A STUDY IN CYTOGENETICS IN THE SHEEP.

by

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A STUDY IN CYTOGENETICS IN THE SHEEP.

General Introduction.

Little is known, even today about the biological mechanism of transmission of all the desirable features that interest breeders of domestic animals. The present high standard of quality of these animals, is largely the result of the improvement in animal breeding begun by Robert Bakewell in England in 1760. Bakewell's theories that "like begets like" and "breed the best to the best" have been the rules of thumb from which husbandmen have worked to produce the domestic animals of today. For centuries prior to Bakewell's time, superstition and mystery had surrounded the transmission of breeding and sex determination. Aristotle, writing on this subject in sheep, stated that "the lambs are white or black according as the veins beneath the tongue of the ram are white or black" and that "sex is determined by the nature of water drunk, or the direction in which the wind is blowing during copulation". He suggested facing sheep into the North wind during mating to produce males. Even Darwin, in 1868, while paying tribute to the breeders of domestic animals for the quality/

quality their skill had produced, failed to offer any solution to this imposing riddle of inheritance.

At the same time as Darwin was expounding his evolutionary postulates, the basic experiments which were to form the foundation of modern genetics were being carried out in an Augustinian monastry by the monk Gregor Mendel (1865). Mendel's work was to lie undiscovered for over thirty years till 1901, when three investigators, De Vries in Holland, Correns in Germany and Techermak in Austria found Mendel's papers and proclaimed their importance. Mendel showed that heredity materials consist of genes that segregate at gametogenesis. This fact, coupled with the experiments and observations of Boveri (1887-1902) and Hertwig 1876, that heredity was transmitted by the nuclei of the egg and sperm left the final problem of solving the association of the nuclear material the chromosome, (Waldeyer 1888) with the genes, the hereditary transmitters. A cytologist, W.S. Sutton in 1902-1903 offered proof of this relationship in his work with the lubber grasshopper. Sutton further confirmed the suggestion of Montgomery (1901), that members of a chromosome group have a characteristic/

characteristic size relationship. The exciting step in the establishment of the role of chromosomes was when McLung (1902), acting on his own and Montgomery's (1901) observation, suggested that the accessory chromosome found in the insect species Orthoptera, was a sex determiner, but that the ovum was still the final selector of sex. Gradually the facts in sex determination have been uncovered by others as well, including Wilson (1905 and 1906) and Bridges (1913 and 1916) until we now know for our higher animals and man that the male is the result of a heterogametic fertilisation and the female a homogametic fertilisation.

The gene chromosome relationship, the determination of sex by chromosomes and finally observations by Morgan (1911) and Sturtevant (1913), showing that the X chromosome in Drosophila not only contained essential factors for sex determination, but other factors which were linked to this chromosome and further that these factors are arranged in linear series, have largely formed the basis of modern genetics.

The study of genetics has grown into two somewhat complementary but not always united subjects. Classical/

Classical genetics as applied to mammalian and human breeding is a highly conceptual subject based on a largely mathematical system of deduction. Cytogenetics on the other hand has been the subject of the cytologist, making his crude but nevertheless painstaking observations on the visible nuclear elements, the chromosomes. The division between these two subjects has been joined on many occasions with studies on plants and insects, which have a much simpler chromosome complex than our farm animals and man.

The classical example of this is shown in the work on the large salivary gland chromosomes of various Dipteran insects. These were first reported by Balbiani (1881), but their true structure was initially demonstrated by Heitz and Bauer (1933), who showed them to be paired homologous chromosomes. Painter (1933, 1934 and 1935), in applying the salivary gland technique to a cytogenetical analysis, was able to show banding of homologous regions between the paired chromosomes. The view was then expressed by Bridges (1934), that there was an association between the salivary gland chromosomes and normal mitotic chromosomes as seen at prometaphase. The enormous amount of work which followed/

followed these findings on Drosophila was carefully recorded and analysed by Bridges (1944) who was able to correlate the banding (genetic loci) with phenotypic expression over some 5,000 characters. This then can be taken as a model of cytogenetics made possible by the freak situation of the giant chromosomes in this insect.

Until recently the greatest barrier to mammalian chromosome studies has been the difficulties involved in making suitable morphological preparations. The extent of this difficulty need barely be emphasised when it is considered that it was not until 1956 when Tjio and Levan reported the human chromosome complement as forty six and not forty eight as had been thought previously. In spite of this difficulty and also the fact that the gene complex of mammals is far more complicated than a simple insect such as Drosophila, a start has been made, and a very good start to unravel some of the mystery associating phenotype with the chromosomal pattern, particularly in man.

It would be unrealistic to claim that the investigation of cytogenetical problems in farm animals/

animals could offer as immediate help to veterinary practice as the study of human cytogenetics has to medicine. However the fact that a start should be made along similar lines on investigation as is being done in human medicine is obvious for several reasons. Firstly, there are so many similarities between our subjects and it is highly probable that veterinary studies could make an assistant contribution to medical studies, as is seen in the great interest shown by medical cytogeneticists in the bovine freemartin for example. (Ohno Trujillo, Stenius, Christian, & Teplitz, 1962; Goodfellow, Strong & Stewart, 1965). Secondly it would be unwise for the veterinary profession to ignore the development of the skills of cytogenetics which could have a useful place in the investigation of many aspects of infertility in particular and developmental disorders of our farm animals (Bishop, 1964; Pakes and Griesemer, 1965).

Medical Cytogenetics.

Many human developmental diseases can now be associated with chromosomal abnormalities, and it is to the great credit of the investigators of these abnormalities/

abnormalities that concurrently a most systematic methodology for investigation has been evolved in so short a time. It is therefore pertinent to survey briefly some of these diseases and retrace the advances made in human chromosomal technique within recent years. Of all the chromosomal abnormalities unearthed in man those involving the sex chromosomes have probably been the most extensively studied and have yielded some of the clearest information as to the cause of abnormal phenotype. Probably the main reason behind the rapid development in this particular field was the discovery of sex chromatin and the recognition of its significance, even before chromosome studies had begun.

The Role of Sex Chromatin.

Nineteen forty nine is usually quoted as the date of discovery of sex chromatin by Barr and Bertram while working on the neurones of the cat. These workers showed that when stained suitably the nerve cells of the cat showed a sexual dimorphism. A small dark staining body was present in cell nuclei of the female cat but not in the nuclei of the male cat. This body found in female cell nuclei only, was originally referred/

referred to as the Barr body, but is now more generally termed the sex chromatin body. While Barr and Bertram first discovered sex chromatin in mammals and subsequently did much to advance our present information on it, the original discovery is due to Smith (1944 and 1945) who found the cells of the larvae of the spruce-bud worm could be differentiated sexually by a dark staining boady in the nucleus of the female cells. Smith (1945), further suggested that this method may be useful in mammals for studying sex ratios before differential mortality could distort the primary sex ratio.

Apart from considerations of the properties of sex chromatin and its relationship to the X Chromosome which will be discussed later in Section I. of this thesis, the fact that sex chromatin is present in female cells and not male cells is one of the axioms on which medical cytogenetics rests.

