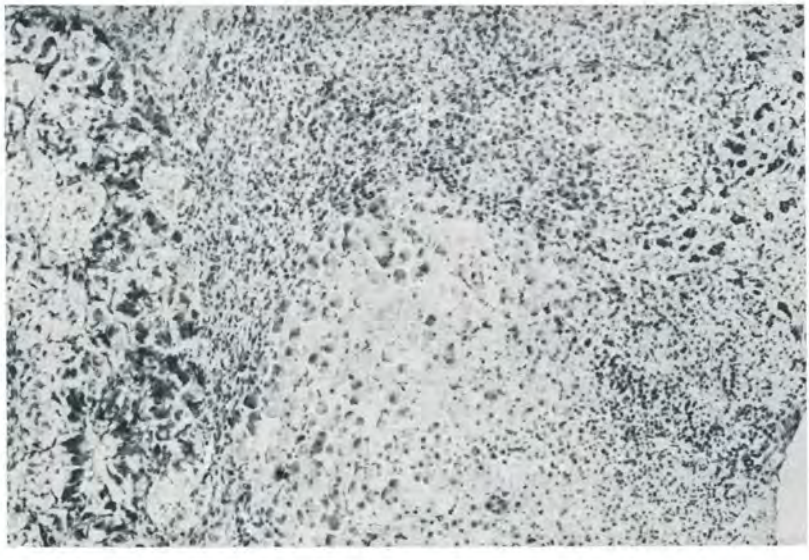

PLATE 9.—Focal cellular hypertrophy as revealed in the adrenal cortex of case II. The zona glomerulosa on the right indicates some atrophy. The medulla on the left is quite normal. Stained H. & E. 95 ×

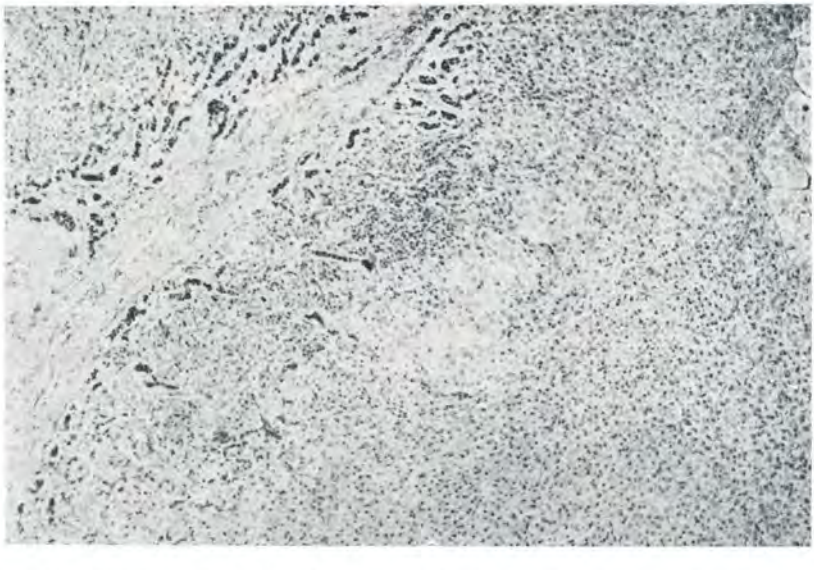

PLATE 10.—The only area of focal cellular hypertrophy that could be located in the adrenal cortex of case III. Stained H. & E. 75 ×

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Case III (3199)

This pig was a sibling of case II and was found to be a right lateral hermaphrodite (Cohr's nomenclature not applicable). It had an *ovotestis on the right side* and a *testis only on the left*. Only the right gonad had descended into the scrotal sac which was unnaturally distended due to fluid accumulation in the right uterine horn attached to the tail of the epididymis. A separate fibrous mass morphologically resembling an immature ovary was situated in a bursa next to the point of emergence of the right ductus deferens, but, contrary to expectation no follicles were present. Rogers (1929) stated that the ovarian portion of the ovotestis was invariably situated on the caudal pole of the testis next to the tail of the epididymis. On the other hand, the ovotestis contained several follicles on its ventral border as well as one mass of luteal tissue—evidence, therefore, that luteinization had occurred. It was impossible to determine whether ovulation had actually taken place and must only be assumed.

The genital ducts and gland systems were much better developed on the right side than on the left. The left uterine horn was not continuous with the right but present as a few saccular dilatations which contained a gelatinous substance, *no* fluid and presented *no* sign of endometritis. Uterine glands were present. The distended right uterine horn contained no uterine glands and its histological appearance was identical to that of case II, i.e. revealing a typical endometritis.

The histological appearance of the testis, glandular systems and ducts was identical to that described in case II.

Only a slight tendency for focal hypertrophy of the adrenal cortex was seen. The hypertrophied cells were identical to those of case II. An occasional mitotic figure was indicative of hyperplasia (Plate 10). Otherwise it was quite normal. The pineal, which unfortunately was not collected in the previous two cases, was of significant interest as the presence of two types of chief cells could be ascertained: one type with a shrunken darkstaining nucleus with increased eosinophilia of cytoplasm; the other type had a large vesicular nucleus with a prominent nucleolus and a nuclear membrane, which was often folded. These cells were decidedly hypertrophied with expanded processes. Intercellular oedema was conspicuous. Even the neuroglia cells had vesicular nuclei.

Bone marrow collected at various intervals yielded a total of 169 spreads, 165 of which had 38 chromosomes, one had 35, one 34 and two had 39 chromosomes. The latter two had an extra dot-like chromosome which most probably could have been an early neutrophil granule. The female sex complement was present in every spread.

The last sample of bone marrow from this pig was collected at 10 a.m., after 400 ml of 5 per cent chloral hydrate had been given intraperitoneally at 9.25 a.m. and 6 ml of nembutal at 9.55 a.m. It was noteworthy that the number of spreads obtained per preparation exceeded 35. Mitoses of haemopoietic cells had apparently been stimulated tremendously by the pretreatment.

Cases IV to VIII were received from a farmer in the Estcourt district and only the significant features of each are discussed.

Case IV (3342)

It was a male pseudohermaphrodite (H. testicularis), about $1\frac{1}{2}$ months old, presenting histological features in both the right and left testes similar to those of the testes of case I, but with the Leydig cells small, scarce and inactive and Sertoli cells small with granular nuclei and apparently not yet fully differentiated. The accessory

genital glands were all present but due to the prepuberal state still relatively inactive. The vesicula seminalis dorsally had two saccular dilatations next to each other, interconnected by a large opening. The uterus was represented only on the right side and lined by simple columnar epithelium with bleb-like secretion. No endometrial glands were present. Slight endometritis was already obvious.

Chromosome analysis revealed 31 excellent spreads all of which were found to have 2n = 38 chromosomes with a normal female sex complement. These findings were substantiated by the presence of Barr bodies in nerve cells and ductus deferens.

The adrenal was completely normal but examination of the pineal revealed cellular hypertrophy and oedema, the same as in the previous case.

Case V (3343)

It was a bilateral true hermaphrodite (H. glandularis bilateralis) about two months old with the ovarian part better developed on the left side and the testis on the right side. This arrangement was reflected in a much greater development, even to the extent of coiling, of the left uterine horn whereas that on the right side was only 6·5 cm long.

Histological examination of the left ovotestis revealed numerous primordial follicles and several normal developing follicles with oöcytes. The testis contained immature Sertoli cells in the tubules, no spermatogonia and poorly developed and scattered cells of Leydig. In the right ovary (situated apart from the testis) follicular development was not as advanced, follicular cavities were absent but numerous atretic follicles in various stages of regression to corpora fibrosa atretica were present. The right testis was larger than, but histologically identical to the left one.

In the uterine horns only occasional glands with cystlike enlargements, were present. Endometritis associated with fibrosis had already commenced. The body of the uterus ended just cranial to a weakly developed vesicula seminalis and prostate. A ductus deferens, accessory glands and enlarged clitoris were present as usual.

The adrenal was normal whereas the pineal had undergone the same cellular hypertrophy as in some of the previous cases.

Chromosome analysis yielded 26 spreads all with 38 chromosomes and a normal female sex complement. This was substantiated by nuclear sexing on nerve cells.

Case VI (3344)

This pig, aged about two months, was a typical case of male gonadal pseudohermaphroditism (H. testicularis), two distinct testes being present without any trace of ovarian tissue. An epididymis, ductus deferens, vesicula seminalis, prostate, urethra, bulbo-urethral glands and enlarged clitoris were present as usual. The uterine horns were present on both sides. Examination of the endometrium revealed no inflammatory reaction. The uterine glands were normal.

The pineal contained a central cystic cavity filled with a clear fluid and lined simply by flattened chief cells. The chief cells were hypertrophied, some revealed folded nuclear membranes while the interstitial tissue was very oedematous (Plates 11 & 12). The adrenal appeared quite normal.

Chromosome analysis on bone marrow yielded 30 spreads all with 38 chromosomes and a normal female sex complement. Nuclear sexing on nerve cells corroborated these findings.

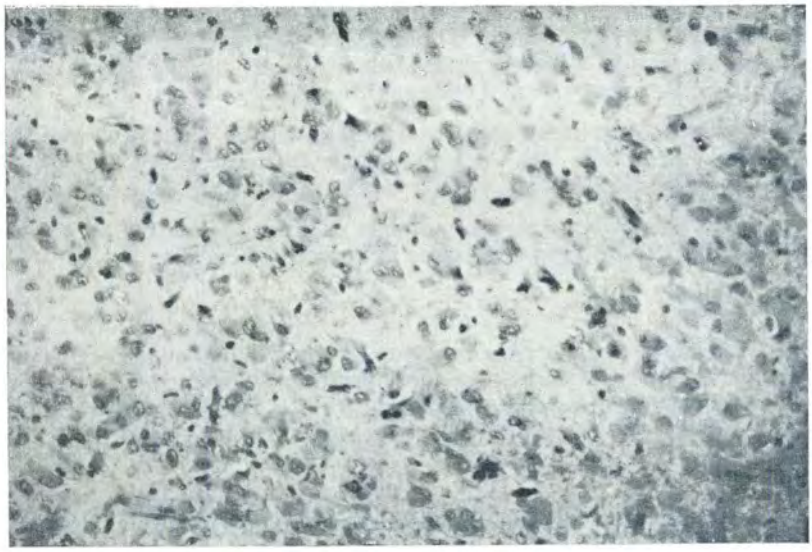

PLATE 11.—An area in the pineal of case VI revealing generalized cellular hypertrophy and oedema. Stained H. & E. 190×

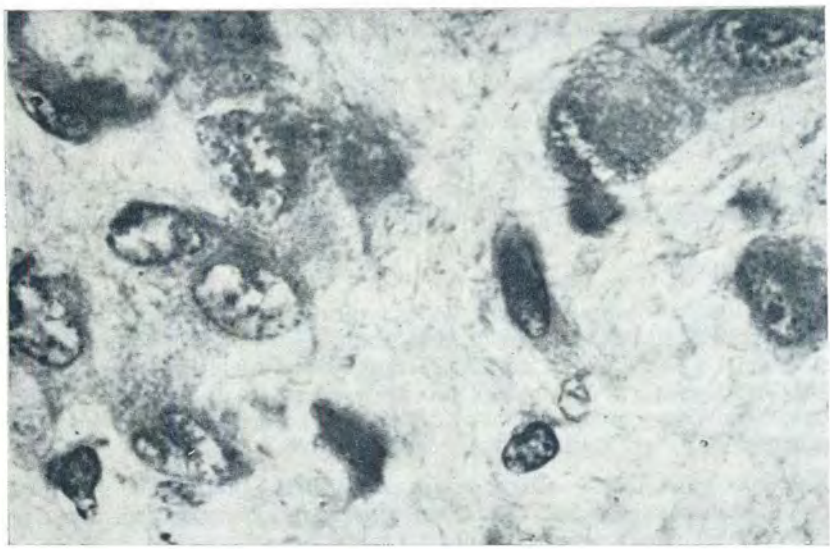

PLATE 12.—Cellular hypertrophy and folding of the nuclear membrane as seen in the pineal of case VI. Oedema of the intercellular spaces is also seen to be present. Stained H. & E. 1200×

Case VII (3345)

This pig aged about two months presented the most typical case of almost complete masculinization: it was a male gonadal pseudohermaphrodite (H. testicularis). The only feminine trait was the presence of an enlarged uterus masculinus. On the right side a small uterine horn was present next to the ductus deferens; it ended blindly 5 cm from the spermatic lymphnode. On the left side it ended blindly 0·5 cm from the bifurcation. The clitoris was enlarged as usual.

The testes were hypoplastic and had the usual appearance histologically with numerous Leydig cells. In the accessory glands, which were all present, there were signs of slight activity.

The adrenal appeared normal. The pineal contained a fluid-filled cyst identical to the previous case. Cellular hypertrophy and oedema were obvious.

Chromosome analysis yielded 17 spreads each with 38 chromosomes and a normal female sex complement. Barr bodies were present in nerve cells.

Case VIII (3346)

This pig aged three to four months, was a gonadal male pseudohermaphrodite (H. testicularis) similar to case VI but Leydig cells were more numerous and apparently active. The enlarged clitoris (=penis) was much better developed than in any of the previous cases and was situated 9 cm ventral to the anus; it could easily be retracted or extended in its sheath. The accessory glands, which were all present, contained appreciable secretion. A uterine horn (3 to 6 mm in diam.) was present only on the left side and there was thus no bifurcation.

The pineal contained a cystic cavity, hypertrophied cells and a fair degree of oedema, as in previous cases. The adrenals were normal.

Chromosome analysis on bone marrow yielded 12 spreads all with 38 chromosomes and a normal female sex complement. Barr bodies in nerve cells substantiated this finding.

3. *Discussion*

(*a*) *Occurrence and morphology*

Intersexuality is a common occurrence in cows, pigs and goats but is considered rare in other animals. As stated previously, it may reach an incidence of up to 20 per cent in isolated herds of pigs. Of the various grades that occur in pigs, male pseudohermaphroditism (H. testicularis) was found to be more frequent than the female type or even true hermaphroditism. This frequency was also noted by Krediet (1936) who considered " . . . . de gevallen van vervrouwelijking zoo in de minderheid, dat er onderzoekers zijn, die zich daarmede niet eens bezighouden ". He of course, being unable to do chromosome determinations still considered the female type to be the result of a feminizing change of a genetic male (and *vice versa*!). Smidt (1962) found male gonads to be present more frequently on the right side.

After studying the organogenesis of ovariotestes from the point of view of sex reversal, Krediet (1939, p. 233), made the following statement showing that even without recourse to chromsome studies he was on the right track: "In all these cases the presence of cortical cords is an indication that one has to do with originally female animals and that a stronger male factor makes its influence felt, which is partly or entirely changing the originally female elements into male ones ".

Regarding male and female pseudohermaphroditism in man, Sohval (1961) states: " In either case the nuclear sex chromatin pattern is concordant with the gonadal sex. Similarly, the sex chromosome complement appears to correspond to the type of gonads present ". It appears from this that in humans the karyotype corresponds to the gonadal sex. Sohval (1963) and also Jones, Ferguson-Smith & Heller (1965). however, have found that in true hermaphroditism testicular tissue does develop in association with an XX sex chromosome complement. In the pig, judging only from the present investigations and from the eight chromatin positive cases mentioned previously, the genetic sex is female, *in direct contrast to the gonadal sex*. This supports Baker's (1925) view that sex-intergrade pigs are masculinized genetic females.

Zuckerman & Groome (1940) on the other hand, in an experimental investigation on a male pseudohermaphrodite pig of the Large White breed, were in favour of the hypothesis that pig intersexes are " . . . . genetic males whose differentiation was altered as a result of a change in their chemical environment following upon the passage of an excessive amount of maternal sex-hormone across the placenta ". This is in support of Witschi's (1939) explanation of human male intersexuality being caused in a similar manner. In view of the present findings, such a hypothesis cannot be supported: it is most likely that Zuckerman's experimental animal was a genetic female similar to those in this investigation. Furthermore, the pig has an epithelio-chorial placenta which makes transplacental transfer of hormones less likely. One would also expect a greater incidence of intersexuality in the pig, were Zuckerman's hypothesis correct.

To obtain greater clarity on the nature of intersexuality in polytocous animals, such as the pig, simultaneous karyological, haemo-immunological and especially biochemical studies must be undertaken on intersexes and their siblings. At present a boar and sow known to produce intersexes are being kept for further study of possible chorio-allantoic anastomoses and embryological development of the intersexual gonads—the latter a subject only partially studied by Krediet (1939) due to lack of known intersex embryos.

(*b*) *Chromosome constitution*

Reports of the chromosome constitution of intersex pigs had at the time of writing not been encountered in the literature. Since then the articles by Makino, *et al.* 1962 and lately that of Hard & Eisen (1965) were noted. Makino's results correspond to mine but those of Hard & Eisen do not. They found their pig to be " . . . a *sterile male with an XX sex chromosome complement*, rather than a true hermaphrodite " (my own italics). As Hard & Eisen had no access to autopsy findings they could possibly have missed suppressed Müllerian duct remnants and therefore, in the light of the findings in this paper, it is also just possible that their pig was a masculinized genetic female. They were also unable to investigate the adrenal or pineal which could possibly have corroborated these findings. In the eight cases studied during this investigation all were genetic females ($2n = 38$, XX) notwithstanding the fact that one (case VII) had practically no internal female genital apparatus.

With the exception of human cases (Overzier, 1963), chromosome aberrations as the primary cause of intersexuality have not been described in mammals. The report of Makino (1950) who is quoted by Young (1961) as having found chromosomes of the male type in a goat intersex, the two mentioned above and the present study, in which intersex pigs were found to be genetic females, are hitherto the only records of karyotype studies on intersexes of domestic animals, apart from those on bovine freemartins.

In this study considerable effort was expended in trying to locate a Y-chromosome in these intersexes—the finding of such a chromosome would have offered a most suitable explanation for the masculinization which is seen to such a varying extent in them. The suggestion of a Y-chromosome being lost from an XXY-zygote as cited by Hard & Eisen (1965) cannot be supported in the light of these findings. The finding of a small dot-like structure in cases II and III in a few spreads could have been regarded as indicative of an XX-XXY mosaic, somewhat akin to the Klinefelter's syndrome in humans. This would have been a most significant finding but unfortunately the dot-like structure was smaller than a normal Y-chromosome and one hesitates to draw specific conclusions therefrom. It is, therefore tentatively interpreted as an artefact, perhaps even a single granule of one of the promyelocytes.

It is therefore concluded from available evidence at hand that intersex pigs are masculinized genetic females.

(*c*) *Possibility of Freemartinism*

If porcine intersexuality were produced by suppression of female development in zygotic females by male stimuli or by the antagonistic action of male and female stimuli as a result of chorionic vascular anastomoses (Hughes, 1929) then all such hormonally determined intersexes should have the female chromosome configuration. [Greene, Burrill & Ivy (1938), found androgens to be more potent than oestrogens in embryonic structures]. Such is the case in bovine freemartins; all are genetic females (Overzier, 1963). In these, the gonads are either rudimentary or resemble testes externally. Germ cells are absent while Wolffian and Müllerian ducts are poorly developed.

There is as yet hardly any evidence for considering the intersex condition in pigs as freemartins. Hughes (1929) and Benoit (1964) are the only authors who have described two cases of chorio-allantoic anastomoses in pigs, the latter with the production of a freemartin condition.

Sex-intergrades have also been born singly or as co-twin to normal females (Baker, 1925). Sex-intergrades can occasionally be fertile (Hulland, 1965; Benoit, 1964) while this is not the case in the bovine fremartin: they are invariably sterile. Consequently the mechanisms of formation of these two conditions cannot be similar. It does not rule out, however, the possibility that freemartins as such can also occur in pigs, but with the exception of Benoit's paper no other evidence has been brought forward for such a condition, nor has a single instance been substantiated where female influence altered male development in monochorionic opposite-sexed twins. Hughes (1929) described one instance in the male co-twin where Müllerian duct enlargement could have been caused by female influence, but this she considered mere coincidence. The male co-twin, also in the case of cattle, always develops normally.

In any case chorio-vascular anastomoses result in blood chimerism as has been mentioned in Chapters II and III. Neither in the cases studied, nor in the literature has any evidence ever been noted of the occurrence of blood chimerism in pigs. It thus appears that choriovascular anastomosis is not the mechanism responsible for intersexuality in pigs. Baker (1964) in a search for monozygotic twins among 29 sets of two litter mates and four sets of three litter mates found none. Histocompatibility was shown to exist between monozygotic sibs of the armadillo.

The fact that pregnancy occurred or that at least ovulation had taken place and corpora lutea formed in these hermaphrodite pigs indicates that female germ cells can develop notwithstanding the presence of testicular tissue. Benoit (1964) postulated the testicular sterility to be due to the higher temperature in the abdominal cavity. But in cases II and III at least one testicle had descended into the scrotal sac and yet it was sterile! Sterility in the case of the testis but not in the case of the ovary must be caused by the absent Y-chromosome and by no other reason. In the case of the XX-complement follicles can of course develop to maturity as is shown by the reported cases of pregnancy in intersexes above. See also (*e*) below.

Although Walker (1961) mentions the fact that undescended testes in a hermaphrodite bitch are apt to reveal a higher incidence of neoplasia no such growths were identified in these studies. As causative factor for such growths he considered higher temperature or possibly inability of poorly functioning testes to fully control the "feed-back" mechanism from the pituitary. Continued gonadotrophic stimulation could induce tumour formation.

(*d*) *Nuclear sexing*

Ever since nuclear sexing has been applied in genotypic studies (Overzier, 1963; Sohval, 1961) sex determination in doubtful cases has become a simple matter. Such determinations have also been applied to pig intersexes.

Cantwell *et al.* (1958) using the Feulgen method, found the nuclear sex of seven intersexual pigs to be female. Their determinations were made on nervous tissue, which they found to be the only suitable material for nuclear sex determinations. Of the seven intersexes, four were male pseudohermaphrodites with anatomical features similar to the one described above, two were true hermaphrodites and one was a case of gonadal dysgenesis.

In support of Cantwell and co-workers' findings, Barr bodies could only be demonstrated in ductus deferens and nerve cells of intersexes. They were particularly prominent in the epithelium of the ductus deferens and of course in nerve cells. In every case the chromosomal sex was confirmed by determination of the nuclear sex.

By means of drumstick determinations in neutrophils, Lüers & Struck (1960) demonstrated that a pig intersex—gonadal type not specified—was female, i.e. chromatin positive. They, however, came to the conclusion that polymorphic sexing was unsuitable as a reliable routine method of sexing pigs, goats and sheep, due to the paucity of drumsticks in neutrophils (2/500 neutrophils). In case I the author came across drumsticks in two boars (1/1000 neutrophils). Their chromosomes, however, were not checked. This observation supports the opinion of Lüers & Struck (1960) that polymorphic sexing is not reliable in the pig. For this reason it was not attempted in the other intersexes.

Lichtner (1961) found that males and cryptorchids were distinguishable from females by nuclear morphology but division of intersexes into types by this means was not possible.

(*e*) *Genetics*

The consensus of opinion is that intersexuality is a hereditary characteristic. Some cases of intersexuality, especially in pigs and goats, are inherited as recessive characters; in pigs the possibility of a single pair of recessive genes and one or more modifiers as primary causes has been considered (Johnston *et al.* 1958). Baker (1925) also considered it to be inherited as a sex-limited recessive with the possibility of modifying autosomal genes. Baker (1925) found intergrade-producing females commoner than intergrade-producing males.

Koch, Fischer & Schumann (1957) quote Jakobiec & Marchlewski, Salerno, Mohr and Crew as all being in favour of the hereditary nature of intersexuality. Hereditary intersexuality has also been reported by Asdell (1956) in Brown Swiss bulls. It is thus possible that modifying autosomal or Y-chromosome genes, apart from adrenocortical hormonal influences discussed later, may influence the normal female chromosome complement to the extent that gonadal male intersexes are produced. These modifiying genes may be in the nature of male determiners as found for instance in *Drosophila melanogaster*. Here male determiners are present on autosomes and female determiners on X-chromosomes. With reference to humans Sohval (1963) states: " Female determiners are thought to be located largely, if not entirely, in the X-chromosomes while male determiners are situated in the Y-chromosome and in the autosomes ". The Y-chromosome of *D. melanogaster* is necessary only for male fertility and has no male-determining influence. If such a situation is at all present in mammals it is likely that more than one modifying gene is present otherwise it would be difficult to explain the variability encountered in intersexes. The possibility also exists that mutations could produce additional male determiners on autosomes thereby causing the sex-determining imbalance in these cases.

Although the Y-chromosome in humans is reported to contain genes which control development of testicular structures (Lauritzen, Froland & Johnsen, 1965; Sohval, 1963) such structures apparently can develop in the absence of a Y-chromosome. This concept is strengthened by the finding that pig intersexes, and especially case II where cultures of testicles and kidneys were made, were all found to contain an XX-complement.

Similar cases of testicular development in the absence of a Y-chromosome have also been reported for humans (Therkelsen, 1964; Sohval, 1961; Conen & Erkman, 1963; Hungerford, Donelly & Nowell, 1964; De le Chapelle, Hortling, Niemi & Wennstrom, 1964), but no single case has been reported where fertility occurred in the absence of a Y-chromosome. Strongly localized mosaicism can, however, be incriminated.

Brogger & Aagenaes (1964) reported on a human ovario-testicular hermaphrodite in which the testes, only after a second biopsy, proved to be an XX-XY mosaic. Cultures from marrow, skin and peripheral blood revealed a normal female karyotype.

In the case of the intersex pigs the more obvious conclusion is that testicular development can occur in the absence of a Y-chromosome presumably as a result of abnormal hormonal control (see below) or possibly as a result of autosomal male determiners. However, for fertility to develop a Y-chromosome appears to be essential.

(*f*) Role of the adrenal cortex

The focal cellular hypertrophy and to a lesser degree hyperplasia of the adrenal cortex observed in pig intersexes have not been recorded before. As such it merits the critical evaluation recent knowledge and observations allow. In the present series of eight cases it occurred extensively in case II (Plate 9) but was limited to only a single group of small nodules in case III (Plate 10). These two siblings were nine and ten months old respectively when slaughtered, while the others in which the phenomenon was not observed, were between two and three months old: an age difference of $\pm$seven months extending over a significant phase of life namely puberty.

The first consideration is to what extent the lesion has any connection with the aberrant genital morphology. This connection is best elucidated by comparison with the adrenogenital syndrome in humans.

Bierich (1963) raised certain aspects in the pathogenesis of the congenital adrenogenital syndrome which are fundamental in realizing this interrelationship:—

1. In humans he found that the embryonal adrenal cortex became functional at a time when genital duct differentiation was almost complete. In the adrenogenital syndrome androgens produced by the functionally aberrant adrenal cortex would, therefore, mostly affect those parts of the genital organs derived from the urogenital sinus and genital tubercle. The degree of virilization was dependent on the time at which the necessary effective threshold level of androgen release was reached by the foetal adrenal. This may even be delayed postnatally in which case no adrenal hyperplasia was noticed.
2. In the aetiology of the congenital adrenogenital syndrome defects of certain enzymes (11- and/or 21-hydroxylases) needed in cortisol synthesis have been found to result in the formation of an inactive intermediate as well as in an inadequate cortisol synthesis (Bierich, 1963; Van Rensburg, 1965). As a result of the low level of plasma cortisol the normal inhibitory feedback mechanism on ACTH release does not exist: continuous ACTH stimulation caused adrenocortical cellular hypertrophy, resulting in increased production of androgens and oestrogens—the action of the latter is suppressed by the former.
3. The resulting high level of plasma androgens suppresses gonadotrophin release by the pituitary with the result that the gonads do not mature.
4. In the adrenogenital syndrome the adrenals tend to increase in size with age and in old age nodules of hyperplasia are often found.

In view of the close interrelationship functionally between the adrenal cortex and the sexual system (Van Rensburg, 1965) and the existence of an established adrenogenital syndrome in man (Bierich, 1963) one is apt to conclude an immediate causal relationship in the pig. It is possible that such a relationship may exist but its pathogenesis must differ considerably from the human adrenogenital syndrome. There are some important differences: in this syndrome in humans we mostly have virilization of the urogenital sinus and genital tubercle (Bierich, 1963), i.e. an external virilization in which gonads and genital ducts are not affected. In pig intersexes the gonads and genital ducts are mostly affected, often without marked external changes being present—intersexes are often only identified on slaughtering. In pigs therefore, an internal virilization or often a practically complete masculinization (case VII) is present which must be initiated at a much earlier stage than in humans.

In humans the adrenogenital syndrome is familial in its incidence while in the pig the intersex condition is inherited as a sex-limited recessive (Johnston *et al.*, 1958). Above all, cases II and III were siblings whereas cases V to VIII originated from the same farm and were probably related.

On the other hand the adrenogenital syndrome has been shown to affect both male and female in humans (Bierich, 1963) while in pigs the intersex condition as far as determined affects females only. The relatively late appearance of the adrenal lesion long after the abnormality of the genital morphology had established itself suggests that it may be secondary and not primary as in humans. In pigs the adrenals

appear normal at birth (Johnston *et al.*, 1958) while those in the human condition reveal bilateral hypertrophy of the adrenal cortex brought about mainly by expansion of the zona fasciculata in both directions. Only in old age are nodules of hyperplasia often found. At least one may therefore state that the position in the pig is reminiscent of an adrenogenital syndrome but the differences are much too significant for confirmation of such a diagnosis.

With the above considerations in mind the pathogenesis of the condition in pigs can be very tentatively considered as occurring by either one of two possibilities: (The influence of the pineal gland as a third possibility is discussed in the next section):—

(*a*) The sex-limited recessive gene and its possible autosomal modifiers could cause the modification or absence of an enzyme in the adrenal cortex (similar to above) resulting in an immediate virilizing effect. For this mechanism to be involved an early effective level of androgen release must be reached in order to induce early testicular development. The foetal adrenal must release secretions not later than the end of the indifferent stage of the gonads. [In humans according to Gillman (1948) the adrenals are highly differentiated before the sex becomes manifest. The same holds true for the pig]. At this stage the adrenal cortex must therefore be assumed to be functionally aberrant although normal histologically. That a very gradual hypertrophy and even slower hyperplasia could exist in the cortex is perhaps borne out to a limited extent by the observation of a generalized but slight degree of cellular hypertrophy of the fascicular zone in case III but not apparent in the others due to their being too young. The impression was also gained that the zona glomerulosa was slightly atrophied and the fascicular zone generally wider than normal. Unfortunately weights and measurements of the adrenals were not taken for confirmation of such an impression as these findings were not suspected initially. If the degree of cellular hypertrophy (cases II and III) be correlated with the state of the gonads then the impression is gained that the degree of cellular hypertrophy has a quantitative significance revealing a more direct interrelationship with testicular development. This becomes apparent if the interrelationship between virilization and hyperplasia as set out in 2, 3 and 4 above is considered.

External virilization, limited practically to hypertrophy of the clitoris, becomes apparent mainly after a couple of months. If the adrenal cortex is primarily involved one would expect more adult indications of virilization as occurs in the human syndrome (Bierich, 1963). It is therefore extremely peculiar that masculinization should seemingly stop abruptly without any further progressive changes becoming evident. Until further evidence become available it is therefore uncertain to decide whether the adrenal lesion can be considered as the late morphological expression of a primarily determined biochemical aberration or whether it is present merely as a secondary effect.

(*b*) As a second possibility recessive gene and autosomal modifiers could directly influence the gonadal medulla to produce its morphogenetic inductor (" Medullarin ", Sohval, 1963). A mutational variation in number of such autosomal modifiers could determine the production of varying amounts of " cortexin " and " medullarin " and thus stimulate presence of ovary and testes in varying degrees. In birds and amphibians

Witchi & Opitz (1963) have indicated that when the genetically determined epistatic gonad inductor is inhibited the weaker and hypostatic inductor is given a chance to become active and may prevail. Once the gonads are formed in the pig interstitial cell, secretions are responsible for the morphological differentiation of the genital ducts (Sohval, 1963).

It can be assumed that if the primary influence is chromosomal the adrenal cortices would also be implicated: possibly by secreting androgens. Results would then be similar to that postulated in (*a*) above. In the pig, contrary to the position found in humans and most other animals the interstitial cells of Leydig only undergo physiological atrophy some time after birth (Gillman, 1948). Although my own observations correspond with those of Johnston *et al.* (1958) in that hyperplasia of Leydig cells occurs, it must until further information becomes available be considered as a normal occurrence. At the same time, however, it must not be forgotten that Leydig cells, being normally male specific cells with an XY complement, in these cases possess an XX complement which immediately suggests aberrant functioning. The seminiferous tubules contain no germ cells and remain hypoplastic.

The increasingly important role ascribed to the adrenal cortex in abnormalities of the genitals, has recently been stressed by Van Rensburg (1965). In cattle with cystic ovaries hypertrophy of the adrenal cortex was encountered. Garm (1949) believed the primary defect in cases of cystic ovaries to be an increased production of FSH and a failure to produce sufficient LH for ovulation. Prolonged secretion of oestrogen stimulated ACTH release which resulted in adrenocortical hyperplasia. Only one case of virilization of a cow with an adenoma of the adrenal cortex has been recorded (Smith & Jones, 1961). She developed a crest on the neck and the bellowing of a bull.

The observations on the adrenal fall in line with cytological evidence from the pineal (see next section) and further implicate hormonal or neuro-humoral imbalance as an aetiological factor. The problems involved are, however, of such dimensions that no definite conclusions can be drawn until supporting histochemical and biochemical determinations together with cytological confirmation upon examination of the pituitary have been made. These fall outside the scope of this work. The problems encountered nevertheless accentuate the importance of serial sectioning of the adrenal glands, of undertaking exact measurements of cortical widths and of weighing the adrenals and comparing the results critically with those obtained on normal glands.

(*g*) *Role of the pineal gland*

Cellular hypertrophy of the pineal gland was observed in all the intersex pigs in which it was examined. This finding is substantiated by the histological investigations of Heiniger (1965) on the pineal gland. In his summary he stated: " Even very old sows, which show big nuclei as well, do not reach the volumes of the cell nuclei in the pineal gland of the intersexual animals ". He was, however, not able to give a functional interpretation of the hypertrophied cells. Frauchiger (1963) had also noticed these morphological differentiating characteristics of the intersex pineal. In my opinion hypertrophy, folding of the nuclear membrane and oedema of intercellular spaces as observed by them as well as in the cases investigated in this work (Plates 11 and 12), indicate increased anabolic activity. This is in accord with the latest concepts on nuclear activity and cell function.

Although cysts have been reported in most animals the incidence in intersexed pigs, namely 44·5 per cent (Heiniger, 1965) is the highest. These cysts are not lined by ependyma but seem to be enlarged tissue spaces—perhaps a result of oedematous changes in the pineal. As such spaces and even hypertrophy of pineocytes normally occur in aged animals the question must be raised whether they can be interpreted as signs of increased activity or merely as indications of ageing and premature cessation of function.

If the inhibitory action of the pineal on FSH secretion and on ovarian atrophy (Steyn, 1966) is kept in mind, the latter consideration, namely ageing and premature cessation of function, is most unlikely. Cessation of ovarian function takes place in old sows and would necessitate a greater inhibiting action by the pineal. The pineal gland, contrary to former opinion, would then remain constant or actually increase in size with old age, as Heiniger (1965) observed. The hypertrophy is more pronounced in the intersex: one may thus assume that there is a greater inhibitory effect in the latter.