Following its original discovery, the knowledge of sex chromatin has increased rapidly. Barr, Bertram and Lindsay (1950) were able to show that a sex difference occurred in human sympathetic ganglion cells and later Graham and Barr (1952) showed that the sex/

sex dimorphism was not confined to nerve cells alone but was present in a high percentage of cases in the cells of many other tissues of the cat. The advance in information of sex chromatin now developed very quickly and it can be said that it became part of medical cytogenetics when Moore, Graham and Barr (1953) showed its presence in female skin biopsies and also that of an hermaphrodite. In 1954 Moore and Barr showed its presence in many other cell types examined in man. As a result of skin biopsy technique, Polani, Hunter and Lennox (1954) and Wilkins, Grumbach and VanWyk (1954) were able to demonstrate that many patients with XO Turner's syndrome (gonadal agenesis) lacked sex chromatin. This added confirmatory evidence that sex chromatin was linked with the X chromosome. When Moore and Barr (1955), and Marberger, Boccabella and Nelson (1955), found that sex chromatin could be demonstrated in buccal mucosal cells, the path lay wide open for extensive survey application to clinical abnormalities associated with sex. Several groups of workers including Riis, Johnson and Mosbech (1956), Jackson, Shapiro, Uys and Hoffenbyrg (1956), Plunkett and Barr (1956), Nelson (1956) Sohval, Gabrilore, Gaines and Soffer/

Soffer (1956), were then able to find that sex chromatin was present in the majority of patients with Klinefelter's syndrome (primary micro-orchidism), (XXY Chromosome complement).

The comparatively simple finding of Davidson and Smith (1954) that a sex difference could be demonstrated in polymorphonuclear leucocytes of peripheral blood added yet another reliable diagnostic precedure to medical cytogenetics. When techniques became available from 1959 onwards for the studying of human chromosomes, a striking correlation was revealed between the sex chromatin picture and the number of X chromosomes in the individual concerned. The presence of sex chromatin is associated with 2 X chromosomes as in the female and its absence with One X chromosome as in the male (Stewart 1960). This correlation between sex chromatin and the number of X chromosomes has been of untold value in checking the chromosomal complement of patients in medical genetics. Further, the rapid advance in sex chromosomal knowledge in relation to both Klinefelter's and Turner's syndromes can be attributed to the fact that by nuclear sexing these cases could be more easily detected./

detected. This latter fact is evident by the large scale surveys using nuclear sexing, carried out on male patients of mental deficiency hospitals by Ferguson-Smith, Lennox, Mack and Stewart (1957), Ferguson-Smith (1958 and 1959), Barr, Shaver, Carr and Plunkett, (1959) and MacLean, Mitchell, Harnden, Williams, Jacobs, Buckton, Baikie, Court-Brown, McBride, Strong, Close and Jones (1962). In these surveys approximately 1% of patients were found to be chromatin positive cases of Klinefelter's syndrome, which was higher than the incidence of two per thousand found among the general population at birth, by MacLean, Harnden, Court-Brown, Bond and Mantle (1964).

Similarly, surveys on female patients of mental hospitals by Fraser, Campbell, MacGillivray, Boyd and Lennox (1960), Sanderson and Stewart (1961), Johnston, Ferguson-Smith, Handmaker, Jones and Jones (1961), MacLean, Mitchell, Harnden, Williams, Jacobs, Buckton, Baikie, Court-Brown, McBride, Strong, Close and Jones, (1962), Hamerton, Jagiello and Kirkman (1962), Davies (1963) and Ridler, Shapiro and McKibben (1963), have shown that the evidence of Turner's syndrome without sex/

sex chromatin was four per ten thousand and incidence of the female XXX triple X syndrome was four per thousand.

It can be said therefore that when improved methods of chromosome examination became available by the development of bone marrow technique of Ford, Jacobs and Lajtha (1958) and now universally accepted leucocyte culture technique of Moorehead, Nowell, Mellman, Battips and Hungerford (1960) the practical application of nuclear sexing had shown clearly the direction in which these more sophisticated techniques could be utilised most effectively.

Chromosome Studies in Man.

Within the space of a year, three important diseases in human medicine were shown to be associated with a chromosomal aberration. In the first case, Lejeune, Gautier and Turpin (1959) demonstrated a specific chromosomal abnormality in mongolism (Down's syndrome), in which one of the small chromosome of the acrocentric group, G or chromosome 21-22 according to some authors, is present in triplicate, (trisomy G). Much information has now been gathered as a consequence of this original finding and many cases of primary non disjunction, Down's syndrome or mongolism (Yunis 1965) have/

have been described. Another type of mongolism, called translocation mongolism was shown by Penrose, Ellis and Delhanty (1960) to have the normal number of chromosomes (46), but an abnormal chromosome in the X6-12 chromosome group. This was interpreted as being a 15/21 chromosome translocation in which the greater part of chromosome 21 had become attathed to chromosome 15. The mongoloid patient had two normal chromosomes 21 in addition, and so was in effect trisomic for chromosome 21. In some cases siblings were affected and it was found that the mother carried the abnormal chromosome but had 45 chromosomes and only one chromosome 21.

While mongolism is an autosomal chromosome aberration, the other two chromosome abnormalities discovered at nearly the same time involved the sex chromosomes. Ford, Jones, Polani, de Almeida and Briggs (1959b), described a patient with Turner's syndrome (Turner 1938), who also had forty five chromosomes. The chromosome missing was an X and the patient was chromatin negative. Klinefelter's syndrome (Klinefelter, Reifenstein and Albright 1942) was the third of these diseases to be found associated with a

chromosomal/

chromosomal abnormality by Jacobs and Strong (1959), who demonstrated forty seven chromosomes and an XXY sex chromosome complement. The finding of these three major chromosomal abnormalities in so short a time has been followed by innumerable reports of cases of similar nature to these and in addition many others have been added and are being added continually to an already formidable list. From a purely chromosomal viewpoint, these cases fall into one of two groups, those in which there is an aberration of one or more of the autosomes and those cases involving the sex chromosomes. It is the latter group which are given more particular study here, since sexual abnormalities of the sheep form a large part of this work.

Sex Chromosome Abnormalities in Man.

Turner's Syndrome.

The work of Lindsten (1963) and Ferguson-Smith, Alexander Bowen, Goodman, Kaufmann, Jones and Heller (1964) and Ferguson-Smith (1965) in particular have shown the wide variety of both phenotype and chromosomal pattern that can be expected in one syndrome. The original syndrome described by Turner in/

in post-pubertal females was characterised by short stature, infantalism, streak gonads and other associated congental malformations. These features are seen in the classical chromosomal case described by Ford et al (1959b) associated with monosomy of the X chromosome. Ferguson-Smith (1965), describes no fewer than fifteen karyotype categories from 307 reported cases of Turner's Syndrome. Within these categories are excellent examples of some of the various types of chromosomal change reported to date in human chromosome studies. These include the XO group, ~~(aneuploidy~~ the XX/XO chromosome mosaic (de Grouchy, Lamy, Frezal and Ribier (1962a) in which two cell lines can be demonstrated, an XO/XX/XXX mosaic series, Carr, Morishima, Barr, Grumbach, Luers and Boschann (1962), with three cell lines and a ring X chromosome mosaic group in which one of the X chromosomes is present in the form of a large ring (Lindsten and Lillinger, 1962). Two groups of cases are present showing the presence of an Isochromosome X representing the transverse division of the X chromosome at the centromere instead of the normal longitudinal division. One of these is an isochromosome X,/

isochromosome X, of the long arms (Lindsten, Franceschi, Ikkos, Kaijser Klinger and Luft (1963), and the other isochromosome X of the short arms. Another two groups of cases of Turner's Syndrome are reported in which there is a chromosomal deletion of either most of the long arm (Grouchy, Lamy, Frezal and Ribier, 1961), or most of the short arm of one of the X chromosomes, (Jacobs, Harnden, Buckton, Court-Brown, King, McBride, MacGregor and Maclean, 1961).