If the more recently determined mesodermal origin of pineocytes (Frauchiger, 1964, Meyburg, 1964 cited by Heiniger, 1965) instead of the older neuroectodermal origin be considered then an early cessation of function is also very unlikely.

The fact that cellular hypertrophy of the adrenal cortex apparently succeeds cellular hypertrophy of the pineal as indicated by the incidence of each in the intersexes of different ages implicates a possible interrelationship. There is some evidence that a hormone adrenoglomerulotropin secreted presumably by the subcommissural organ, but possibly also by the pineal (Steyn, 1966), seems to affect aldosterone secretion by the adrenals. In anencephalic human monsters Potter (1952) invariably found extreme hypoplasia of the adrenals. She could not associate this with the fact that the hypophysis in such cases also tended to be hypoplastic because in the normal foetus she found the adrenal gland to have a greater size, before the hypophysis could be expected to exert any hormonal effect, than in mature anencephalic monsters. The question therefore arises whether congenital absence of the pineal is perhaps not the true cause of such hypoplastic adrenals. Hypoplasia of the hypophysis is to be expected in such cases if its dependence on neurohumors is considered. Is the development of the adrenal not also then dependent on neurohumors especially if its close association with a neuroectodermal derivative, the medulla, is considered?

Cellular hypertrophy of the pineal associated with the gonadal disturbance and delayed nodular hyperplasia of the adrenal cortex suggests an interrelationship in which the pineal probably plays a primary role. This may mean that the sex-limited genetic effect is primarily exerted through the pineal. However, the position may not be as simple as this if the feedback mechanism of control existing in endocrine and neurohumoral control be considered. The final settling of this question once again requires extensive physiological and biochemical determinations for which the intersex pig appears to be well suited.

(h) Origin of the uterine fluid

The origin of the yellowish-brown fluid which fills the uterus is not easy to assess. According to Arthur (1959) it is undoubtedly a uterine secretion formed under the progestational effect of testicular androgens. In the distended uteri of cases II (Plate 8) and III no glands were present in the mucosa (they are present when not distended) and yet the amount of fluid gradually increased as could be seen by the enlargement of that part situated in the scrotal sacs and visible externally. It is

most unlikely, therefore, that the fluid had originated from the uterine glands. In case III the left uterine horn was not continuous with the right and therefore inaccessible to urine. There was no endometritis but only some gelatinous substance and no fluid; histologically it was perfectly normal. The endometritis of the right horn had evidently been caused by some irritating substance, such as urine, which could have gained entrance through the utero-vaginal opening. As the bladder contained a fluid similar in appearance to that of the uterus such a deduction appears justified. It must also be kept in mind that the genital organs were abnormal, that the cervix was poorly developed and that the urethral opening was situated on the dorsal side of the enlarged clitoris with the result that, as is general for intersex pigs, urination occurred in dorsally-directed spurts, thereby causing a certain amount of back flow. These facts, together with the absence of a micturating mechanism in the uterus and the rather small utero-vaginal opening facilitated urine retention in the uterus. Urine, being foreign to the uterus caused irritation, endometritis and gradual distension of the uterus. The conclusion is therefore made that the bulk of the uterine fluid consists of urine with small initial contributions from the uterine glands and additions at a later stage of some cellular debris and perhaps tissue fluid.

(*i*) *Proposed new nomenclature of intersex conditions*

Due to uncertainty which arises with the existing nomenclature regarding the exact nature and presence or absence of the gonads I would like to suggest a modification of Cohrs' (1962) nomenclature. As the gonads are the primary and most significant structures which actually control the development of the ducts and gland systems their presence or absence should be stressed in the terminology. The position on the left should be mentioned first and a hyphen inserted between left and right positions when written. The nature of the genital ducts is left out of account as their morphology is very variable, and can be forecast within certain limits from the gonads present.

All intersexes are listed as hermaphrodites and are subdivided according to presence or absence of testis, ovary or ovotestis. The terms unilateral or bilateral are used to indicate presence on one or both sides respectively; the terms sinister and dexter to indicate left and right sides; the prefix ovario- for ovary and ovo- for ovotestis.

The following categories, which are self-explanatory, are then suggested:—

Hermaphroditismus testicularis bilateralis.
Hermaphroditismus testicularis unilateralis (sinistra or dextra)
Hermaphroditismus ovarialis bilateralis.
Hermaphroditismus ovarialis unilateralis (sinistra or dextra).
Hermaphroditismus ovotesticularis bilateralis.
Hermaphroditismus ovotesticularis unilateralis (sinistra or dextra).
Hermaphroditismus testiculo-ovarialis or *vice versa*.
Hermaphroditismus testiculo-ovotesticularis (right gonad an ovotestis) or *vice versa*: H. ovotesticulotesticularis.
Hermaphroditismus ovario-ovotesticularis or *vice versa*: ovotesticulo-ovarialis.

(*j*) *Sexual instincts of sex-intergrade pigs*

Generally speaking the sexual instincts are male. The enlarged clitoris can become erect and protruded. Copulation of females is often attempted.

Some do show signs of heat. They are apt to show fits of rage caused by seeing females on heat. During these fits of rage their tusks become worn down in a characteristic manner (Baker, 1925). These fits of rage are apparently caused by the desires provoked by the male hormones which cannot be satisfied. It suggests that male hormones are produced. The Leydig cells, especially in the older individuals, appear to be active. This corresponds to the observations of De Regt (1935) that abnormal quantities and ratios of sex-hormones exist in the urine of an intersex pig.

4. *Conclusions and summary*

1. All intersex pigs investigated were genetic females notwithstanding male sexual instincts and almost complete absence of female genital tracts in at least one. A modal number of 38 chromosomes was present, all with the normal female sex complement. Hypoplastic testicular development is found to occur in complete absence of a Y-chromosome. Klinefelter-type syndrome is definitely ruled out.

2. Nuclear sexing in intersexes is applicable to nerve cells and epithelium of the ductus deferens. Polymorphic sexing was found unreliable in the pig as drumsticks also occasionally occur in males.

3. The primary aetiological factor is considered to be a sex-limited recessive gene possibly associated with autosomal modifiers. These modifiers could be in the nature of male determiners as in *Drosophila melanogaster*.

4. Chimerism as an aetiological factor in intersex development is excluded. So far, only one case of freemartinism in pigs has been mentioned in the literature whereas the incidence of intersexes may be as much as 20 per cent in some herds.

5. The cellular hypertrophy of the adrenal cortex found in two cases is considered as reminiscent of an adrenogenital syndrome. A comparison is made between the condition in the pig and the human adrenogenital syndrome. Biochemical determinations are needed before final conclusions can be made.

6. The pineal gland, which undergoes cellular hypertrophy, is undoubtedly concerned in the expression of the recessive gene. A suggested interrelationship exists between the pineal, the adrenal cortex and gonads. Its precise physiological role needs further investigation. The intersex pigs are considered as favourable subjects for experimental procedures on pineal function.

7. The bulk of the uterine fluid consists of urine with small initial contributions from the uterine glands and additions at a later stage of some cellular debris and perhaps tissue fluid.

8. A modified nomenclature, in which the presence, absence and nature of the gonads are clearly and unambiguously indicated is suggested. It is further suggested that it should replace the non-informative terminology of the existing systems.

CHAPTER III

CYTOGENETICS OF THE SHEEP=*Ovis aries* LINNAEUS

A. THE NORMAL PICTURE

1. *The karyotype*

(*a*) *Review of literature*

Earlier investigators, using difficult and less rewarding techniques, have reported different and, as has been shown subsequently, some incorrect chromosome numbers for the sheep.

Wodsedalek (1922) was the first to study the chromosomes of the sheep and found the number to be $2n = 33$ in spermatogonia of the male and $2n = 34$ in oögonia of the female. Krallinger (1931), found 50 to 60 chromosomes (60 being the most probable number) in the spermatogonia of the Merino sheep and 30 bivalents in the anaphase of the first meiotic division of primary spermatocytes. Shiwago (1931) found sheep amnion cells to contain $2n = 54$ chromosomes. Butarin (1935), studying two kinds of sheep in Siberia found 54 to 56 chromosomes in the spermatogonial metaphase of the Arkhar sheep and 52 and 54 chromosomes for the same stages in testicular tissue from the fat-rumped ram.

Novikov (1935), reported $2n = 60$ in hybrids of the wild European Moufflon and Merino sheep. He noted the chromosomes to be rod-shaped and could not identify any sex chromosomes in the diploid set. Bruce (1935), confirmed these findings of $2n = 60$ in Merino sheep. So did Pchakadze (1936) find $2n = 60$ in Georgian fat-tailed sheep.

Berry (1938) reported $2n = 54$ for amnion cells from Rambouillet sheep and in 1941 reported the same number in testicular tissue. Ahmed (1940) and also Makino (1943) found the same chromosome number in testicular tissue of sheep.

Melander (1959) using acetic-orcein squashes of lung tissue of half grown sheep embryos found the Karakul breed to have $2n = 54$. He identified the three large submedian pairs as a class separate from the autosomal acrocentrics. Nakanishi & Mizutani (1959) working on lung cultures from a male lamb came to the same conclusion of $2n = 54$. Bomsel-Helmreich (1959) using the Feulgen method and squash technique on pieces of testes found a variation of 46 to 60 but a greater frequency of 54 chromosomes in various rams (Merino, Southdown, Ile de France). The more recent investigators such as Borland (1964) and McFee, Banner & Murphree (1965) have used sternal bone marrow spreads and leucocyte cultures respectively. Their methods have given magnificant results. Nevertheless, the position of the X-chromosome is still doubtful. Shiwago (1931), Bruce (1935), Butarin (1935), Berry (1941), Nakanishi & Mizutani (1959) consider the X-chromosome to be one of the larger of the medium-sized acrocentrics whereas Makino (1943) took it to be a long rod-shaped element. Melander (1959) found the X-chromosome unidentifiable. McFee, *et al* (1965) consider the X-chromosomes to be the largest of the acrocentric members, whereas Borland (1964) considers them to be amongst the smallest group.

Wodsedalek (1929) claimed the ram to lack a Y-chromosome. Ahmed (1940) considered the Y-chromosome to be acrocentric. Other earlier investigators considered the Y-chromosome to be the small chromosome but none could determine the position of the centromere. Melander (1959) found the centromere to be median, Borland (1964) considered it to be acrocentric while McFee, *et al.* (1965) in excellent spreads found it to be " probably submetacentric ".

(b) Results

The normal karyotype of the sheep (Merino, Karakul and Afrikaner breeds) was found to be $2n = 54$ (Plate 13). The autosomes consist of six large submetacentrics, forming group 1 to 3 and 46 acrocentrics (group 4–26) which are easily arranged in descending order of length. The two X-chromosomes are also acrocentrics belonging to an intermediate group and on account thereof it has not been possible to distinguish them convincingly from the autosomal acrocentrics. The Y-chromosome is a very small submetacentric and therefore readily identifiable—mostly as a small square.

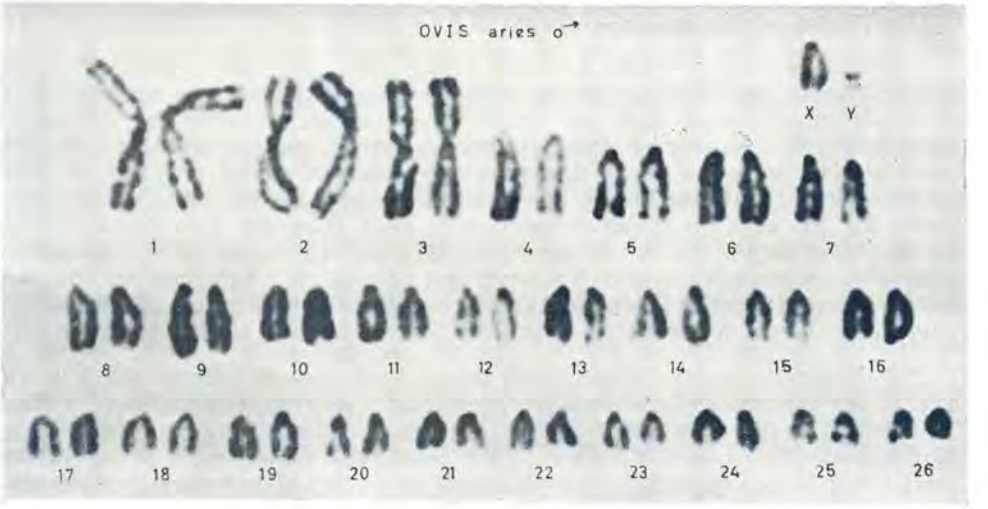

PLATE 13.—Karyogram of sheep chromosomes

(*c*) *Discussion*

The karyotype as found for normal sheep (Plate 13) supports the results of all recent authors (McFee *et al.*, 1965; Borland, 1964; Melander, 1959) as well as those of some earlier workers (Berry, 1938, 1941; Ahmed, 1940; Makino, 1943). The initial discrepancies of the modal number of the sheep appear mainly to be due to misinterpretations of the three pairs of long submetacentric chromosomes. If the crude methods of earlier investigators are considered, such difficulties in interpretation are quite understandable.

The only difficulty in the karyotype of the sheep is the identification of the exact position of the X-chromosome. It is to be hoped that in the near future the use of tritiated thymidine for identifying the late-replicating X-chromosome will eventually settle this question.

The results obtained by earlier investigators may give the impression that various breeds of sheep may differ in their chromosomal numbers. As these earlier results are unreliable due to inferior technique, one could still regard the matter as controversial.

However, the finding of 54 chromosomes in the following breeds: Merino, Southdown, Ryeland Dorset horn, Border Leicester, Romney Marsh, Cheviot (Borland, 1964); Suffolk and some crossbreds (McFee *et al.*, 1965) Rambouillet (Berry, 1938); Karakul (Melander, 1959); Limousin, Southdown, Merino, Ile de France (Bomsel-Helmreich, 1959) Corriedale, Merino, Karakul (Makino, 1943) and the Afrikaner and Persian in the present investigation, indicates a modal number of 54 as generally accepted for all sheep breeds. Makino (1943) could find no racial difference. This is supported by these figures.

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2. *Cytological sexing*

(*a*) *Review of the literature*

(1) *Nuclear sexing*: Sachs & Danon (1956) were apparently amongst the first investigators to apply nuclear sexing to sheep tissues. They found epithelial cells of new-born and aged male and female sheep to lack a clear sex difference. Schmidtke (1957), using two rams, two ewes, a wether, one ram lamb and one ewe lamb, could distinguish sexual dimorphism in neuronal nuclei provided all Barr bodies except those attached to the nucleolus were considered. Of 1,200 male nuclei 3·2 per cent contained chromocentres away from the nucleolus and 77·6 per cent none at all. In 900 female nuclei 72·1 per cent contained chromocentres away from the nucleolus and only 6·6 per cent none at all. Paranucleolar chromocentres were not considered significant as they occurred in both sexes. Struck (1961) found buccal smears completely unsuitable for determining sexual difference.

(2) *Polymorphic sexing*: Kraft (1960) found 3 per cent of polymorphs in seven ewes to contain drumsticks whereas two males had none. Schuchardt (1960) studied 25 ewes and 25 rams: he observed the frequency of drumsticks to be 6 and 1·5 per cent respectively. Lüers & Struck (1960) considered polymorphic sexing in the sheep not specific enough due to the occasional occurrence of drumsticks in males and general low count of drumsticks in the ewe (3/500). According to them the sheep has a low value for all types of appendages. Sessile dropforms (drops without handles) were included under typical drumsticks. Sessile nodules, small clubs and occasional rackets were approximately equal in both sexes.

Böhme (1962) also obtained low drumstick counts (1 to 26/500 polymorphs in females and rarely one in males) but by statistical methods he could arrive at a positive result. According to the formula of Kosenow & Scupin (1956) ewes were indicated by $\frac{A+B}{C} > 0{\cdot}1$ and rams by $\frac{A+B}{C} < 0{\cdot}1$. A + B gave a figure of 2 to 4 per cent in ♀ and 0 to 0·8 per cent in ♂.

Colby & Calhoun (1963) had to count an average of 73 $\pm 9{\cdot}63$ polymorphs to find one drumstick. Of the animals they investigated (cow, pig, horse, goat, sheep, dog, cat) they found the lowest count in sheep.

(*b*) *Results*

Nuclear sexing in skin was found inapplicable as chromatin granules in both sexes are rather numerous. An approximate figure of five drumsticks per 500 neutrophils was obtained in the case of ewes, in males one per 500, by counting 2,000 neutrophils in each of three ewes and three rams. Neuronal nuclei were found most suitable for nuclear sexing in the sheep.

(*c*) *Discussion*

Because in males a few polymorphs may also contain drumsticks, the method lacks absolute specificity in the sheep. However, an indication as to the sex is obtained when at least two to three thousand neutrophils are counted. In judging results, the clinical picture (lymphoid or myeloid increase or decrease) as well as degree of lobation may influence the final outcome. In the sheep neutrophils are found to have 4, 5 and 6 lobes as a rule but nuclei with 7 to 10 lobes may also occur (Lüers & Struck, 1960). Small lobes are often present and cause confusion. The low drumstick count is therefore not influenced by a high degree of lobation.

It is obvious that nuclear sexing is only applicable to tissues whose nuclei tend to be vesicular. That is why nerve cell nuclei are so ideally suited. Barr bodies are present in the other nuclei but cannot be distinguished from the chromatin granules, some of which are situated next to the nuclear membrane. Of the cell investigated in the sheep so far, only neurons were suitable for nuclear sexing.