It has further been found from studies on Turner's Syndrome that the abnormal X chromosome is the inactivated chromosome and sex chromatin forming (McLean 1962). It is still partly genetically active however and by careful comparisons between the wide variety of phenotypes and chromosomal patterns presented in Turner's Syndrome, a distinct association can be drawn between certain of the Turner stigmata and parts of the X chromosome. There is suggested evidence (Ferguson-Smith, 1965) of areas of homologous loci between the X and Y chromosome in man.

Klinefelter's Syndrome.

Of all the human chromosomal diseases, the incident of Klinefelter's syndrome is the highest.

The/

The incident is quoted as high as 3% of males attending an infertility clinic (Ferguson-Smith et al., 1957). The most constant clinical feature is sterility associated with primary micro-orchidism. Severe stigmata as seen in Turner's Syndrome do not appear to occur, but there would appear to be a higher incidence among the mentally subnormal (Kaplan, 1961). The chromosomal pattern can be represented by an XXY karyotype, an XXY/XX mosaic pattern (Ford, Polani, Briggs and Bishop 1959), or an XXXY (Carr, Barr, Plunkett, Grumbach, Morishima and Chu 1961), or even XXXXY (Fraccaro, Kaijser and Lindsten 1960). The sex chromatin picture corresponds with the number of X chromosomes, but one, present in each of these cases and as many as three sex chromatin bodies have been seen in XXXXY cases. The discovery of the chromosomal pattern in Klinefelter's Syndrome has added much to the knowledge of the Y chromosome in man. The evidence is very strongly in favour of the fact that the Y chromosome is male determining and responsible for testicular formation (Ferguson-Smith 1963) and that the germ cell dysgenesis seen in Klinefelter's syndrome is due to the genetic inbalance caused by the additional X chromosomes.

Trisomy/

Trisomy X. (The Triple X Syndrome).

Trisomy X in Drosophila as described by Bridges (1921) produces what is termed a super female fly which is usually non-viable. In man however trisomy X is compatible with life (Jacobs, Baikie, Court-Brown, McGregor, McLean and Harnden 1959), and that in many cases only a mild phenotypic effect was produced (Close 1963), as compared with the autosomal trisomies. As contrast to both Turner's and Klinefelter's Syndromes in which all cases are sterile, some cases of trisomy X are known to have normal ovarian function and bear children (Fraser, Campbell, MacGillivray, Boyd and Lennox, 1960; Stewart and Sanderson, 1960). As pointed out, most cases of triple X or trisomy X have been found by screening tests carried out on patients of mental institutions. As with Turner's Syndrome, and Klinefelter's Syndrome, cases of mosaicism have also been reported (Day, Larson and Wright 1963), and in other cases four or five X chromosomes have been reported (Carr, Barr and Plunkett 1961; Kesaree and Wooley 1963).

The Y Chromosome Abnormalities in Man.

The Y chromosome is the bearer of masculinizing factors/

factors in man. Evidence from Klinefelter's syndrome and more recently a case of true hermaphroditism (Brogger and Aagenaes 1965), suggest that it is responsible for inducing testicular differentiation in the primitive gonad. Numerical aberrations have been reported in the Y chromosome. One case with two Y chromosomes was fertile and phenotypically normal (Sandberg, Koepf, Ishihara and Hauschka 1961), and another had Klinefelter's Syndrome (Carr, Barr and Plunkett 1961). Structural abnormalities also occur in the Y chromosome and great poly-morphism in size has been demonstrated without any obvious phenotypic effect (Jacobs 1964). There are however, reported cases of structural abnormalities of the Y chromosome with abnormal phenotype, such as the Y-Y translocation reported by Yunis (1965) and a deletion of the long arm, (Vahuru, Patton, Voorkess and Gardner 1961).

Hermaphroditism and the Sex Chromosomes.

Several cases have now been reported in which both male and female cell lines are present in one or more tissues within the one individual (Gartler, Waxman and Giblett 1962; Josso, Grouchy, Auvert, Nezelof/

Nezelof, Jayle Moullec, Frezal, Lamy and de Casaubon 1965) and a variety of intersex phenotypes have been presented as a consequence. No further reference will be made at this stage since a full discussion on the sex chromosomes and hermaphroditism is found under part 4. The use of chromosome studies in the study of intersexuality in man has however added a further diagnostic tool for determining the future treatment of these cases.

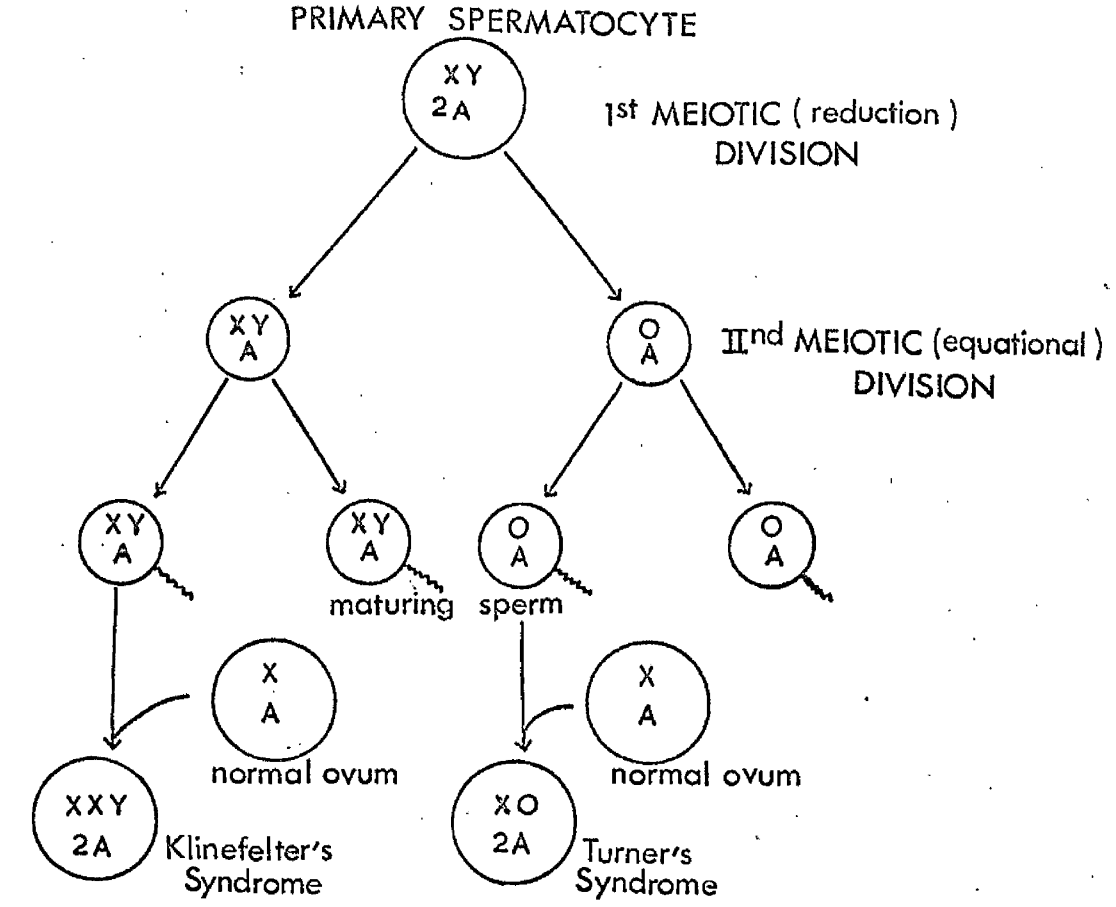
Non-Disjunction of the Sex Chromosomes.

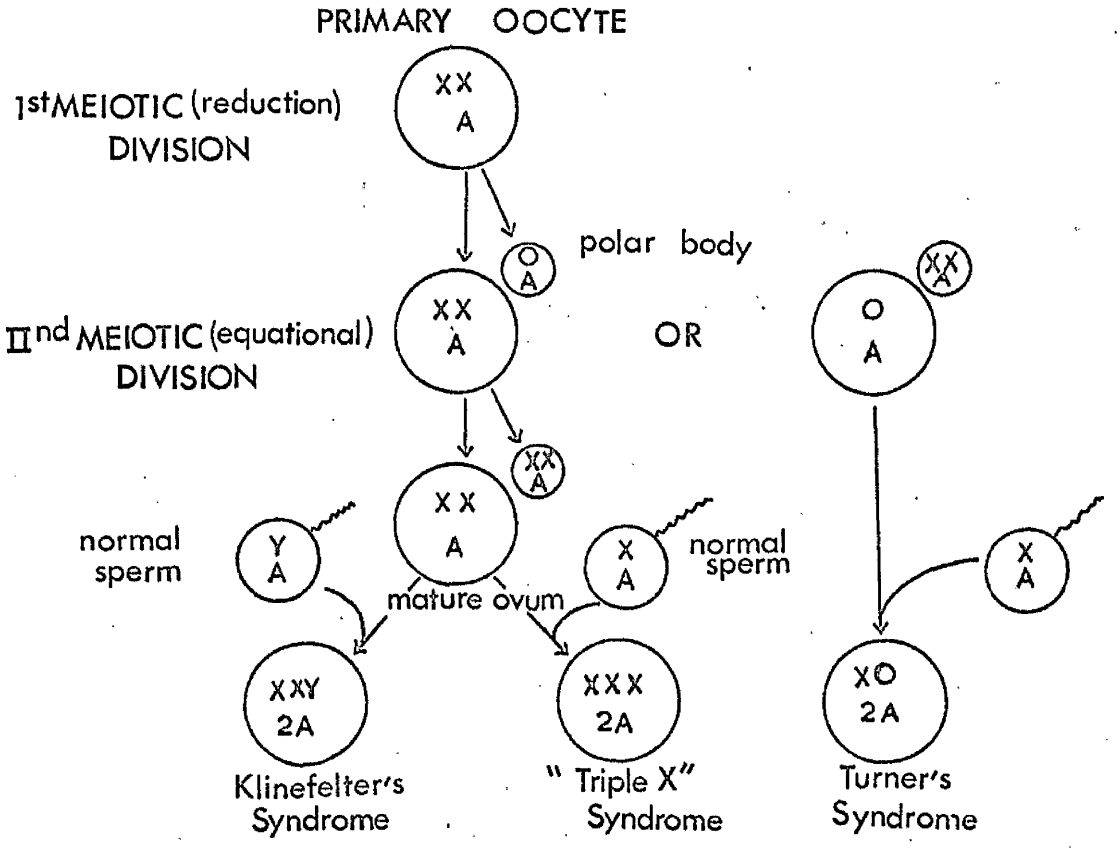
Although morphologically abnormal chromosomes have been reported on many occasions, in the majority of cases of the diseases described, the chromosome aberration is one of number. Either hyperdiploidy as in Klinefelter's Syndrome, triple X and ~~familial~~ regular mongolism, or hypodiploidy as in Turner's Syndrome. Changes involving number are collectively termed aneuploidy. The origin of aneuploidy is not difficult to postulate, and is best described by the mechanism of non-disjunction (Bridges 1913b). Non dis-junction implies an abnormal cell division in which the newly divided chromosomes fail to go to the opposite poles of the cell spindle, but remain attached to one another and pass to the same pole, so that on the/

the two newly formed cells one has one less chromosome and the other has an extra chromosome. It is possible that non-disjunction could occur at one of two places (Ferguson-Smith, 1961; Lennox, 1960), either at the first cell division of the zygote in a female cell and the result being XXX and XO cells, one of these could be eliminated and trisomy X or Turner's Syndrome result. On the other hand, non-disjunction during gametogenesis offers a simple explanation of many of the numerical chromosome abnormalities.

The results of fertilisation of these abnormal gametes by normal gametes is clearly summarised in Fig. 1.

Fig. I. Diagrammatic Representation of Meiotic Non-Disjunction of the Sex Chromosomes (After Ferguson-Smith).


PRIMARY SPERMATOCYTE
XY 2A
1st MEIOTIC (reduction) DIVISION
XY A
O A
IInd MEIOTIC (equational) DIVISION
XY A
XY A
O A
O A
maturing
sperm
X A
X A
normal ovum
normal ovum
XXY 2A
Klinefelter's Syndrome
XO 2A
Turner's Syndrome


PRIMARY OOCYTE
1stMEIOTIC (reduction) DIVISION
XX A
O A
polar body
IInd MEIOTIC (equational) DIVISION
XX A
OR
O A
XX A
XX A
normal sperm
Y A
mature ovum
X A
normal sperm
X A
XXY 2A
XXX 2A
XO 2A
Klinefelter's Syndrome
" Triple X" Syndrome
Turner's Syndrome

Chromosome Studies on Abortuses and Stillborn Infants.

The recent association of chromosome abnormality with some cases of abortion in humans should raise considerable interest with veterinarians, who are showing increasing interest both in embryonic death (Boyd 1965) and unexplained causes of abortion and apparently irreducible level of infertility in livestock (Bishop 1964).

Schmid (1962), showed an abnormal karyotype in one parent of a couple who had had two unsuccessful pregnancies due to abortion. Clendenin and Benirschke (1963), noted three cases out of ten abortions studied to have abnormal chromosome pictures. Thiede and Salm (1964), reported almost 60% abnormal karyotypes among spontaneously aborted foetuses, including one XO karyotype and two cases of tetraploidy of the X chromosome. The main work in this field however has been carried out by Carr (1961, 1963 and 1965). The last paper by Carr emphasised the high incidence of chromosome abnormalities in spontaneous abortions in man. In this study he showed that in an analysis of 200 successful cultures of tissues from previable spontaneous abortions 44 had chromosome abnormalities. These 44 cases included 11 specimens in which a sex chromosome was missing, the chromosome anomoly being the/

the same as that found in subjects with Turner's syndrome. A second group included specimens with three instead of 2 chromosome sets, resulting in a chromosome count of 69 instead of the normal 46, this latter anomoly being known as triploidy. In the third group of 22 cases, cultured cells showed the presence of one extra chromosome (trisomy). Seven of these had an extra chromosome in group E., six cases in group D., five cases in group G., two cases with an extra element resembling members of group C., and finally two others with an extra group B., chromosome.

From this work Carr emphasised that chromosome abnormalities in aborted foetuses are frequently associated with structural abnormality of the foetus and that the average period of gestation of such foetuses was shorter than chromosomally normal abortions.

Chromosome Studies Associated with Autosomal Aberrations.

Apart from many cases of regular mongolism and translocation mongolism, other types of mongoloid cases are described involving trisomy of other chromosome groups (Edwards, Harnden, Cameron, Cross/

Crosse and Wolff, 1960; Hecht, Bryant, Motulsky and Giblett, 1963b), and translocation of a member of the group D chromosomes (Jacobsen, Dupont and Mikkelsen 1963; Hamerton, Giannelli and Carter 1963). Isochromosome formation has also been reported in an autosome of the D group in man (Therman, Patau, De Mars, Smith and Inhorn 1963). An example of chromosome deletion in an autosome has now been described on several occasions in man as seen in the "cri du chat" syndrome, in which the short arm of a B group chromosome is missing (Lejeune, Lafourcade, Berger, Vialatte, Boeswillwald, Seringe, and Turpin 1963; Patau, Therman and Inhorn 1964). Many other individual cases of phenotypic abnormality in man associated with chromosomal disease are listed by Yunis (1965).