B.—The intersex condition in the sheep

(*a*) *Review of literature*

Even before the establishment of the Roman Empire, freemartinism—the term is so old that its etymology is uncertain—had been known to occur in heterosexual bovine twins. Its existence in sheep, doubted at first, has been established tentatively in recent years. Roberts & Greenwood (1928), Ewen & Hummason (1947) and Slee (1963) have reported cases in sheep morphologically resembling the bovine freemartin.

Essential to the development of freemartinism is the presence of chorio-vascular anastomoses between heterosexual twins. Their occurrence was denied by Lillie (1922), but first described by Rotermund (1930) in sheep (in one out of eleven heterosexual twins). Stormont, Weir & Lane (1953) assumed their presence in heterosexual twins (with a suggested frequency of 5 per cent) on the grounds of erythrocyte mosaicism. Recently Alexander & Williams (1964) illustrated such anastomoses in one heterosexual pair. Moor & Rowson (1958) found tissue tolerance to exist in one out of the five pairs of heterosexual twins examined; the ewe of the pair in question was thus considerd to be a freemartin.

Chromosomal evidence for bovine freemartins has been established by Ohno *et al.* (1962) and recently confirmed by Kanagawa, Muramoto, Kawata & Ishikawa (1965). No karyotyping has been reported in the case of the ovine freemartin.

(*b*) *Results*

Chromosome studies were carried out on aspirated sternal bone marrow cells as outlined in Chapter I. The tissue cultures were made from one testicle and one kidney from an Afrikaner X Persian crossbred animal. The Karakul was subjected only to chromosome counts.

The chromosome number and morphology corresponded to that of a normal sheep, viz. $2n = 54$ (Plate 13). In the Afrikaner X Persian cross 41·5 per cent of the spreads were of 2A–XY constitution (Plate 15 b), whereas the remainder (58·5 per cent) were 2A–XX (Plate 15 a). A total of 77 excellent spreads was examined. Chromosome preparations from the tissue culture material established the somatic sex unequivocally as female. In the 76 spreads examined from the Karakul no Y-chromosomes were found.

The results of polymorphic sexing by blood smear examination are given in Table 4.

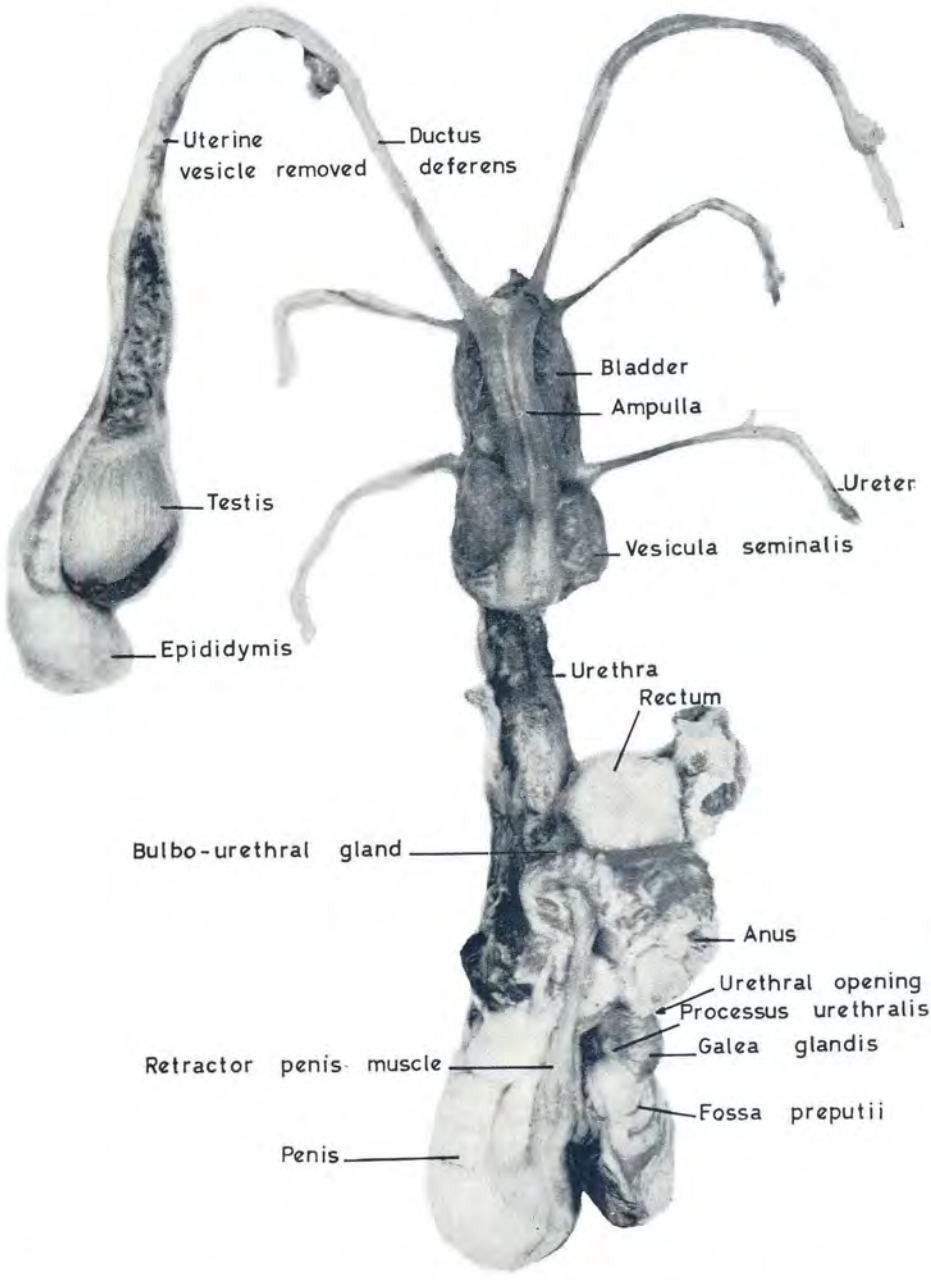

PLATE 14.—The genital organs of the Afrikaner × Persian freemartin. The right testis was removed for tissue culture

TABLE 4.—*The results of polymorphic sexing on blood smears of the two intersexes*

Sheep	Neutrophils counted	Drumsticks	Sessile Nodules	Small clubs	Rackets
Afrikaner × Persian cross.....	1000	11	25	30	1
Karakul.....	1000	26	40	25	0

Only occasional sex chromatin bodies of Barr were encountered in buccal smears from both cases. This is of no significance as buccal smears are considered by Struck (1961) to be unsuitable in the sheep. The polymorphic sex, however, was indicative of a female configuration.

The genital tract of the Afrikaner X Persian cross represented features characteristic of a gonadal male intersex (Plate 14). A male tract had developed without the formation of any definite female structures. Two distinct, but small testes with epididymes (distinctly divisible into head, body and tail regions), pampiniform plexuses and ductus deferentes were situated in peritoneal evaginations in the inguinal region. They could be felt underneath the skin, were encased in a tunica vaginalis, but no scrotum was present. Histologically the testes consisted of ill-defined seminiferous tubules, mostly collapsed, lined only by scattered Sertoli cells containing lipoid droplets. Interstitial cells of Leydig were scattered, often in small groups and most in an inactive form: pycnotic nuclei, condensed eosinophilic cytoplasm with fine lipoid droplets.

A small, pea-sized vesicle at the cranial pole of the pampiniform plexus, next to, but not attached to the ductus deferens, upon histological examination proved to be a remnant of the uterus. It was the only structure present indicative of any internal female genitalia (Plate 14). No evidence of ovarian tissue was found at all. All male accessory glands, with possible exception of the pars disseminata of the prostate, were present but smaller than normal. The bulbo-urethral gland was small and covered by the musculus urethralis. The M. retractor penis and M. retractor ani were present but insignficant. The penis was very tortuously arranged, small, with its tip covered by the galea glandis and continued as a solid processus urethralis. It projected about one inch beyond the rudimentary fossa preputii. The preputium and tip of the penis were visible externally and situated just below the anus. The normal developmental extension of the penis to a subabdominal position had not taken place. The pelvic urethra was encased by the M. urethralis. The urethra opened just ventral to the anus and was not continued on to the penis. It was not possible to determine whether a strip of urethral mucosa extended along the penis, without disturbing the topographic relationships of the specimen.

The genital organs of the Karakul were an almost exact replica of the foregoing except that the urethral opening was much larger and closely simulated a vulva. Even the pea-sized vesicular remnant of the uterus was present. No indication of any primordial germ cells was seen. Subepithelial lymphocytic infiltrations associated with numerous plasma cells were found in the epididymis and especially in the ampulla. The body and tail region of the epididymis contained melanotic pigment.

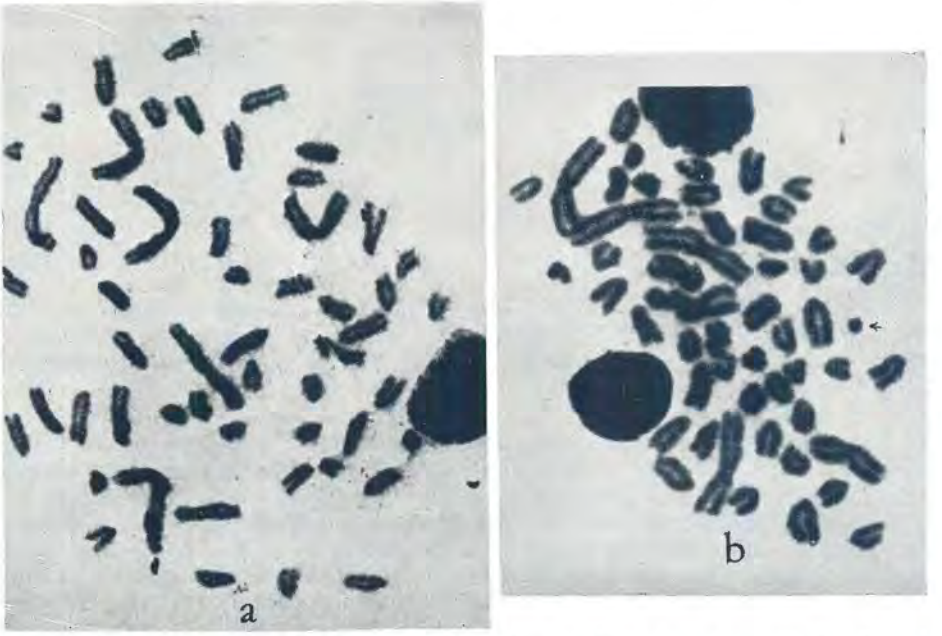

PLATE 15.—Male (*b*) and female (*a*) karyotypes from the same bone marrow preparation from the Afrikaner × Persian cross showing distinctly the Y-chromosome in b but absent in a

(*c*) *Discussion*

The establishment of bone marrow chimerism, which differs from mosaicism in that mixed cell populations are derived from different genetic sources, in the female twin of a heterosexual pair (Plate 15) considered together with previous reports of homograft acceptance, erythrocyte mosaicism and vascular anastomoses, finally removes all doubt as to the existence of freemartinism in sheep. In the Afrikaner X Persian cross, polymorphic sexing of blood smears and karyological examination of testes and kidney cultures proved the somatic sex to be female. Polymorphic sexing pointed to a similar conclusion in case of the Karakul.

It is assumed that 2A–XY cells had become established in the bone marrow of the crossbred animal during the presomite stage as a result of vascular anastomoses. [Such anastomoses are established in cattle and marmosets during the presomite stage—Ohno *et al.* (1962)]. Lillie (1922) found these anastomoses usually to occur in bovine embryos of about 22 mm although the foetal membranes were shown to fuse already at the 3·75 mm stage. In twin pregnancies in sheep he found membranes fusing without vascular anastomoses becoming established. By contrast, vascular anastomoses have been described by the authors Rotermund (1930) and Alexander & Williams (1964) cited previously. Petskoi established the incidence of vascular anastomoses in heterosexual sheep twins in 8 per cent of cases, and Kurnasov found it to be 0·8 per cent [both authors cited by Alexander & Williams (1964)]. Slee (1963) came to the conclusion that anastomoses between male and female embryos probably results in freemartinism of the female although not always overtly. He found no statistical difference in fertility between 187 ewes that had been born twins to females and 167 ewes that had been born twins to males.

It is apparent that immunological tolerance to these donor cells had remained during adult life in the Afrikaner X Persian cross; no evidence of such cells was found in the Karakul.

Since sex determination is genetically established but sexual differentiation generally accepted to be hormonally controlled, it has been assumed [hormonal theory of Lillie (1922)] that testicular hormones formed prior to ovarian hormones are responsible for the inhibition of female development in the freemartin. Bouters & VandePlassche (1964), however, pointed out that in the bovine foetus no such testicular steroid hormone could be identified between two to four months gestation age. In fact, androgens experimentally injected directly into the foetus or mother only partly produce the typical deviations seen in freemartins (Jost, Chodkiewicz & Mauleon, 1963). They are of the opinion that the masculinizing influence must originate from enzymes set free into the circulation by the Y-chromosomes. They also pointed out that the homogametic sex (in this case the female) is more susceptible to influences from the heterogametic sex (the male) than *vice versa*. This is further substantiated by the stronger masculinizing effect of the Y versus XX chromosomal complement seen in abnormal human karyotypes: XXY (Klinefelter's syndrome) and XXXY. They conclude: " Deze verschillende reacties bij zoogdieren, vogels en amphibieën tonen aan dat de labiliteit van de gonaden en van het ganse geslachtsapparaat niet afhankelijk blijkt te zijn van het phenotypisch geslacht, maar wel van de aan- of afwezigheid van een Y-chromosoom ".

That such an influence had existed during early foetal stages in these two ovine freemartins is indicated by the practically complete absence of any typical female genitalia. It can, therefore, safely be assumed that the enzymes from the Y-chromosomes of the donor cells were potent enough to switch the development of the target organs, i.e. genital system (for discussion of possible mechanisms, see page 277) which at that time should have been extremely susceptible to induction, into a direction opposite to that determined at fertilization. Various grades of intersexuality [the three so far described anatomically (Roberts & Greenwood, 1928) are practically identical] could thus be explained as a result of variation in the time of establishment of anastomoses or strength of virilizing influence.

It is apparent that the sexual metamorphosis of a genotypic female in the male direction under influence of the Y-chromosome cannot attain the normal mature expression because of the presence of the XX complement in the tissues constituting the genital system. It is this complement which reduces the competence of the organs to undergo normal organogenesis.

Due consideration must be given to hormonal influences. It is conceivable that the endocrine system may similarly be rerouted into a male direction under influence of the Y complement, and so secondarily influence the sexual change. Overzier (1963) states: " No significant change in the excretion of neutral 17 ketosteroids, oestrogens or the pituitary gonadotrophins was found in a variety of conditions that constitute this group of pseudohermaphrodites ". In view of this pronouncement, the most logical explanation for the sterility and incomplete development of the genitalia is, that a source of Y-chromosome enzymes from the donor cells in the bone marrow is only able to produce the initial sexual change (in what manner is still doubtful), and that Y-chromosome enzymes specifically produced in the target tissues are necessary for development to be maintained and completed. Wiesner (1934–35) mentions that day-old male mice, when castrated, fail to develop accessory genital structures but females on ovariectomy do. This indicates that the female is the neutral sex and that in absence of androgens and even oestrogens female organs

develop. But where the male hormonal stimulus is present and the organs stimulated possess a female chromosome complement (as in the crossbred under discussion), tissue specific chalones (Bullough, 1963) cannot be produced and this hampers development to maturity.

The Karakul intersex, although according to the owner not one of a twin, had male genitalia practically identical to those of the first, but showed no bone marrow chimerism. The adrenal and hypophysis were histologically normal. No biochemical determinations were done. Hence one of the logical conclusions would be that it could have been a freemartin. Accurate observation of individuals on a large ranch is wellnigh impossible, hence the owner could well have been mistaken. Bone marrow chimerism could have been established initially but the donor cells must have died out. It has been suggested that even erythrocyte mosaicism may eventually disappear (Slee, 1963). There is also a tendency for the body to remove exogenous cells over a number of years without formation of antibodies (Bouquet, 1963). According to Sachs (1963), however, intersexes often occur in Karakuls even in good breeding flocks and may possibly be due to inbreeding. If the Karakul then had truly not been one of a heterosexual twin, its intersex condition may have been inherited as a sex-linked recessive similar to that described in the pig (see discussion, Chapter II). It must, however, be considered very peculiar that the morphological effects of chimerism and sex-linked inheritance on the genital organs of these two cases are absolutely identical. If, after further investigations this perhaps turns out to be a general phenomenon it would help to elucidate the aetiology of the sex-linked type of intersex condition by thus indicating a basic similarity in mode of expression with the chimeric type.

The above postulated enzymatic induction of male instead of female genitalia appears to be a sound hypothesis as far as the bovine, sheep and possibly pig, are concerned (see discussions Chapters II and IV). As is so frequently the case in biology, there are exceptions. Benirschke, Anderson & Brownhill (1962) and Benirshke & Brownhill (1962) have found marrow chimerism to exist in marmosets without any indications of freemartinism. The same applies to the human. The two cases described (Booth *et al.*, 1957; Nicholas, Jenkins & Marsh, 1957) both had three children. Ryan, Benirschke & Smith (1961) still postulating gonadal hormones as causing freemartinism, showed that in the case of primates the placenta is able to convert some male gonadal hormones to oestrogens. This according to his preliminary tests was not the case in the cow.