Many workers in medical cytogenetics have directed their attention to the study of chromosomal change associated with malignancy. The finding of the Philadelphia chromosome and its association with chronic myelogenous leukamia by Nowell and Hungerford (1960) has been responsible for directing much attention to this field. The Philadelphia chromosome and its/

its association with chronic leukaemia has subsequently been shown by many workers (Sandberg, Ishihara, Crosswhite and Hauschka, 1962b; Tough, Court-Brown, Baikie, Buckton, Harnden, Jacobs and Williams 1962). In acute leukaemia and malignant tumours as well, a variety of chromosome changes have been reported (Sandberg, Ishihara, Crosswhite and Hauschka, 1962a), and different cell lines are frequently observed (Lubs and Clark 1963). Studies on these changes may lead to a better understanding on their significance in malignant diseases. The effect of ionizing radiations on the chromosome picture in man has received a good deal of study, the interest being not only effect of atmospheric radiation but also in the effect of radioactive drugs (Boyd 1965) and the effect of exposure to X Ray therapy (Tough, Buckton, Baikie and Court-Brown, 1960).

Veterinary Cytogenetics.

The application of current cytogenetical techniques in veterinary medicine is at a very early stage. The chromosome number of each of our domestic animals is known (Gimenez Martin and Fernandez, Lopez Saez 1962; and Pakes and Griesemer 1965), and culture techniques have been applied to producing/

producing karyotypes and some preliminary studies in the following species. Cat, (Chu, Thuline and Norby, 1964), cattle, sheep and goat (Basrur and Gilman, 1964; Biggers and McFeely, 1963; McFee, Banner and Murphree, 1965). Pig (Antonio and Torlone, 1964). Dog (Gustavsson, 1964; Fraccaro, Gustavsson, Maj. Hulten, Lindsten, Mannini and Tiepolo, 1964) and horse (Benirschke, Brownhill and Beath, 1962; and Trujillo, Stenius, Christian and Ohno, 1962).

Very few abnormalities associated with chromosome pleomorphism, particularly sex chromosome changes, have been discovered in domestic animals. As mentioned previously, some work has been done on the bovine freemartin, which will be discussed more completely under section 4 (Goodfellow et al., 1965, Ohno et al., 1963 and Kanagawa, Muromoto, Kawata and Ishkawa, 1965).

An infertile bull carrying a percentage of cells with a suspected additional autosome was reported by Basrur, Gilman Coulbrough (1963), and a translocation between two autosomes has been reported in a subfertile boar (Henricson and Bachstrom, 1964).

Two/

Two recent publications, one by Nes (1966), reported a case of testicular pseudohermaphroditism in three out of eight of the offspring of the N.R.F. breed of cattle. These had female external genitalia, but a male XY karyotype from skin cultures. The other was the case of McFeeley (1966) who showed that in chromosome studies of eight pig blastocysts, seven had the normal diploid chromosome number, while the eighth had cells which were predominantly triploid. He reported a further case in a Yorkshire gilt, in which the chromosomes of one blastocyst showed a deletion in one of the chromosomes in group D in nine out of eleven cells studied. There are several reports on chromosome studies in infertile cats (Thuline and Norby (1961). Thuline and Norby (1963), reported the first case of hermaphroditism in a cat with both male XY and female XX cell lines, while Chu, Thuline and Norby (1964) discuss a case of triploid-diploid chimerism in a tortoiseshell cat. Some of the cells examined in this case had a 2A-XXY complement likening it to Klinefelter's Syndrome in man. The classical case of the horse-donkey hybrid, the mule, has been extensively reported. The mule is a sterile hybrid

and/

and chromosome studies have confirmed it to have an intermediate diploid chromosome number, 63, the horse (equus cabalus) 64 and the donkey 62 (equus asinus), (Trujillo, Stenius, Christian and Ohno 1962.)

Cytogenetical studies have been undertaken on two neoplastic diseases of domestic animals, Bovine lymphosarcoma (Basrur, Gilman and McSherry, 1964; Hare, McFeely, Abt and Frierman 1964), and Japanese workers have reported that the karyotypes of neoplastic cells of dogs with transmissible venereal tumour differ markedly from the normal dog karyotype,(Makino, Sofuni and Takayama, 1962).

In 1964 very little reseach had been done on either sex chromatin or the mitotic chromosomes of the sheep and although several publications have appeared during the period of this study, they are very limited in extent. The main object of this thesis was to examine the use of some current cytogenetical techniques including sex chromatin, leucocyte culture method and chromosome identification in the sheep, and to apply these in an examination of some sexually abnormal sheep.

PART I.

SEX CHROMATIN IN THE SHEEP.

PART I.

SEX CHROMATIN IN THE SHEEP.

In view of the proven value of sex chromatin studies in medical genetics, a brief survey of the properties of sex chromatin, its relationship to the X chromosome and its position in the sheep and other members of the same taxon is important.

General Properties.

The sex chromatin body is small and well defined, staining intensely with nuclear dyes such as cresyl echt violet (Moore and Barr, 1955), thionin (Klinger and Ludwig, 1957) and haemotoxylin (Barr, 1955) The fact that it is stained by the Feulgen technique following acid hydrolysis and has an affinity for methyl green rather than pyronin (Lindsay and Barr, 1955) indicates that it composed of chromosomal material, namely D.N.A.. Sex chromatin can be seen in living cells also, by use of phase microscopy (Miles, 1960).

The size of the chromatin body in man has been estimated at between 0.7μ - 1.2μ (Moore and Barr, 1954 and 1955) and is usually described as having several different shapes depending on its relationship/

relationship to the nucleur membrane, or its position in the nucleus.

Sex chromatin has not been reported in zygotes, but it has been reported in cells of a late cat blastocyst (14 days gestation) (Austin and Amoroso, 1957), and human trophoblasts at about the twelfth day (Park, 1957). The behaviour of the sex chromatin body is also different in germ cells. While it is normally present in the female somatic cell, in the oocyte both X chromosomes are isopycnotic (negative heteropycnosis), while in the spermatocyte the XY bivalent is heteropycnotic (Ohno and Makino, 1961; Ohno, Makino, Kaplan and Kinosita, 1961).

Sex Chromatin and the X Chromosome.

Chromosome studies in man have shown that there is a close association between the number of X chromosomes found in patients and the number of sex chromatin bodies seen in the nuclei of non-dividing cells. As well as a number relationship there is also a size relationship (Jacobs, Harnden, Court Brown, Goldstein, Close, MacGregor, MacLean and Strong, 1960). These authors reported finding smaller/

smaller than normal sex chromatin bodies in patients who also had deleted X chromosomes as seen in karyotype preparations. Similarly larger than normal X chromosomes have been found associated with large sex chromatin bodies (Fraccaro, Ikkos, Lindsten, Luft and Kaijser, 1960).

The association between sex chromatin bodies and the X chromosome is further strengthened by autoradiographic work using tritiated thymidine. These have demonstrated that the sex chromatin body corresponds to a late replicating X chromosome (Grumbach and Morishima, 1962; Morishima, Grumbach and Taylor, 1962; German, 1962) and in patients with three or more X chromosomes there are two or more late labelling X chromosomes (Grumbach, Morishima and Taylor, 1962). The abnormally large X chromosome found in some patients is also late labelling (Muldal, Gilbert, Lajtha, Lindsten, Rowley and Fraccaro, 1963).

These findings confirm the idea first formulated by Ohno, Kaplan and Kinosita (1959), that the sex chromatin body is a single X chromosome and that it is genetically inactive and superfluous as suggested by Stewart (1960).

Lyon (1961, 1962 and 1963), however, proposed that while genes on the X chromosome are inactive the inactivation occurs at random. She further suggested that the decision as to which X chromosome is to be inactivated occurs in early embryonic life, so that in some cells of the body the inactive X chromosome is from the male parent while in others it is from the female parent. Lyon's hypothesis is of great importance and while her first observations were made on piebald female mice which were heterozygous for sex-linked genes, it provides an explanation for dosage compensation as described by Müller (1932). A good example of dosage compensation is shown in the gene controlling glucose-6-phosphate dehydrogenase in the red cells of man. Glucose-6-phosphate dehydrogenase occurs in equal amounts in both men and women (Marks, 1958), and yet the gene controlling its production is located on the X chromosome, so that obviously only one of the X chromosomes must be fully active in women. A further example of dosage compensation was demonstrated in the XO female mouse which is both viable and fertile (Welshons and Russell 1959).

By comparison with what is known of sex chromatin/

chromatin behaviour in man, information on the sheep and related species is scarce.

Review of Literature on Sex Chromatin in the Sheep and Related Animals.(Artiodactyla).

Sex chromatin was first shown in nerve cells of the female goat and deer, members of the cloven-hoofed group of animals by Moore and Barr, (1953). Further information of another member of the same group was produced when Moore, Graham and Barr (1957) showed the nuclear sex of the bovine free-martin as judged from nerve cells, was the same as that of a female calf, while in the bull calf no sex chromatin body was seen. These authors also suggested from this study that sex chromatin was not affected by male hormonal influence. These findings of a sex difference in nerve tissue between male and female cattle was in agreement with Osuchowska and Siminski (1957) who, while not being able to distinguish sex chromatin in female calf liver cells were able to show a sex difference in the oral epithelium of male and female cattle. They reported 68.5% of epithelial cells with an intensely hetero pycnotic grain in the nuclear membrane of cows and in only 21% of cells of the bull.

This/

This latter finding was contrary to the observations of Struck (1961) who in a detailed study of the sex chromatin of buccal smears of several animals, concluded that in cattle, sheep and goats, the nuclei of both sexes contain several coarse chromatin granules, which showed no differences between sexes.

Lang and Hansel (1959) demonstrated sexual dimorphism in three tissues of cattle. They described a mildly chromotropic planoconvex mass found predominantly in females as being sex chromatin. The tissues studied were liver, pancreas and adrenal, and the sex chromatin appeared in 68%, 62% and 61% of these female cells respectively and less than 10% of male cells.

This difference, however, was only demonstrable following acid hydrolysis with 7N. HCl and staining with thionin. Nineteen out of twenty unidentified tissues were classified correctly according to sex.

Moller and Neimann-Sorensen (1957) studied 106 preparations of bovine amniotic allantoidic fluid and 28 whole mounts of embryonic membrane using cresyl echt violet as a stain. On 65 fluid sample preparations which were suitable for diagnosis, seven were incorrect but on the twenty-eight whole mount preparations the sex was diagnosed correctly in each

each case. A distinctive chromocentre described as sex chromatin, was seen in 52%-68% of female cells and only 8%-20% of male cells. These workers concluded that it is unrealistic to talk about prenatal sex determination by this method because of the inherent damage possible to the foetus but agreed with Fuchs and Riss (1956) that antenatal sex determination if possible, might be of practical value in cattle breeding and veterinary practice. Sachs Serr and Danon (1956) showed that a correct prenatal diagnosis of sex can be made in human amniotic cells. Two of these workers, Sachs and Danon (1956), decided that a prenatal sex diagnosis could not be made in cattle and sheep by the determination of the percentage of amniotic fluid cells with a chromocentre at the nuclear membrane. This decision was based of the examination of epithelial tissue of male and female cattle placentae, and from 6 week, 8 week and 16 week old male foetuses and from a 12 week old female foetus. The cells in these specimens had several chromocentres and no clear sex difference as in humans. Similar examinations in the sheep showed no difference between the sexes.

Colombo/

Colombo and Tosi (1958) in a study on bovine foetal membrane cells concluded that it is not possible to diagnose the sex of the foetus from the deposit of amniotic or allantoic fluids, because of the scarcity of cells, but it is possible to determine cytological sex on the chorionic membrane cells. Using Feulgen stain with fast green counterstain and cresyl violet, these authors claimed in 32 specimens examined that they diagnosed sex correctly in 28 cases.

Hoshino and Toryu (1958) were able to establish a clear sex difference in nerve cells taken from various regions of the nervous systems of cats, pigs, goats, cattle and horses. This chromatin mass occurring in 80% of female cells was Feulgen positive and no comparable mass in size or shape was seen in the male nuclei. The sex chromatin in nerve tissue was not divided into three types as was done by Moore and Barr (1953), adjacent to nucleolus, adjacent to nuclear membrane and free in the nucleoplasm. They were not able, however, to distinguish a sex difference in any other tissue in cattle and only in the epithelial cells and smooth muscle cells of the duodenum in the horse.

The/

The findings in cattle of Hoshino and Toryu (1958) were in agreement with Moore and Aiyede (1958), that bovine nerve cells show sex dimorphism but sex chromatin was not recognisable in other tissues. These authors also concluded that neuronal nuclei of the pig are different in male and female, but in other cells multiple chromatin masses obscure any sex difference. This is again confirmed by the findings of Hay and Moore (1961) in the pig. Cantwell, Johnston and Zeller (1958), who also worked on the pig, showed that it was possible to determine genetic sex of males and female swine intersexes using the Feulgen staining method, on nerve tissue only. Non-nerve tissue did not reveal the distinguishing sex chromatin, due to the presence of other coarse chromatin masses. All the intersexes examined were genetic females. Non-nerve tissues examined included liver, heart, kidney, thyroid, pancreas and adrenal.

Schmidtke (1957) examined squash preparations of grey matter brain cells from cattle, pig, sheep, horse, hen, rat, mouse and golden hamster. In the cattle, sheep and pig, this author described nuclei of several types within sexes, but clearly described a/

a sex difference between male and female, young and old, for the three species.

In the sheep, Schmidtke (1957) commented on the number of nucleoli seen in resting nuclei, particularly in young sheep. This number was found to vary from one to four and tended to decrease with maturity.

Although Schmidtke (1957) showed that there is a discernible sex difference in nerve cells of the ewe and the ram, his description of these was not as clear as that of Moore and Barr (1953) for the goat and deer. Further, since only one area of nerve tissue was examined, it was considered that the position of sex chromatin in the nerve tissue of sheep should be re-examined.

In this study, no attempt was made to pursue in the sheep the findings of Davidson and Smith (1954), of the accessory drumstick on a percentage of polymorphonuclear blood cells of women. The reason for this was that an extensive study by Colby and Colhoun (1963) showed that of the female domestic animals studied, including cow, goat, sheep, pig, horse and dog, the frequency of this accessory nuclear lobule was lowest in the sheep.

An/

An average of 73±9.63 cells had to be counted to obtain a single cell with an appendage. Such a test was concluded by these authors to be of dubious value as a method of sex diagnosis in the sheep.

THE EXAMINATION OF NERVE TISSUE IN THE SHEEP FOR SEX CHROMATIN.