(d) Conclusions and summary

Two gonadal male sheep intersexes are described. In one of them bone marrow chimerism was determined. This is considered as final proof of the freemartin condition occurring in the female member of some heterosexual sheep twins. Enzymes believed to be produced by the Y-chromosome of the donor cells are considered to be the active agents in switching genital development. As chimerism was not indicated in the other intersex it may have been inherited as a sex-linked recessive characteristic similar to that in the pig. It was, however, found most peculiar that effects of chimerism and that of presumably sex-linked inheritance produced practically identical malformed male genital organs in the two intersexes. Testicular hormones from the male twin are considered of no initial causal significance in the development of freemartinism.

CHAPTER IV

CYTOGENETICS OF THE OF THE BOVINE—*BOS TAURUS* LINNAEUS

A. THE NORMAL PICTURE

1. *The Karyotype*

(*a*) *Review of the literature*

The first attempts at determining the chromosome number of the bovine was by Von Bardeleben (1892) who established the count as $2n = 16$ in the spermatogonial mitosis. Schoenfeld (1902) found 20 to 25 in the spermatogonial division and 12 in meiotic divisions, Von Hoof (1913) $2n = 20$ to 24 in spermatogonial divisions and 12 in the first meiotic division, and Masui (1919) $2n = 33$ in the spermatogonial divisions and 17 in the second meiotic division. Wodsedalek (1920) reported 37 in the male and 38 in the female. The first to report the correct number of 60 was Krallinger in 1927, 1928 and 1931. Makino (1943, 1944) also counted 60. He could detect no racial difference. The above determinations were all done using the squash technique on testicular tissue. The modal number has been confirmed by later workers: Melander & Knudsen (1953), Knudsen (1954); Leuchtenberger *et al.* (1956); Postiglioni-Grimaldi (1957) and Melander (1957) (all cited by Melander, 1959).

In 1962 Sasaki & Makino revised the work on somatic cells *in vitro*. The object of this work and that of later workers has been to develop improved technical procedures such as preparation of cultures from blood and other somatic tissues, e.g. Biggers & McFeely, 1963; Ulbrich & Weinhold, 1963; Kanagawa *et al.*, 1965. These improved methods have led to the study of chromosomal aberrations, e.g. Basrur & Gilman, 1964.

Yosida & Lamartain (1964) reported the chromosome number of dwarf cattle also to be $2n = 60$. They considered the characteristic morphological diminution in size to be controlled by an autosomal gene.

The X-chromosomes were first mentioned by Masui (1919) who failed to recognize a Y-chromosome. Wodsedalek (1920) also found the male constitution to be XO. Krallinger (1927) was the first to identify the Y-chromosome and therefore the XY mechanism.

(*b*) *Results*

The chromosome number of the bovine in numerous excellent bone marrow spreads was substantiated as 60 in Jerseys, Frieslands and Afrikaners. The autosome are all acrocentrics, easily arranged in descending order of length, whereas the X-chromosome is a large submetacentric with the Y-chromosome as a small metacentric.

Evidence of a diurnal haemopoietic cycle was found to exist in the bovine. During the summer months peaks were obtained between 8.30 and 9 a.m. and at $\pm$12 a.m. During the rest of the day spreads were mostly very scarce or absent. During the winter months mitotic peaks were somewhat later, between 9 and 10 a.m. and between 12 and 1 p.m.

(*c*) *Discussion*

In the karyogram of cattle the heterochromosomes are the only metacentrics present, the autosomes all being acrocentric. This fact together with the difference in size of the X and Y-chromosomes makes sex determination of specific chromosome spreads, especially in chimeras such as the freemartin, a relatively simple matter. I found the same arrangement in the dog: the heterochromosomes differ in size and are both submetacentrics, the autosomes are all acrocentrics.

Again as in the case of the sheep and pig it appears as if all breeds of *Bos taurus* have chromosomes identical in number and morphology. Jerseys, Frieslands, Afrikaners, Swedish S.L.B. breed, Santa Gertrudis cattle and even dwarf cattle have all been reported to have a modal number of $2n=60$. This also applies to some bovine somatic tissues other than blood, e.g. skin, lung, liver and gonads (Kanagawa *et al.* 1965).

2. *Cytological sexing.*

(*a*) *Review of the literature*

(1) *Nuclear sexing.*—As in the case of the sheep, Sachs & Danon (1956) were apparently the first to attempt nuclear sexing in cattle. They found epithelial cells of newly born and old male and female animals completely lacking in sexual dimorphism. Struck (1961) came to the same conclusion due to her inability to identify Barr bodies because of the large number of chromatin granules present. The buccal smear test for sexual dimorphism is therefore not applicable to cattle.

Neurones were found most suitable for identification of Barr bodies by Moore, Graham & Barr (1957) and Schmidtke (1957). The latter author observed the frequency of paranucleolar chromocentres to be about the same in males and females and thus of no value for purposes of sex determination. Those between the nucleolus and nuclear membrane were more sex specific. Those present in males amounted to only 14·5 per cent (bulls)—18·3 per cent (bull calves) whereas in females they amounted to 95 per cent (cows)—94·7 per cent (heifer calves) of cells examined.

Lang & Hansel (1959) could establish sexual dimorphism in liver, pancreas and adrenal in 68, 62 and 61 per cent of cells examined respectively but only in 10 per cent or less of male cells, provided they hydrolysed the cells in 7 N HCl for 15 minutes to remove obscuring chromocentres from the nuclei.

(2) *Polymorphic sexing.*—Rentsch *et al.* (1960), after studying smears from six cows and four bulls regarded polymorphic sexing unsuitable in the case of cattle. Kraft (1960) observed 4 to 5 per cent of polymorphs to possess drumsticks in cows and 1 to 2 per cent in males of the 24 animals used. Benda (1961) established a definite statistical difference between 60 bulls and 60 cows: 0 to 0·8 per cent polymorphs in females and 0 to 0·6 per cent in males which was inapplicable to the individual case for the determination of sex.

Schuchardt (1960) using Kosenow & Scupin's (1956) formulae on data from 25 cows found $\frac{A+B}{C} > 1{\cdot}85 = ♀$ — a positive result. He also found drumsticks in both sexes —2·6 per cent of polymorphs possessed drumsticks in the females and 0·8 per cent in the 25 males examined. Colby & Calhoun (1963) only saw two drumsticks per 2500 female cells. In males they found none. They quoted Arrieta & Rosales (1960), who also failed to establish sufficient difference to employ as a practical method for cytological sex determination.

Statistically the most significant findings were those of Sampath Kumaran & Iya, (1965). After examining blood smears for drumsticks (200 polymorphs for each animal) from 913 cattle of three different breeds: the Red Sindhi (215); Sahiwal (337) and Tharparkar (361) they concluded that: sex chromatin occurs in the female polymorph but is predominantly of the sessile type (B); that it very rarely occurs in males (only eight calves indicated the presence of sex chromatin whereas only four females had none); that there is a steady increase in number of polymorphs with sex chromatin from birth to eight years and thereafter a decline and that the maximum number of sex chromatin bodies were found in the group of cows yielding 3000 to 3199 Kg of milk.

(*b*) *Results*

Nuclear sexing with the routine staining methods was possible in cattle only in the case of nervous tissue.

Polymorphic sexing (on five animals of each sex) revealed 2 to 6 drumsticks per 500 polymorphs (including eosinophiles) in females and an average of 0 to 4 (2) in the male. The average value of $\frac{A+B}{C}$ was found to be 4·5 in the cow and 1·6 in the bull. Drumstick sexing therefore, although possible, is not as clear-cut in cattle as one would wish it to be.

(*c*) *Discussion*

The data obtained so far reveal that nuclear sexing in the bovine species except that on neuronal nuclei, does not yield unequivocal results. Special techniques such as acid hydrolysis may facilitate matters by removing the excessive number of chromatin granules. In the buccal mucosa the degree of keratinization appears to play a decisive role. In carnivores and humans, where no keratinization of buccal mucosa takes place, sex chromatin is easily distinguished. In the process of keratinization it appears as if more heterochromatin (= chromocentres) is formed prior to eventual death of the cell.

From this one may induce that specific specialization of a cell is accompanied by formation of increased amounts of heterochromatin—generally accepted as the inactive form—up to a definite limit. This may account for the numerous chromocentres seen in the nuclei of various tissues.

Polymorphic sexing equally yields ambiguous results. Low and high values for the formula $\frac{A+B}{C}$ definitely indicate male and female sex respectively but the borderline cases ($\pm$ 1·8 to 2·0) leave one in doubt. Values for A +B alone do not have any significance. The wide variation of numbers of drumsticks attached to polymorphonuclear leucocytes as given by different workers—0 to 5·0 per cent in females and 0 to 2·0 per cent in males—is not only indicative of the variability of occurrence of this nuclear appendage, but also of the differences in the interpretation accorded them by observers of experience. If the figures of Kumaran & Iya, (1965), who stressed the predominance of the sessile type of nodule in cattle, be included, then the variation would be even wider. Their figures for sex chromatin of the various age groups of females vary from 5·5 to 8·1 per cent. Figures for males were omitted. The differences which exist in interpretation render sufficient proof of the equivocal nature of the polymorphic sexing in cattle.

B. The bovine freemartin

1. *Review of the literature*

The term freemartin signifies a sexually imperfect, always sterile female partner of a pair of heterosexual twins (Swett, Mathews & Graves, 1940). About 95 per cent of the heifers of such heterosexual twins are freemartins.

Moore *et al.* (1957) speculated on the etymology of the term " freemartin ", which is obscure, despite or probably because of the fact that the condition has been known for over 2000 years. They also reviewed the earliest literature. Some of this information is given here. The prefix " free " appears to be a simplification of the term " farrow " which in Scotland and adjoining territory is related to infertility. It may also be connected with the Flemish " varvekoe "—a cow not giving milk—or the West Flemish " varwekoe "—a cow no longer capable of giving off-spring.

" Martin " may be derived from the Irish and Gaelic word " mart " meaning heifer or cow. It may also be associated with Martinmas day (November, 11th or day of St. Martin) a day on which it was customary in England and Scotland to slaughter cattle and salt their meat for winter use. The word " mart ", " maert ", " mert " or " mairt " was used for such fattened cattle.

Hunter (1779) after having had his interest awakened by Jenner, was probably the first person to describe a freemartin anatomically. Previously, in 1692 Valsalva and Baglivi, pupils of Malpighi, had discussed anatomical features of the freemartin in correspondence (Belloni, 1952). Spiegelberg (1861) first showed the gonads to resemble testes histologically. The reproductive system of freemartins was fully described histologically by Chapin (1917) and Willier (1921).

The aetiology of the freemartin condition had given rise to some interesting speculations (see Swett *et al.*, 1940), but in the last fifty years it has been convincingly shown that the freemartin is a genetic female, a member of dizygotic or fraternal twins (or triplets, etc.) (Lillie, 1917, 1922) and is actually a naturally occurring chimera formed as a result of chorio-allantoic anastomoses between heterosexual individuals. Lillie (1917, 1922) was responsible for the widely accepted but erroneous concept that suppression of development of the female gonads and genital tract was caused by male sex hormones gaining entrance into the female through the anastomoses.

Keller & Tandler (1916) at about the same time as Lillie postulated that a chromosomal enzyme derived from somatic cells was the true aetiological factor. Due to the immense importance attached to hormones in the years after Lillie's findings his hormonal theory found greater acceptance than the chromosomal enzyme theory of Keller & Tandler and superseded it. It is only recently in 1963 that the original Keller & Tandler theory has been modernized by Bouters & VandePlassche (1964), Fecheimer, Herschler & Gilmore (1963) and Stewart (1965). This became possible after Ohno *et al.* (1962) had demonstrated that freemartins, although genetic females, harbour varying numbers of male somatic cells in their myeloid tissue: they are thus chimeras (rather than mosaics, which term is applied to situations where atypical cells develop from a single zygote).

2. *Results*

In the course of this investigation, three pairs of heterosexual twins were investigated for chimerism.

Case I: Examination of chromosome spreads prepared from the sternal bone marrow of both individuals of a heterosexual pair of Jersey calf twins revealed typical chimerism, as shown in Table 5.

TABLE 5.—*The relative percentages of male and female cells as indicated by chromosome determinations on the bone marrow of the Jersey twins, Case I*

Age	Sex	Percentage ♂ cells	Percentage ♀ cells	Total number of spreads counted
±2 weeks	♂	61	39	23
±2 weeks	♀	46	54	56

The evidence for chimerism was substantiated by blood group determinations: both calves had identical blood groups indicating a mixing of blood during embryonal development (see Table 9). The results of polymorphic sexing can be seen in Table 6. As these calves were sold to a butcher, no further observations could be made.

TABLE 6.—*The various values of* $\frac{A+B}{C}$ *and drumsticks per* 1000 *polymorphs as obtained in the three cases of heterosexual twins*

Cases	$\frac{A+B}{C}$		Drumsticks/1000 polymorphs		Total No. of polymorphs counted (incl. eosinophils)
	♀	♂	♀	♂	
I	5·25	1·67	5	3	2000
II	3	—	13	—	2000
III	7·5	5·5	5 (4 in eosinophils)	3	2000

Case II: These were Jersey heterosexual twins obtained just after birth. Both were chimeras as determined by cytogenetic studies (Table 7) and substantiated by blood group determinations. These were identical (see Table 9).

TABLE 7.—*The percentage distribution of male and female cells as determined for the Jersey twins—Case II—*

Age	Sex	Percentage ♂ cells	Percentage ♀ cells	Total number of spreads counted
3 weeks	♂	68	32	44
3 weeks	♀	43	57	60
15 months	♀	67	33	119

The male calf died at the age of six months and no further determinations could be made. The freemartin was killed at the age of 16 months. The results of polymorphic sexing are shown in Table 6.

The genital system of the freemartin (Plate 16) was characterized by complete suppression of uterus, cervix, uterine tubes and ovaries (represented only by fibrous tissue mostly) and only partial suppression of the caudal ends of the Wolffian ducts.

These ends showed rudimentary branching tubules which could be interpreted as primordial seminal vesicles. An interesting feature, often seen in cows other than freemartins, was the persistence of part of the septum between the caudal ends of the Müllerian ducts (Plate 16).

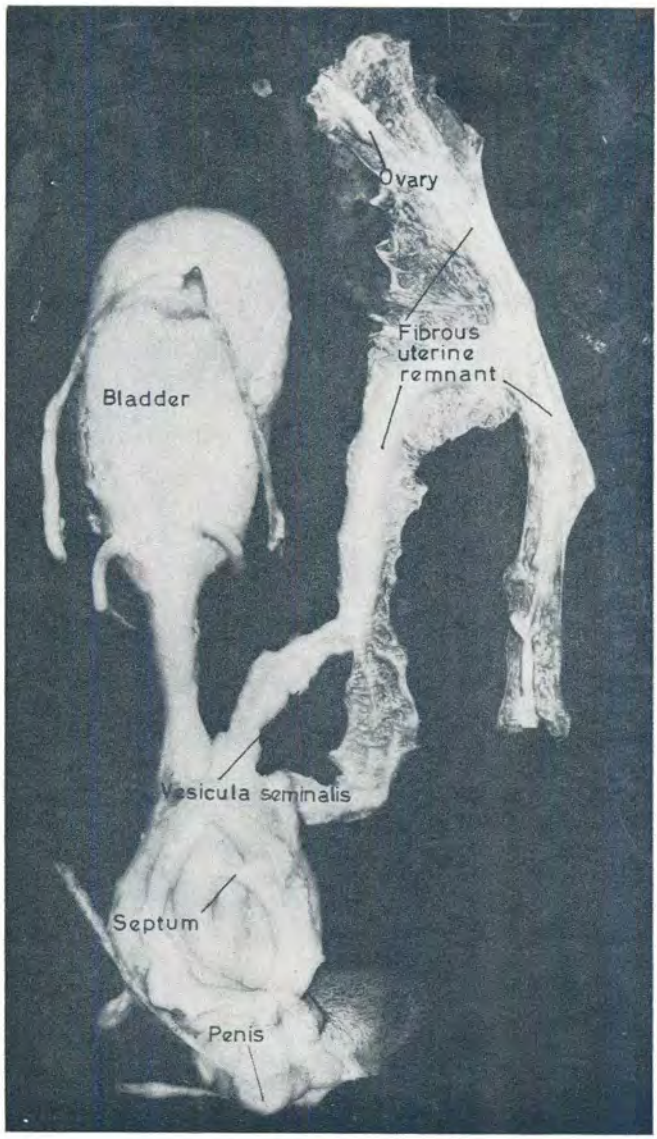

Plate 16.—The genital tract of the Jersey freemartin of Case II revealing complete regression of the uterus, fallopian tubes and the nodular remnant of the ovary. The enlarged clitoris, the persisting septum (see text) and the primordial seminal vesicles are distinctly seen

The ovarian remnants consisted of a very well developed tunica albuginea, a considerably atrophied cortex with small persisting cell groups surrounded by a thin glassy membrane (interpreted as remnants of Pflüger's cell cords) and a medulla in which the rete ovarii was well developed, almost resembling immature seminiferous tubules (Plate 17). Numerous interstitial cells resembling Leydig cells were present. They appeared active. The impression gained was that of early suppression of ovarian development without stimulation of male development. Vascular anastomoses in these heterosexual twins must have been established at a very early age. Examination of the pineal and adrenal glands revealed no abnormalities. The hypophysis, apart from possible relative variation of cell types which was not investigated, appeared quite normal. Swett *et al.* (1940) found " . . . . that the freemartins had a larger number of grams of pituitary body for each unit of empty-body weight than did the heifers of single births, although actually the pituitary bodies of the freemartins were smaller ". Hall (1938) found the cellular structure of the hypophysis to be intermediate between that of a castrated animal and a normal cow.

Case III: These were a pair of heterosexual Friesland twins obtained after birth.