Procedure.

The object of this study on sex chromatin in the sheep was to examine its position in both adult and juvenile nerve tissue of both sexes. A further study was made on the multiple chromocentres reported in amnion cells (Sachs and Danon, 1956) and buccal cells (Struck, 1961), to see if in fact a sex difference did occur and to what extent these tissues could be used as an adjunct to sex chromosome studies. The animals used in these examinations included four Scottish Blackface sheep, two ewes and two rams; also three grey face lambs, one male and two female.

The above animals were destroyed by intravenous injection of 15 m l pentobarbitone (3 gr. per m l)* and nerve tissue form the following regions was collected within a few minutes of destruction/

* Euthetal (May & Baker)

destruction and fixed in 10% formal saline.

(1) Cerebellum.

(2) Spinal cord from the region of the second cervical vertebra.

(3) Spinal cord from the region of the second thoracic vertebra.

(4) Spinal cord from the region of the second lumbar vertebra.

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

Fixation of tissues was for thirty-six hours, after which they were taken through the routine embedding process ready for sectioning.

This process included the following steps.

(1)	50% ethyl alcohol.	2 hours.
(2)	70% ethyl alcohol.	2 hours.
(3)	90% ethyl alcohol.	2 hours.
(4)	Absolute alcohol(3 changes)	each 2 hours.
(5)	Chloroform (3 changes)	each 2 hours.
(6)	Benzene.(Crystalisable G.P.R.)	2 hours.
(7)	Paraffin Wax. (Filtered paraffin M.P. 54.56 57°C).	4 hours
(8)	Embedded in paraffin wax.	

Sections/

Sections were then cut from each area of nerve tissue at 10μ thickness on a hand operated rotary microtome.

Cut sections were stained with cresyl echt violet (Coleman and Bell).

As a stain for sex chromatin, cresyl echt violet has proved very popular and its use is described by a large number of workers including Graham and Barr (1952), Moore and Barr (1953) and Klinger and Ludwig (1957).

It was found, however, that staining sections of male and female tissues mounted together, as used by Moore and Barr (1953), was not practical as the time required in cresyl echt violet was very short. Sections so treated in this study were invariably too deeply stained in one or other of the sexes. The sections of each sex were therefore stained separately to give better control on the degree of staining. The following modification of the procedure recommended by Moore and Barr (1955), for oral mucosal cells was found satisfactory.

(1) Xylol.

(2) Absolute alcohol.

(3) /

(3) 70% ethyl alcohol.

(4) 50% ethyl alcohol.

(5) Distilled water, 2 washes.

(6) Cresyl echt violet (Coleman and Bell) 2% 1 - 2 minutes.

(7) 95% ethyl alcohol, 4 minutes.

(8) 95% ethyl alcohol, dip.

(9) Absolute ethyl alcohol, dip.

(10) Xylol, 2 changes, 10 minutes each.

(11) Mounted in D.P.X.

All microscopic examinations were made using a Wild M20 microscope. Cells were studied closely, using a X100 oil immersion objective and X10 eye pieces. For each of the seven animals, as many cells as possible were drawn from each of the five different levels of nerve tissue, scoring a maximum of 250 cells from cerebellum and 108 from spinal cord sections. It was not always possible to record the same number of cells as in some spinal cord sections, only very small numbers of suitable cells were present.

Scoring was done only on cells in which a clear nucleolus could be seen. The verner position for each cell drawn was recorded and sex chromatin body, if/

if present, was scored as being in one of three places, viz. at the nucleolus, free in the nucleoplasm or at the nuclear membrane.

Results.

The results of these examinations are shown in Table 1, which shows the relative numbers and percentages of cells showing sex chromatin in four female animals. Figs. 2 to 6, with explanations indicate the nature of cells and various positions of the sex chromatin body as scored in the adult ewe and ewe lamb. Figs 7 and 8, show the nerve cells of the ram and ram lamb in which no sex chromatin body was seen.

Discussion of Results.

As seen in Table 1 the average percentage of cells in the cerebellum showing sex chromatin, of three ewes examined was 91.72% from 640 cells. This figure is in close agreement with the results of Moore and Barr (1953) who reported 94.8% of cells of the female goat and 92.0% of cells for the female deer, as having sex chromatin. It is also similar to the 85.5% of cells reported as showing sex chromatin in the cerebral hemisphere sections of the sheep, (Schmidtke 1957). None of the 1,363 nerve cells/

cells examined in the three male animals showed a chromatin body comparable in size to the sex chromatin body seen in ewes.

A similar result was obtained from the spinal cord cells of the four ewes, 85.13% of cells from the four regions studied, showing clear sex chromatin bodies in one of the three positions recorded. This again was in close agreement with the results of Moore and Barr (1953) for the goat and deer. The percentages of cells for their study were 91.8% for the goat and 82.0% for the deer.

As seen also from Table 1 there was no significant difference between the evidence of sex chromatin in lambs and in adult sheep. A feature recorded in lambs however, was the multiple nucleoli seen in nerve cells, both of the spinal cord and cerebellum (Fig. 6). This was as observed by Schmidtke, but in no way affected the scoring of sex chromatin.

It can, therefore, be concluded that Purkinje cells from the cerebellum and ventral horn cells of the spinal cord are ideally suited for scoring sex chromatin bodies in the sheep. Further, the

percentage/

TABLE 1.

SEX CHROMATIN IN NERVE CELLS OF 2 EWES and 2 EWE LAMBS from 2344 CELLS

	Nucleolus	Nucleoplasm	Periphery	None	Total	% Nucleolus	% Nucleoplasm	% Peripheral	% Total
				CERE BELLUM					
EWE I	186	41	36	19	282	65.95	14.54	12.77	93.26
EWE II			Not Satisfactory						
EWE LAMB I	56	3	37	10	106	52.83	2.83	34.90	90.56
EWE LAMB II	77	28	123	24	252	30.55	11.11	48.80	90.46
TOTAL	319	72	196	53	640	Mean % of cells with sex chromatin			91.72
				CERVICAL SPINAL CORD					
EWE I	78	21	3	13	115	67.82	18.26	2.60	88.68
EWE II	71	6	5	7	89	79.77	6.74	5.55	92.06
EWE LAMB I	8	3	18	7	36	22.22	8.33	50.00	80.55
EWE LAMB II	53	22	17	15	107	49.53	20.56	15.88	85.97
TOTAL	210	52	43	42	347	Mean % of cells with sex chromatin			87.90
				THORACIC SPINAL CORD					
EWE I	55	22	6	20	103	53.40	21.36	5.82	80;58
EWE II	91	2	4	13	110	82.72	1.81	3.64	88.17
EWE LAMB I	16	4	7	9	36	44.44	11.11	19.44	74.99
EWE LAMB II	47	17	24	18	106	44.34	16.04	22.64	83.02
TOTAL	209	45	41	60	355	Mean % of cells with sex chromatin			83.10