TABLE 8.—*The relative percentages of male and female cells as indicated by chromosome determinations on the bone marrow of the Friesland twins—Case III. The arrows indicate stage of infestation with* Paramphistomum microbothrium

Age	Sex	Percentage ♂ cells	Percentage ♀ cells	Total number of spreads collected
±1 mnth.	♂	53	47	62
±5 mnth.	♂	79	21	95
±6 mnth.	♂	60	40	20
6 mnth.	♂	69	31	75
3 weeks.				
1 mnth.	♀♀	49	51	94
±5 mnth.	♀♀	59	41	68
±6 mnth.	♀♀	58·3	41·7	108
± 6 mnth.	♀♀	69	31	65
3 weeks.				

Both these calves were heavily infested with *Paramphistomum microbothrium* Fischoeder between the ages of five and six months as part of another experimental procedure. These calves were killed at the age of ± 7 months. The results of polymorphic sexing are shown in Table 6. Due to the worm infestation the circulating eosinophils were increased and, remarkably, four of the five drumsticks encountered were present in eosinophils. Even more remarkable are the identical percentages of male and female cells obtained in both twins at the age of six months and three weeks.

The genital system was also almost completely suppressed but nevertheless had a few more female characteristics than the previous case. It had a vestibulum of about 5 cm long (two fingers could go in freely) which rapidly narrowed down to 1 cm and eventually ended blindly 1·5 cm from the uterus. The horns of this poorly developed uterus were 4 cm long, ended abruptly and were continued as fibrous bands to which an atrophic ovary was attached on either side. A vesicular remnant of the fallopian tube was present near the left ovary. The histological appearance of the ovaries was

similar to that of the previous case (Plate 17). A well developed tunica albuginea, an atrophic cortex with remnants of cords of Pflüger and a medulla with a well developed rete ovarii resembling seminiferous tubules were characteristic. The clitoris was enlarged, 0·5 cm thick and convoluted as in the case of pig intersexes.

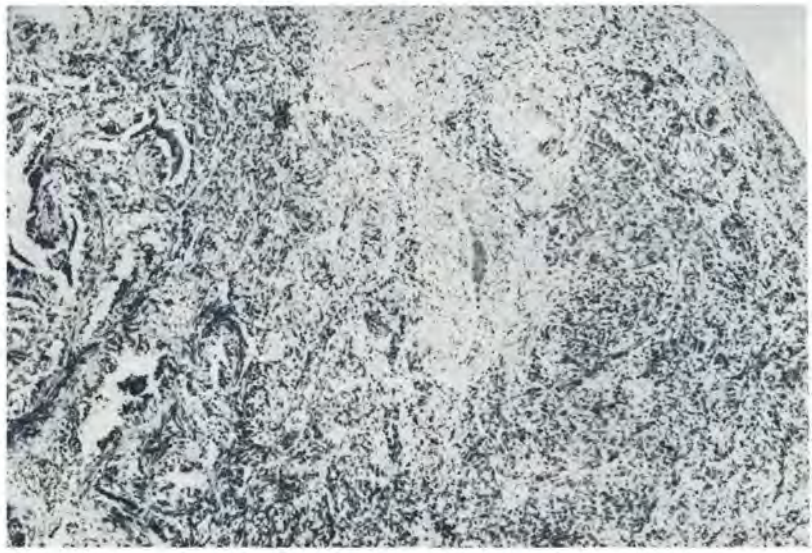

PLATE 17.—The ovary of the freemartin of Case II revealing a well developed tunica albuginea, an atrophied cortex with small cell groups representing atrophied follicular cells and on the left, the medulla with the well developed rete ovarii. Stained H. & E. Magn. 75×

The uterus although atrophied had all the normal histological constituents. Adrenals, pineal and hypophysis appeared normal histologically. Although the chief cells of the pineal contained beautiful single chromocentres next to the nuclear membrane, they were present in the male also and therefore of no differential significance.

3. *Discussion*

It is obvious from these findings (and as discussed in the previous chapter) that the freemartin condition in the bovine is formed as result of cellular interchange and not hormonal interchange between two heterosexual individuals. It remains to be determined whether the cells that participate in the interchange are embryonic haemopoietic cells only, or whether primordial germ cells are also concerned. The former cells must become established in the haemopoietic organs soon after chorio-allantoic anastomoses are formed (i.e. from 3·75 mm to 20 mm CR length) and there act as mother cells for future generations of leucocytes or red blood cells. That this is the case is undoubtedly established by the fact that such donor cells can be shown to be present years after birth and may even increase in proportion (as in cases II and III). Whether it is a case of " survival of the fittest " or whether it is a mere coincidence as to which cells divide, is not known. Donor cells are received by both twins but it is only in the case of the female that suppression of genital organs is achieved for reasons set out in the previous chapter. This suppression of female organs had already been noticed at 32 mm CR length (i.e. one month's pregnancy) (Lillie, 1917, 1922).

TABLE 9.—*The results of blood grouping tests on the first two cases indicate reciprocal mixing of blood—see ± reactions. These results indicate: (a) the red cell antigens determined in ten (A—SA) blood group systems, each system indicating the blood factors or blood groups which are determined by genes at the same locus and most probably on the same chromosome. (b) Haemoglobin types (Hb;animals of Case II were too young for sutch determinations) and (c) Transferrin types (Tf; beta-globulin)*

		A	B	C	FV	J	L	M	SU	Z	SA	Hb	Tf
Case I.....	Freemartin.....	AH/	B ± G ± K ± I ± O_1 ± Y_1 ± A^1_2 ± B^1 ± E^1_2 ± I^1 ± K^1 ± O^1 ± Y^1	C_2 ± W ± R ± X_2	F/F	–/–	L/	–/–	± S_1/	Z/–	–/–	AB	AD
	Bull calf.....	AH/	B ± G ± K ± I ± O_1 ± Y_1 ± A^1_2 ± B^1 ± E^1_2 ± I^1 ± K^1 ± O^1 ± Y^1	C_2 ± W ± R ± X_2	F/F	–/–	L/	–/–	± S_1/	Z/–	–/–	AB	DD
Case II.....	Freemartin.....	AH/	B ± O_1 ± A^1_2 ± D^1 ± E^1_1 I^1 ± O^1	C_1 W	F/V	–/–	L/	–/–	± S_2/	± Z/–	–/–	fet.	DD
	Bull calf.....	AH/	B ± O_1 ± A^1_2 ± D^1 ± E^1_1 I^1 ± O^1	C_1 W	F/V	–/–	L/	–/–	± S_2/	± Z/–	–/–	fet.	DD

If primordial germ cells are concerned they would most certainly have become established in the gonads to which they are specifically attracted. If established in any other tissue, they could exert no foreign influence there and would either degenerate or perhaps change into tissue specific cells (if that were at all possible)! From the statement of Kanagawa *et al.* (1965): " Organs of endodermal and mesodermal origin generally consisted of 2A–XX cells and 2A–XY cells in both freemartins and co-twins, but in those of ectodermal origin no XX/XY chimerism was detected ", it almost appears as if the change of primordial germ cells to tissue specific cells were possible! Their organ chimerism must, however, be attributed to haemopoietic cells present in those organs (as explained lower down) at the time of collection for cultures. Such should also be the case in the ectoderm. They apparently were fortunate in having no migrating haemopoietic cells present in the ectoderm nor in the attached connective tissue.

Ohno *et al.* (1962) found evidence of 2A–XX cells in the testes of two bulls, twins to freemartins. They could not ascertain whether these XX cells could function as spermatogonia or not. Nor could they find any XY cells in freemartin gonads. Kanagawa *et al.* (1965), nevertheless presented some proof of 2A–XY cells in the gonads from two freemartins. In one case they found the ratio of male to female cells as 2 to 136 and in the other as 3 to 192 respectively. Apart from considering these figures as unequivocal proof of chimerism they attached no other significance to them. Ohno and co-workers mentioned the fact that individual haemopoietic cells in the testicular tissue could have acted as precursors for their 2A–XX cells. This in my opinion is a far greater possibility than that they were female germ cells transplanted via the anastomoses. It must be remembered that lymphocytes and monocytes are the most active cells in blood cultures and may also be present as wandering cells in tissue spaces. In cultures from the testis such wandering cells would occasionally be present and could, due to their proliferative ability, be expected to even predominate in the process of regeneration. Even if the blood had been flushed from the blood-vessels [which Ohno and co-workers as well as Kanagawa *et al.* (1965) have apparently omitted] one would not be able to exclude completely the odd chance of a haemopoietic donor cell, present in the tissues, from growing in such a culture. This is the apparent explanation for the low number of male cells found in gonad cultures by Kanagawa and co-workers. The only true evidence for germ cell migration would thus be their presence (i.e. XX cells) in spermatogenesis—as Ohno *et al.* (1962) rightly state: " since the recovery of even one XX-spermatocyte in meiosis would conclusively confirm germ cell chimerism ". In the case of the freemartin such evidence would be even more difficult to obtain, as a matter of fact even be impossible because of its sterility. Some further supporting evidence for germ cell chimerism was found by Moore & Owen (1965) in chicken heterosexual twins hatched from double-yolked eggs. They found female cells in male gonads, but could not find male cells in female gonads.

It is therefore obvious that Y-chromosome containing cells are present in gonads of the freemartin, whether as haemopoietic cells or as germ cells does not matter at this stage. Y-chromosome enzymes are produced at the site and age where and when their influence is most effective, i.e. at the stage when induction determines the direction of gonad differentiation—the female being the neutral sex and subordinate to masculine influence.

According to Young *et al.* (1961) only male hormones are necessary for sex differentiation. In their absence, as shown by castration and cultivation *in vitro*, female sex primordia develop autonomously. In the freemartin the presence of X-chromosomes in the tissues prevents Y-enzymes from exerting their full effect

namely that of masculinization. This is an obvious deduction from the statement of Young and co-workers (p. 139): " Under identical experimental conditions the effect of a male hormone on the growth of a particular male structure was always greater in male embryos than on the homologous structure in females, and *vice versa* ". The Y-enzymes are nevertheless sufficient to suppress normal female organogenesis as is so clearly seen in the cases described. The possibility may of course still exist that male hormones produced by the testicles of the male co-twin (as advocated by Lillie, 1917, & 1922) could equally well suppress female genital development or even stimulate male development. However, due to complete lack of positive evidence for such hormones (see page 265) this possibility must be discarded.

The effectivity of the male complement in haemopoietic cells may be questioned as these cells are continually subject to specific differentiation with consequent suppression of the presumed activity of the Y-chromosome. It must be remembered, however, that at that early stage haemopoietic cells are relatively undifferentiated, and the male complement, to all logical reasoning, still in a state to exert an effect. As undifferentiated amoeboid elements, these haemopoietic cells could move in and out of tissues with relative ease and thus exert a masculinizing influence throughout the body of the freemartin; the gonads would possibly be the only organs capable of response.

Inhibition of female genital development due to the presence of male haemopoietic cells appears to be a sound hypothesis but if compared to the position in the intersex pig (see chapter II) where incidentally no Y-chromosome (and thus no chromosomal masculinizing influence) could be found with nevertheless almost complete virilization in some cases, then doubt arises as to the true effect of the Y-chromosome: is the freemartin condition caused by Y-chromosome enzymes directly inhibiting gonad differentiation the correct solution; is atypical gonad development due to abnormal hormonal influence from the adrenal cortex, hypophysis or perhaps even pineal initiated primarily by the Y-chromosome enzymes a possible explanation or is some other as yet unsuspected mechanism the more correct interpretation? Although no cytological proof of these possibilities was found, biochemical evidence alone would be the more reliable criterion. The mechanism of development of the freemartin condition must be different from that of the intersex condition in the pig: in the former there is mostly suppression of Müllerian duct and ovarian development mostly without apparent masculinization; in the intersex condition virilization takes place which only in extreme cases completely suppresses female development (as in Case VII, Chapter II).

Some authors (Lauritzen *et al.*, 1965) believe the Y-chromosome to initiate development of Leydig cells in the gonads. These cells subsequently produce steroid hormones under influence of gonadotrophic hormones and thus cause differentiation in the male direction (Young *et al.*, 1961). This is apparently not always the case as Leydig cells reveal considerable hyperplasia in the testes of pig intersexes—*in the absence of a Y-chromosome*. A practically identical position has been described in a human phenotypic male with 46/XX chromosomes (de la Chapelle *et al.*, 1964). The role of the Y-chromosome in the establishment of the male gonads is therefore not as simple as originally presumed. Its presence in mammalian testicular tissue and spermatogenic cells is undoubtedly essential in producing fertility because in its absence the testes of gonadal male intersexes are always sterile. In undescended testes of intersexes the higher abdominal temperature connot take all the blame as sterility also occurs in scrotal testes of male pseudohermaphrodites.

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Considering the mechanism of reciprocal blood exchange through chorio-allantoic anastomoses of considerable calibre acting over an extended period of time, one would expect an approximately equal distribution of blood cells between the two partners. The percentage of " takes " may, however, be variable (Tables 7 & 8). Variation in phenotype of freemartins can be explained in a similar fashion (Goodfellow, Strong & Stewart, 1965). Observations on cases II and III suggest that the relative proportions of male and female cells would gradually become altered during life of the freemartin in favour of male and not, as one would expect, female cells. Goodfellow and co-workers have reported a predominance of male cells in the peripheral blood of two freemartins (70 per cent and 56 per cent respectively). The three freemartins reported upon by Ohno *et al.* (1962) on the other hand, had more female than male cells, yet one of their male twins (Animal F) had a greater percentage of female than male cells. Kanagawa, *et al.* (1965) found more female than male cells in five freemartins and more male than female cells in six. Their figures for the male members of heterosexual twins reveal a majority of male cells in four and of female cells also in four individuals, whereas one male had equal percentages of each cell type. Extreme variation in cell counts are revealed by their figures: $\pm$ 97 per cent male cells/$\pm$ 3 per cent female cells in a freemartin and $\pm$ 91 per cent female/$\pm$ 9 per cent male cells in one male member of a heterosexual twin. As all these figures were obtained from cultures they are not reliable as an indication of the living state as a possible differential growth potential and chance could have favoured this extreme shift. The same applies to the figures given by Goodfellow and co-workers. At this stage it is not possible to determine with certainty what factor (or factors) is responsible for these discrepancies in relative percentages obtained in the actual living animal. To all appearances it is merely a matter of chance. No detrimental effect is evidenced in the case of males nor in the adult female, but in the developing female the degree of suppression can to some extent be correlated with the relative percentage of male cells at birth.

From a statistical point of view the evidence at hand is too meagre to allow any definite conclusions to be drawn. Due to the immense amount of work involved in a statistical analysis of variations caused by technical errors or even mitotic peaks, such analyses were not attempted. The slight evidence obtained should be considered only as pointers for future directions of research.

Hermaphroditism may therefore be the result of chimerism caused by placental anastomoses. Chimerism caused by double fertilization at zygosis can also cause true hermaphroditism (Waxman, Gartler & Kelley, 1962 and Josso *et al.*, 1965). On the other hand gonadal development can be quite normal provided chimerism through placental anastomoses develops later during embryogenesis (Woodruff, Fox, Buckton & Jacobs, 1962). For other exceptions see page 266. In the fowl Armstrong & Marshall (1964, p. 299) indicate that embryonic gonads produce substances which can alter sex differentiation.

4. *Conclusions and summary*

It is concluded that Y-chromosome enzymes are necessary to suppress initial ovarian and Müllerian duct development either directly or by initiating applicable gonadotrophic hormones. A certain degree of testicular differentiation may also result in some cases. As only male hormones are apparently necessary for initial sex differentiation (in their absence the female genital system develops) the presence of even small amounts of male-determining enzymes (produced by donor XY cells) may be sufficient to inhibit female genital development or, if more XY cells are present,

testicular development may actually occur. Whether male hormones with a similar inhibiting action are produced by the developing testis of the male co-twin has not yet been finally settled.

Y-chromosome enzymes are derived from haemopoietic cells present in the freemartin as a result of chorio-allantoic anastomoses. The question whether male primordial germ cells (as another source of Y-chromosome enzymes) are present in the gonads of the freemartin is still unsettled.

Some doubt is thrown on the above postulated role of the Y-chromosome when its absence in the intersex condition in pigs is considered: are Y-chromosome enzymes truly the inhibiting or even virilizing agents; is their expression perhaps exerted via the endocrines or is some other unknown mechanism responsible? Y-chromosome is apparently only necessary for fertility: in its absence sterility supervenes as is always the case in gonadal male intersexes. Hypoplastic testicular development in the absence of a Y-chromosome also occurs in humans.

Variable degrees of chimerism are seen in adult heterosexual bovine twins. The number of male and female cells need not be in an equal ratio and may even become altered during adult life. This change in incidence of male and female cells is probably a matter of chance. The ratio existing at birth may possibly be directly related to variations in phenotype of the freemartin.

Chimerism may differ in its effect upon genital development in different species.

CHAPTER V

Chromosome Analyses of Abnormal Animals other than Intersexes

Introduction

In a survey by Anderson *et al.* (1964) of 1,662 mentally defective human beings the frequency of sex chromatin aberrations was found to be 6·6 per 1000 for males and 5·6 per 1000 for females. On pooling the data from the literature they obtained an overall frequency of 9·2 per 1000 for males and 4·5 per 1000 for females.

These figures cannot be extrapolated with any certainty to apply to general chromosomal aberrations in terata whether human or animal. In a recent symposium McKeown & Record (1960) presented the incidence of congenital malformations in humans as being 11·2, 12·2 and 17·3 per thousand births in Sweden, Japan and Birmingham respectively as identified soon after birth. The figures included the following types of malformations: anencephalus, spina bifida, hydrocephalus, mongolism, cardiac malformations, cleft lip and/or palate and talipes. Karyological examinations were apparently not undertaken on these cases.

With regard to domestic animals, in contrast to human beings, no organized mechanism exists for referring malformed animals to an institute where they may be studied scientifically. In addition, most grossly malformed animals are either born dead or die soon after birth and thus of little value for chromosome studies. It depends purely on the interest and initiative of the animal owner and of the veterinarian in attendance (if there be one), as well as on the scientific policy of the institute concerned, whether appropriate cases are studied or not. Consequently this aspect, depending as it does on fortuitous coincidence, has received scant attention. This is also indicated by the absence of figures indicating the incidence of malformed births in animals.

CYTOGENETIC INVESTIGATIONS ON NORMAL AND MALFORMED ANIMALS

The occasional references to chromosome aberrations in domestic animals encountered in the literature indicate that studies in this direction have virtually only commenced, and have been concerned with only two types of anomalies, namely hermaphroditism and neoplasia.

A Klinefelter-syndrome (XXY) was established in male tortoise-shell cats (*cit.* Overzier, 1963). A case of mosaicism i.e. both XX and XY containing cells in cultures of the peripheral blood was established in a true hermaphrodite cat (Thuline & Norby, 1963): freemartinism could therefore be considered as a possibility in the cat.

Gustavson & Rockborn (1964) have reported 59 chromosomes in three cattle with lymphatic leukaemia. Reduction in number was probably caused by centric fusion of a large and a small acrocentric chromosome or by a translocation, the exact nature, however, still being obscure.

Basrur, Gilman & McSherry (1964) have found that the predominant cell in 24 hr old cultures from the prefemoral lymphnode of a 19 months old heifer that died from lymphosarcoma had 61 chromosomes. The extra chromosome was a submetacentric resembling an X-chromosome. Two Barr bodies were present in 35 of 50 nuclei.

Henricson & Bäckström (1964) found a heterozygous translocation in cells cultured from the peripheral blood of a boar with 56 per cent lowered fertility. The descendants of this case are still being studied.

Chromosome spreads prepared from cultures of transmissible venereal tumours of the dog consistently revealed 59 to 60 chromosomes including 16 to 17 metacentric ones—apparently resulting from fusion of acrocentrics, thus producing fewer chromosomes (*cit.* Pakes *et al.*, 1965).

As a fair proportion of South African animal owners are aware of the work of the Veterinary Research Institute and the Faculty of Veterinary Science, an encouraging number of cases are submitted, although the incidence cannot be assessed percentagewise.

It was considered reasonable to undertake chromosome analyses on all cases submitted and these are reported upon. The same technique was used as on the other cases in this study.

Case Reports

1. A young (2-tooth) Karakul ewe (1924/64) with congenital atresia ani and an ano-vestibular fistula had difficulty in defaecation, which eventually caused its death. Karyological analysis on twenty spreads revealed a normal modal number of 54 with a normal female (XX) sex complement. No microscopically visible chromosome aberrations were found.

2. An Afrikaner-Persian crossbred ewe (1926/64) with atresia ani and an ano-vestibular fistula almost similar to the above also eventually died as a result of obstipation. Karyological analysis on 28 spreads revealed a normal chromosome number of 54, a normal female sex complement and no visible aberrations.

3. A Jersey-Red Poll crossbred ox (3469; 1937/64) with congenital absence of its right front leg and part of the scapula was otherwise clinically quite normal; it was killed for dissection at the approximate age of eighteen months. Thirty-two spreads were located, each contained sixty chromosomes. No aberrations at all were found.

4. A bull dog, (1958/64) was referred to this department as a typical case of hypospadias. It had a very slovenly gait, rather long legs and a very dull facial expression, as if mentally affected. Nuclear and polymorphic sexing confirmed its masculine sex: no drumsticks were found per 500 neutrophils examined. The chromosome picture as seen in 91 spreads was that of a male: with a modal number of 2n=78 (Table 10). Microscopic study on the best of these spreads revealed no morphologically visible aberrations.

TABLE 10.—*The numerical chromosome distribution in* 91 *spreads prepared from a hypospadic Bull Dog—Case* 4

Chromosome No	55	69	71	72	75	76	77	78	79	80	81
No. of spreads...	3	1	1	1	2	2	1	73	4	1	2

5. A Jersey heifer (1962/64) was presented with front legs sprawled out, and too weak to stand. This condition was probably caused by a nutritional disturbance: 31 spreads all contained 60 morphologically normal chromosomes. Three spreads had only 59 chromosomes, probably due to loss during preparation.

6. A Merino ewe (18093; 1990/65) with a branchiogenic cyst containing teeth and situated just ventral to the right ear, had 54 normal chromosomes as seen in 20 spreads.

7. A Jersey heifer (1976/64) was diagnosed as a typical case of diprosopus, with four eyes, four nostrils and two mouths. On sucking, its lower jaws moved in unison, both tongues were also synchronized, but tongues and jaws moved independently. The middle nostrils were non-functional: breathing took place through the lateral ones only. The two central eyes always moved in unison but in a direction opposite to the lateral ones. The calf was killed after 48 hours because of its inability to suck properly.

Karyological analysis on 69 spreads revealed no morphological or numerical aberrations.

8. A Merino (18094; 1964/64) born with six legs had an extra pair of hindlegs dangling from the caudoventral region of the pelvis. The hind parts of the legs were directed caudally.

Twenty-one spreads with 54 chromosomes each and three with 52, 53 and 56 chromosomes revealed a normal male karyotype.

9. A normally proportioned Merino ewe lamb (2001/65) with severely stunted growth notwithstanding adequate nutrition was considered to represent a case of dwarfism. It presented normal chromosome morphology and number as revealed by 21 spreads examined.

10. A long-tailed Persian ewe (2002/65) was born with the left front limb absent. Thirty-seven spreads all with 54 chromosomes were examined; no aberrations were visible. Two spreads had 51 and two had 52 chromosomes.

11. A Merino ram (2-tooth) (2022/65) possessed only the right horn, 15 cm in length. The left testicle was smaller than the right, each was situated in its own scrotum which was poorly developed and only about three cm long. The penis was about 5 cm long and projected about 10 cm below the anus. The urethral opening was small.

Twenty-five spreads revealed a karyotype which was perfectly normal with a male sex complement.

12. Six Jerseys were all affected with hereditary laminitis in all four feet. These animals were born apparently normal and the condition developed progressively within the first year of life. The hooves were typically deformed and the painfulness of the condition forced the animals to walk on their knees.

The chromosomes of all these animals were morphologically quite normal (Table 11).

TABLE 11.—*Results of chromosome investigations on Jerseys affected with hereditary laminitis*

Sex	Animal	No. of spreads	Result
♀	2113	36	Normal
♀	2032	30	Normal
♀	2112	39	Normal
♀	3738	56	Normal
♂	B 39	21	Normal
♀	B 38	18	Normal

13. Two Karakul ewes from South West Africa were presented with a history of prolonged gestation. The excessive sizes of the lambs gave rise to dystocia. As a number of such cases had occurred, frequently with death of the lambs and even of the mother, a hereditary condition was suspected and chromosome analyses were undertaken (Table 12).

TABLE 12.—*Results of chromosome investigations on two Karakul ewes with a history of prolonged gestation*

Animal	No. of spreads	Results
18081	34	31 with 54 chromosomes 3 with 53 chromosomes } normal
18078	23	54 chromosomes—normal

Preliminary results from subsequent investigations undertaken by P.A. Basson and J. Morgenthal of this institute point toward a toxic effect from certain plants in their grazing.

14. Two Jerseys cows and a heifer presented facial curvature (deviation to left or right, socalled " skew face "), a suspected hereditary condition which had developed progressively with age. The first case was that of an imported Jersey bull, and the cases reported upon were from its offspring.

There was a unilateral growth inhibition affecting the region of lacrimal, zygomatic and posterior part of the maxillary bones along the longitudinal skull axis, (The condition is being studied at the Institute for Animal Husbandry Research. Irene and the department of Veterinary Anatomy of the University of Pretoria).

It resembles a condition seen in avian gynandromorphs mentioned by Hutt (1964). Unfortunately no tissue cultures were prepared; this method would have been the only way to establish whether a similar phenomenon was responsible here. Examination of the chromosome preparations prepared from the sternal marrow revealed a morphologically normal karyotype in all three cases (Table 13).

TABLE 13.—*Results of chromosome investigations on Jerseys affected with hereditary "skew face"*

Animal	No. of spreads	Results
J 2—cow	58	Normal
J 112—heifer	77	Normal
J 96—heifer	17	Normal

The one X-chromosome was generally more contracted than the other in these preparations. This was considered normal and evidence of the "Lyon" hypothesis (Lyon, 1962).

15. A Jersey calf born without a tail had a morphologically normal karyotype as determined by karyological analysis of 24 spreads.

16. A black and white mottled indigenous Bapedi heifer (2014/65) was born with a double face but single mandible. It was similar to case no. 7 above but not identical to it. The jaw and eye movements were similar but it was able to suck milk much better. Consequently it was kept alive for 47 days during which time the right mouth, being the most active, developed more rapidly than the left and was larger.

The chromosomes investigated in 43 spreads were all normal.

17. An aged Boer goat ewe (2003/65) had an abnormal attitude and mode of locomotion: it held its head high and slantingly to the left, and circled continuously to the right with a goosestep gait. It was reported to have been so since birth. In all other respects it was clinically normal and healthy but never bred. In the eleven spreads obtained no aberrations were discovered. On killing the animal the brain was found to be macroscopically and microscopically normal.

Discussion and conclusion

The malformed or physiologically abnormal animals investigated during the course of this work did not suffer from any microscopically visible chromosome aberrations. The numerical aberrations listed in some of the cases were considered to be of no significance as a small percentage of spreads ($\pm$ 5 to 10 per cent according to various authors) are always aneuploid. This is mostly due to technical errors but occasionally also to faulty mitoses. Polyploids are occasionally encountered. These are considered to be early developmental stages of megakaryocytes.

As these determinations were done on bone marrow cells only, it does not exclude the possibility of localized mosaicism as aetiological factor in some of the deformities, e.g. the diprosopus or "skew face" cases. Such studies would naturally have required the preparation of tissue cultures.

An important consideration is the fact that morphological abnormalities of the animal may be the result of induction disturbances during embryological development. These in turn could be due to single gene mutations which are not morphologically determinable with present methods at our disposal. A morphological and numerically normal karyotype does not exclude the chromosomes from being responsible for the primary causative factors in the form of gene mutations.

It can therefore be concluded that although chromosome aberrations be considered as decisive and incorrigible aetiological factors in specific disturbances such as Kllnefelter's and Turner's syndromes, these preliminary studies indicate that a prolonged and wide search requiring endless patience is needed before similar conditions are uncovered in animals. It would seem imperative that tissue cultures of abnormally affected regions also be undertaken. Such studies require a tremendous amount of routine work and microscopic screening or preparations. A negative result under such conditions is but a poor compensation and but a poor incentive to further investigations. More success would perhaps be obtained in neoplastic conditions.

From preliminary observations it appears to be apparent that the Robertsonian effect (Plate 18) may turn out to be quite a common phenomenon amongst wild animals in South Africa. Wallace (1965, personal communication) has reported it to occur in the impala and observations on the blue wildebeest indicate it to occur in many spreads. From an evolutionary point of view these observations may perhaps indicate one of the possible ways of creation of a new species.

CHAPTER VI

Karyological Studies on some Wild Animals

1. Introduction

In South Africa a large variety of wild animal species, protected mostly in numerous parks and game reserves, offers a unique opportunity and practically an open field for cytogenetical research. Work done at the Veterinary Research Institute from time to time on certain species as potential carriers of disease or parasite hosts, further enhance this field.

In game parks and reserves it is necessary at times to reduce the numbers of animals artificially by shooting in order to avert overpopulation. Recently hippopotami and blue wildebeest in the Kruger National Park, had to be so reduced; the opportunity was seized to undertake cytogenetic studies on these two species. Blood and tissue cultures were not attempted as conditions in the veld regarding transport and storage were unpredictable.

With the recently developed immobilizing drugs such as M99 and the easy method of application (by the use of cross-bows or " Cap-chur " guns), killing of wild animals for such investigations has become unnecessary: bone marrow (and also blood) can be aspirated from the sternum of the subdued animal, without any danger to the investigator. Above all, the equipment needed is inexpensive compared to most branches of scientific research.

Besides a karyological study of the hippopotamus (Gerneke, 1965) and blue wildebeest, that of the yellow mongoose was also undertaken. The results are reported in the ensuing part of this chapter.

2. The Chromosomes of the Blue Wildebeest, *Gorgon Taurinus* (Burchell)

Introduction

The blue wildebeest is a common inhabitant of the Kruger National and Kalahari Gemsbok Parks. Its taxonomic position is as follows:

Order: Artiodactyla
Suborder: Ruminantia
Infraorder: Pecora
Superfamily: Bovoidea
Family: Bovidae
Subfamily: Hippotraginae

In a review of the literature no previous chromosome determinations of the blue wildebeest were encountered.

Material and method

The first determinations on the chromosomes of the blue wildebeest were done on a trip to the Kruger National Park during which preparations from males alone were made. With the kind permission of the Director of the National Zoological Gardens in Pretoria, Dr. D. J. Brand, and the willing cooperation of the veterinary officer, Dr. L. E. Smit, further preparations were made from a male and a female. For collection of sternal bone marrow these two animals were subdued with an immobilizing drug, M99. The bone marrow aspirates were treated as described previously.

Results

The results of polymorphic sexing and chromosome counts are given in Tables 14 and 15. A limited number of spreads revealed the Robertsonian effect (Plate 18). This is seen to result in fewer chromosomes but more meta- or submetacentrics. The normal karyogram (2n=58) is seen in Plates 20, 21 & 22 and a normal metaphase spread in Plate 19.

Table 14.—*Results of polymorphic sexing in the blue wildebeest*

Sex	Drum-sticks	Sessile nodules	Small nodules	Rackets	Total
♀	11	4	0	0	500
♂	0	1	9	0	1500

Table 15.—*The chromosome number as indicated by the most frequent number of chromosomes found in various spreads prepared from several male and a female blue wildebeest*

Sex	54	56	57	58	59	60	Total
♀	—	2	—	63	—	1	66
♂	4	7	1	94	2	4	112

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Discussion and conclusion

The diploid chromosome number of the blue wildebeest was determined as 2n=58. They are arranged as follows (Plates 20, 21 & 22):—

Group 1: A single pair of subtelocentric autosomes very much resembling the sex chromosomes of the bovine.

Group 2—29: This group is represented by 28 pairs of acrocentric chromosomes. The sex chromosomes are included. On arranging the autosomes in descending order of length identical karyograms for male and female are obtained (Plates 20 & 21). The difference in length between adjacent acrocentrics is so slight that almost any chromosome pair can be picked out as the sex chromosomes and the rest arranged to form the remaining 27 pairs (Plate 22). The sex chromosomes represented in Plate 22 therefore merely illustrate one of many possibilities. No claim to authenticity is made.

The chromosomes of the blue wildebeest are therefore peculiar in revealing no sharply defined morphological sex differences. Tritiated thymidine would have to be used to identify the X-chromosomes with certainty. The fact that polymorphic sexing is applicable, at least indicates that sex chromosomes are present as in all other vertebrates.

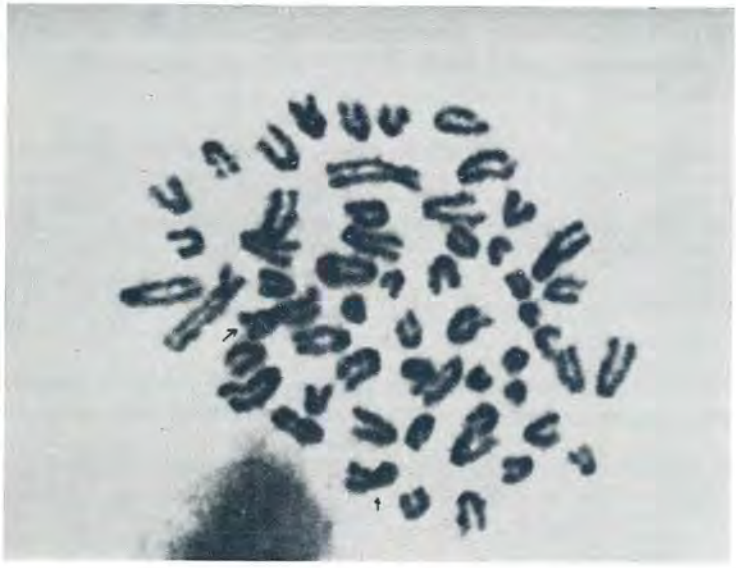

PLATE 18.—The Robertsonain effect seen in a spread from a female blue wildebeest

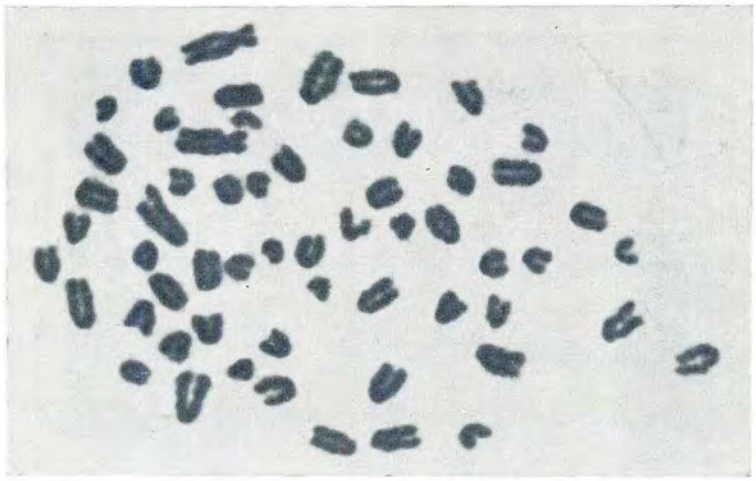

PLATE 19.—A metaphase spread from the bone marrow of a blue wildebeest cow

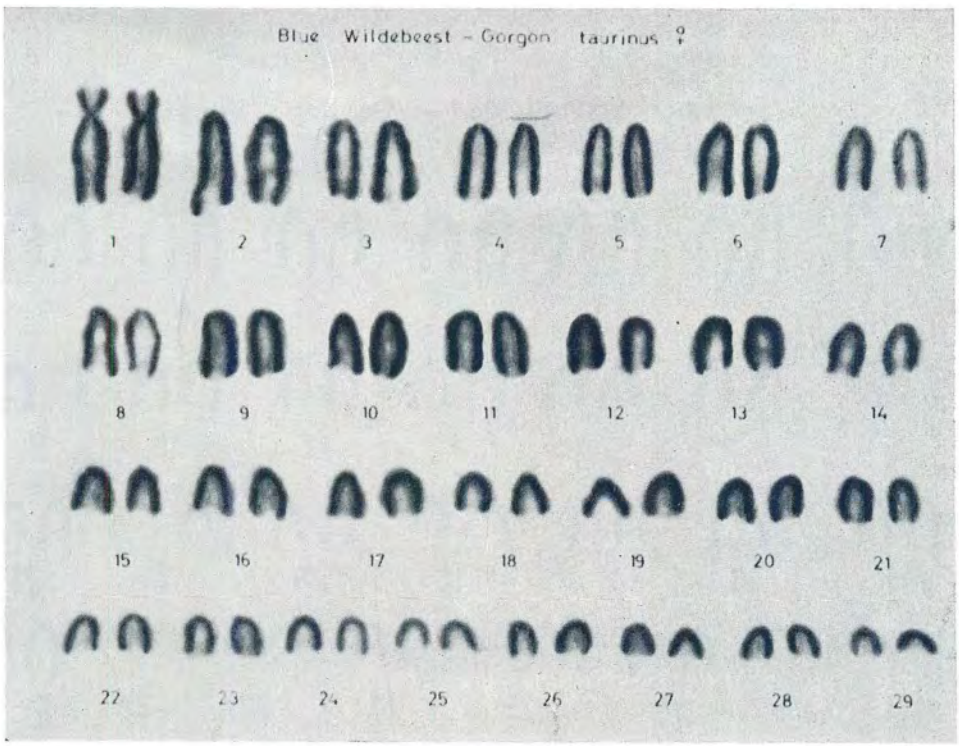

PLATE 20.—A karyogram compiled from the spread in Plate 19. X-chromosomes have not been isolated

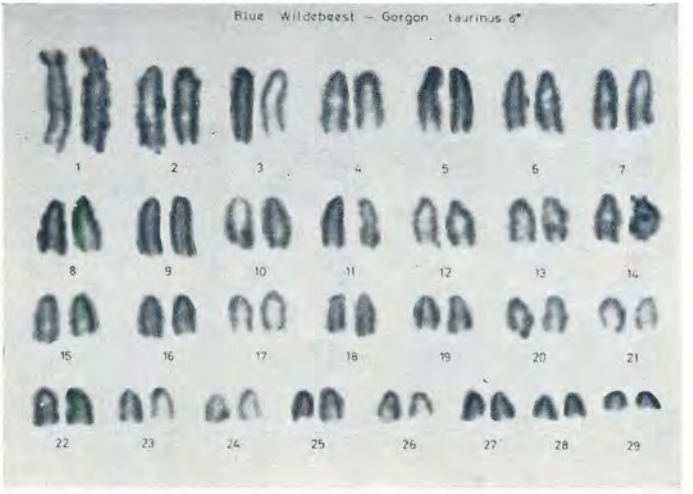

PLATE 21.—A karyogram compiled from a bone marrow spread of a blue wildebeest bull revealing an identical morphology to that of the female. No distinct Y-chromosome is seen

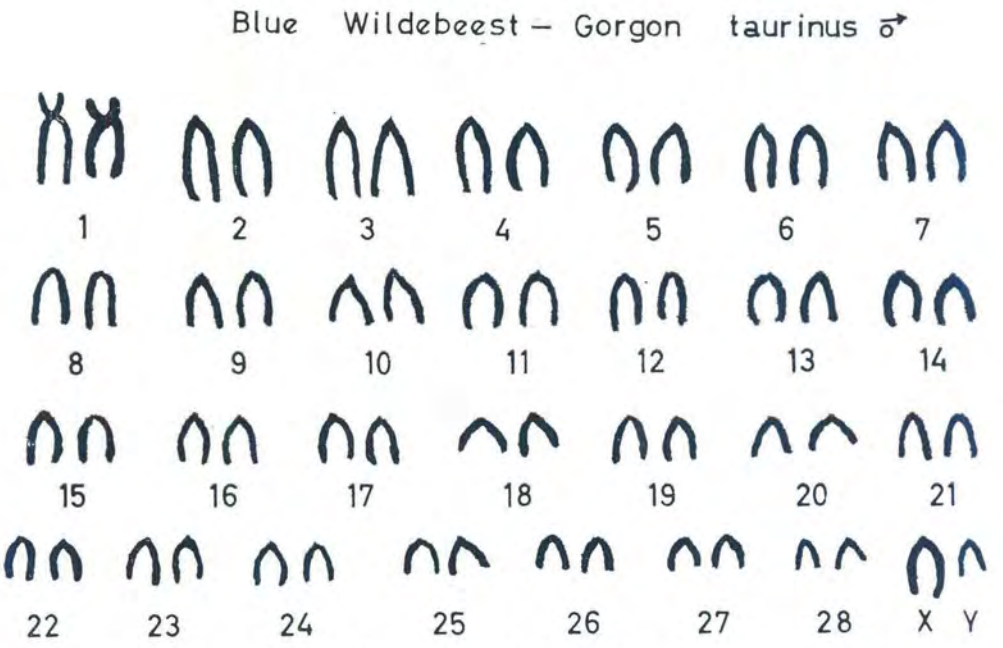

PLATE 22.—A karyogram compiled from a camera lucida drawing of a metaphase spread from the bone marrow of a blue wildebeest bull. A possible pair of sex chromosomes have been picked out. No absolute certainty exists as to the correctness of the choice

3. THE CHROMOSOMES OF THE YELLOW MONGOOSE, *Cynictis penicillata* (G. CUVIER)

Introduction

The yellow mongoose, also referred to as the red (yellow) meercat, is a common inhabitant of the African veld. Its taxonomic position is as follows:

Order: Carnivora
Suborder: Fissipedia
Superfamily: Feloidea
Family: Viverridae

In a review of the literature it became apparent that chromosome determinations have rarely been done on any of the Viverridae. Jordan, (1914) (cit. Makino, 1951) found the haploid number of chromosomes of the mongoose, *Mungos ichneumon* [=*Herpestes ichneumon* Linnaeus] to be 24 in the first spermatogonial division. He could not find a male (Y) chromosome. Fredga (1965) found the chromosomes of the Indian mongoose, *Herpestes auropunctatus* Hodgson to be 2n=36 in the female and 2n=35 in the male: a Y-chromosome was apparently absent. To explain this anomaly he assumed that part of the Y-chromosome had become translocated to an autosome to form a trivalent which was identifiable. Fredga also mentions other aberrant sex-determining mechanisms, which he divides into:

(1) " Species with an XY_1Y_2/XX mechanism, and

(2) Species with an odd number of chromosomes (in either one or both sexes) and having a complicated or unknown mechanism for sex determination ". The XY sex-determining mechanism is therefore not universal amongst animals.

Material and method

By the kind collaboration of Dr. Anna Verster of this Institute, two mongooses, one of either sex, were obtained for chromosome studies. Bone marrow spreads were made according to the method described previously. Camera lucida drawings were prepared from the best spreads obtained and a male and female karyogram (Plate 23) set up.

Results

The number of chromosomes was found to be 2n=36 in the yellow mongoose (Table 16).

TABLE 16.—*The chromosome number as indicated by the most frequent number of chromosomes found in various spreads prepared from a male and female yellow mongoose*

Sex	32	33	34	35	36	37	Total no. of spreads
♂	1	—	—	1	50	1	53
♀	—	1	1	2	109	—	113

Discussion and conclusion

The results as indicated in Table 16 reveal a modal chromosome number of 2n=36 for somatic cells of the yellow mongoose. When arranged in descending order of length the chromosomes can be conveniently grouped as follows (Plate 23):

Group 1—2: These are the largest chromosomes. Both pairs are subtelocentric, the centromere of the former being slightly nearer one end than in the second pair.

Group 3—4: Both these are submetacentric, the third pair being very closely metacentric.

Group 5—9: All five pairs are subtelocentrics, pairs five and six having their centromere nearer one end than the other three.

Group 10—13: Chromosomes 10 and 13 are very closely metacentric while the others are subtelocentrics.

Group 14—17: All are submetacentric although often appearing metacentric especially when they are extensively contracted.

The X-chromosome is a submetacentric fitting in between chromosomes 14 and 15. The Y-chromosome is the smallest one present and is distinctly metacentric.

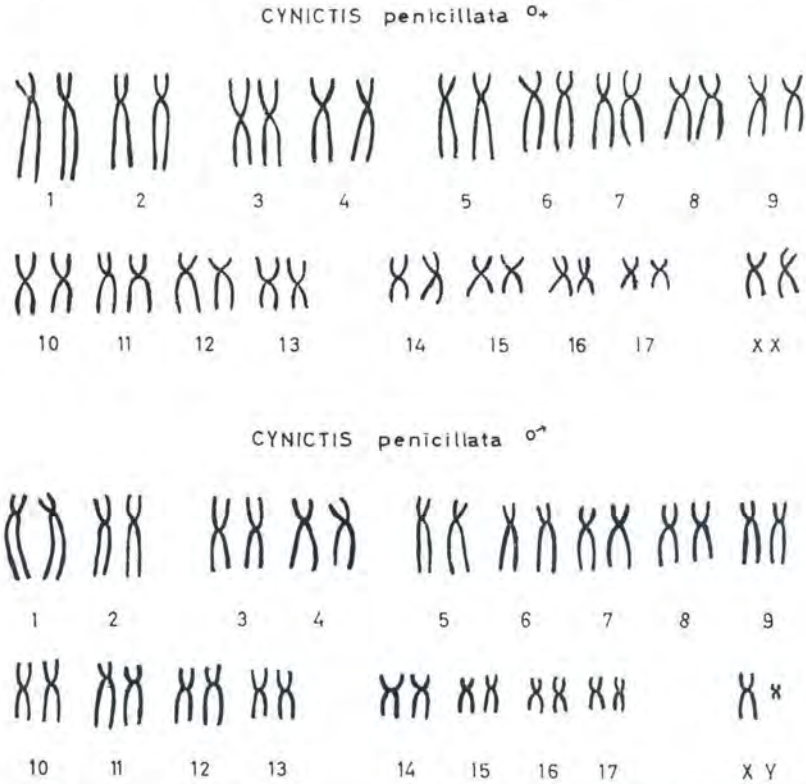

PLATE 23.—Karyograms compiled from camera lucida drawings of metaphase spreads from bone marrow of a male and female yellow mongoose—*Cynictis penicillata*

CHAPTER VII

SUMMARY

Recent advances in technique have greatly facilitated cytogenetic studies and consequently, the undertaking of this work. The object of this project was to determine the normal karyogram of certain mammalian species in which it was either unknown, in doubt or a matter of controversy, to evaluate various methods of determining the sex of an animal with reference to particular domestic species and to investigate the occurrence of chromosomal aberrations possibly associated with congenital malformations in these species.

By way of introduction the latest concepts on chromosome structure and replication including some proposed models are reviewed. Methods of cytological sexing, which include polymorphic sexing performed on blood smears and nuclear sexing, applied to sections or mostly buccal smears, are briefly discussed. In view of aberrations in chromosomal constitution discovered in certain intersexes, a brief comparative review of intersexuality in vertebrates is given.

The technique of making chromosome preparations was based on hypotonic treatment of bone marrow aspirates, subsequent fixation in 1:3 acetic-alcohol, optional treatment with 45 per cent acetic acid for additional swelling and finally, preparation of drop spreads which were then rapidly dried by careful heating. Additional methyl alcohol fixation of spreads and their subsequent staining in alkalinized 10 per cent aqueous Giemsa solution completed the procedure. Screening of preparations was done directly, without mounting. Brief immersion in oil of cloves followed by a rinse in xylol was often necessary for removal of stain deposit.

The diploid chromosome number of the pig was determined as being 38. They are divided into six groups of autosomes with a distinct X and a small Y-chromosome. Nuclear sexing was found to be effective on neurones and buccal smears. In pig intersexes nuclear sexing revealed the presence of Barr bodies in nerve cells and epithelial cells of the ductus deferens. Polymorphic sexing was possible, but useless for critical determinations as a small percentage of drumsticks also occur in the boar. All porcine intersexes investigated were genetic females, notwithstanding the presence of male gonads, male sexual instincts, suppression of female genital tracts and almost complete replacement of the latter by male organs in at least one individual. A Klinefelter-type syndrome as well as chimerism were definitely ruled out. A sex-linked recessive gene possibly associated with autosomal modifiers (in the nature of male determiners as in *Drosophila melanogaster*) is considered to be the primary aetiological factor. No Y-chromosome was ever found in bone marrow spreads prepared from the intersexes.

The adrenal cortex of two intersexual siblings (9 to 10 months old) revealed focal cellular hypertrophy. This was not seen in younger intersexes. This finding makes the intersexual condition of pigs reminiscent of the human adrenogenital syndrome. A comparison is made. Biochemical determinations, however, are needed before final conclusions can be drawn.

In all intersexes investigated the pineal gland was found to undergo oedema and cellular hypertrophy which indicated an increased activity with possibly an increased inhibitory function on female gonadal development. It appears therefore that the pineal is undoubtedly concerned in the expression of the recessive gene. A suggested interrelationship between the pineal, adrenal cortex and gonads is discussed. The

precise physiological nature needs further investigation. Increased cyst-formation of the pineal is also more often encountered in intersexual pigs. They are considered favourable subjects for experimental procedures on pineal function.

The uterine fluid, causing considerable uterine distension and endometritis in aged individuals, consisted mostly of urine with small initial contributions from the uterine glands and subsequent additions of some cellular debris and perhaps tissue fluid. The abnormal nature of the urethra and the absence of a micturating mechanism in the uterus was responsible for this urine accumulation.

It is suggested that the existing non-informative nomenclature of intersexual states be replaced by a suggested modified terminology in which the presence, absence and nature of the gonads are clearly and unambiguously indicated.

The normal karyotype of the sheep was found to be $2n=54$ consisting of six large submetacentrics and 48 acrocentrics of descending order of length. The Y-chromosome is submetacentric and easily identifiable as the smallest chromosome. The X-chromosome is difficult to pinpoint due to its being one of the acrocentrics. Nuclear sexing is only applicable to neuronal nuclei: buccal smears are considered unsuitable. The occurrence of drumsticks in polymorphs from ewes was found to be of low frequency varying from 0·06 to 6 per cent. Occasional drumsticks are present in rams. Only by using the Kosenow-Scupin formula can a positive indication of the sex be obtained.

Two gonadal male sheep intersexes are described. In one of them bone marrow chimerism was determined. This is considered as final proof of the freemartin condition in the female member of some heterosexual sheep twins. Enzymes believed to be produced by the Y-chromosome of the donor cells are considered the active agents in switching genital development. As chimerism was not indicated in the other intersex, the condition may have been inherited as a sex-limited recessive characteristic, similar to that in the pig. It was, however, found most peculiar that the effect of chimerism and that of presumably sex-limited inheritance produced practically identical malformed male genital organs in the two intersexes. Testicular hormones from the male twin are considered of no initial causal significance in the development of the ovine freemartin.

The diploid number in the bovine is $2n=60$. The X-chromosome is a large submetacentric; the Y is a small metacentric. Autosomes are all acrocentrics. Neurones and cells of liver, pancreas and adrenal have been found suitable for nuclear sexing. Drumsticks occur in 0·8 to 8 per cent of polymorphs in females and 0 to 2 per cent in males. Sessile nodules are considered to be sex specific in the bovine. As in the sheep, the Y-chromosome enzymes are considered, for given reasons, to be the aetiological factor in bovine freemartinism.

The ratio of male and female cells existing at birth in heterosexual twins may be related to variations in phenotype of the freemartin. This ratio may gradually change in any direction probably as a matter of chance. Chimerism may differ in its effect upon genital development in different species.

In view of the findings in pig intersexes and in bovine and ovine freemartins it appears that the Y-chromosome is not always necessary for hypoplastic testicular development (species difference?), that it must be present in all gonadal cells for fertility to develop and that its presence in some blood cells may cause suppression of internal female organs or even stimulation of internal male organs. In the former and latter possibilities, sterility of testicles always results. Chromosome analyses on 17 cases of malformations revealed no morphologically visible aberrations.

The chromosomes of the blue wildebeest were found to be 2n=58, with sex chromosomes not morphologically recognizable and autosomes all acrocentric except two large ones, which are subtelocentrics. These chromosomes are therefore peculiar in not representing any clearcut morphological sex differences.

The karyotype of the yellow mongoose was found to contain 2n=36 chromosomes arranged in five groups with the X-chromosome submetacentric, and the Y small and metacentric.

ACKNOWLEDGEMENTS

I wish to thank Prof. B. C. Jansen in his capacity as Chief of the Veterinary Research Institute, Onderstepoort, for permission to carry on this work: to Prof. S. J. van Heerden, Head of the Gynaecology Department for submitting some of the experimental animals and to Prof. H. P. A. de Boom, Head of the Anatomy Department, for his general support and enthusiasm throughout the course of this study and especially for submitting most of the animals. I would also like to thank him for his expert guidance and constructive criticism in preparing the manuscript.

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