LUMBER SPINAL CORD

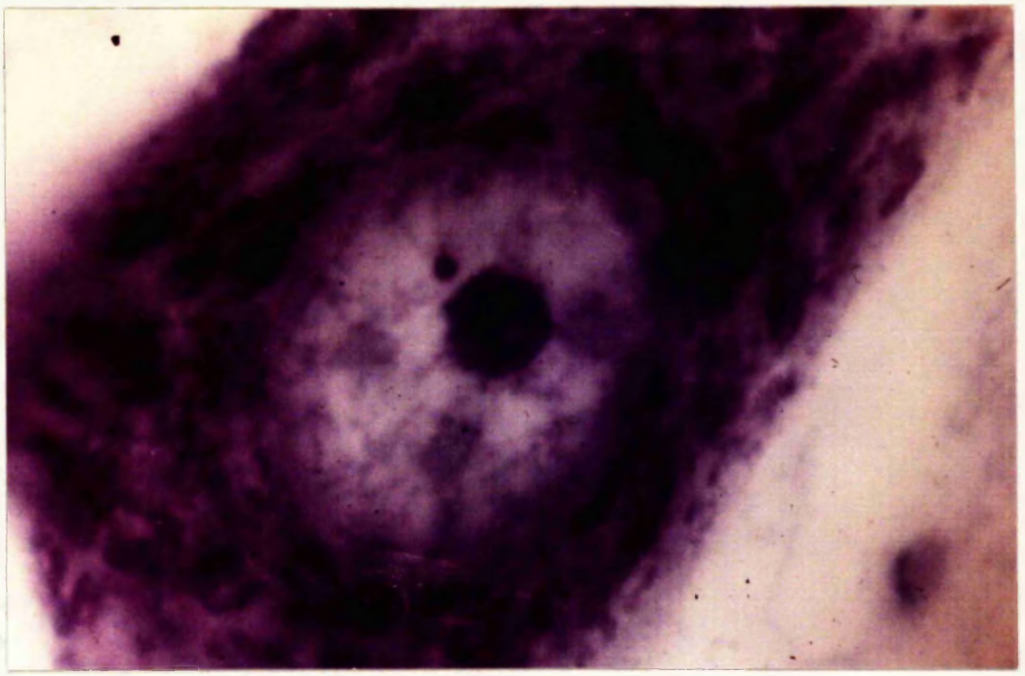
EWE I	87	5	6	7	105	82.85	4.76	5.71	93.32
EWE II	62	16	1	2	81	76.54	19.75	1.23	97.55
EWE LAMB I	32	21	36	19	108	29.63	19.44	33.33	82.40
EWE LAMB II	44	12	33	18	107	41.12	11.22	30.84	83.18
TOTAL	225	54	76	46	401	Mean % of cells with sex chromatin			88.53

SACRAL SPINAL CORD

EWE I	27	45	2	24	98	27.55	45.92	2.04	75.51
EWE II	80	4	4	20	108	74.10	3.70	3.70	81.50
EWE LAMB I	76	6	15	10	107	71.03	5.61	14.02	90.66
EWE LAMB II			Not Satisfactory						
TOTAL	183	55	21	54	313	Mean % of cells with sex chromatin			82.75

Fig. 2. Adult Female Nerve Cell (Spinal Cord), showing Sex Chromatin Body Free in Nucleoplasm (X 1000).

Fig. 3. Adult Female Nerve Cell (Spinal Cord), showing Sex Chromatin Body at Peripheral Position (X 1000).



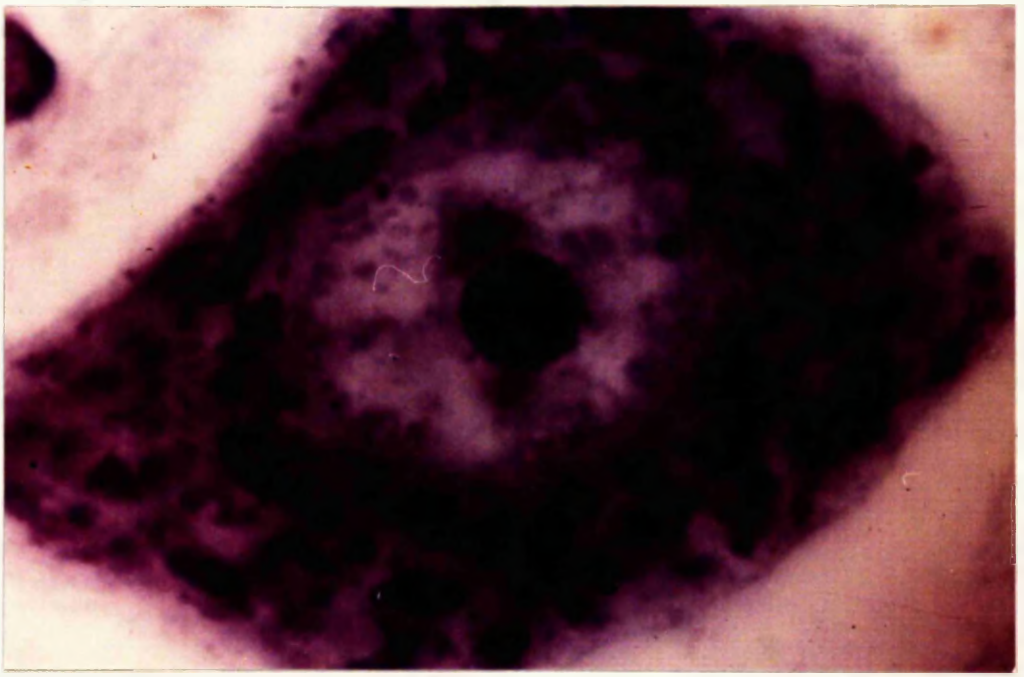


Fig. 4 Adult Female Nerve Cell (Spinal Cord) Showing Sex Chromatin Body in Contact with Nucleolus (X 1000).

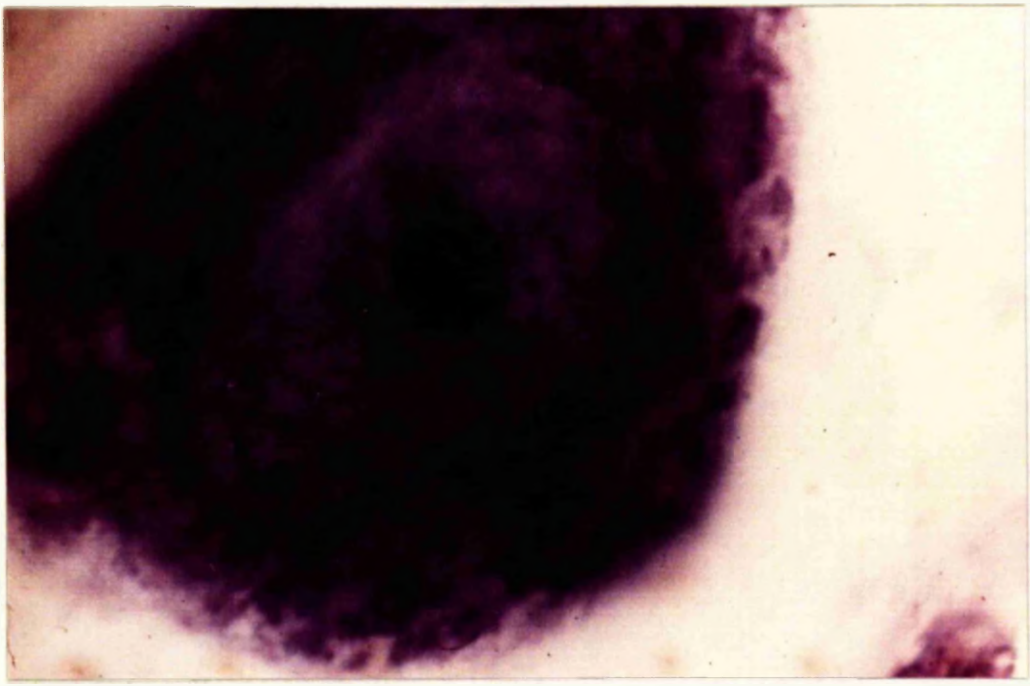
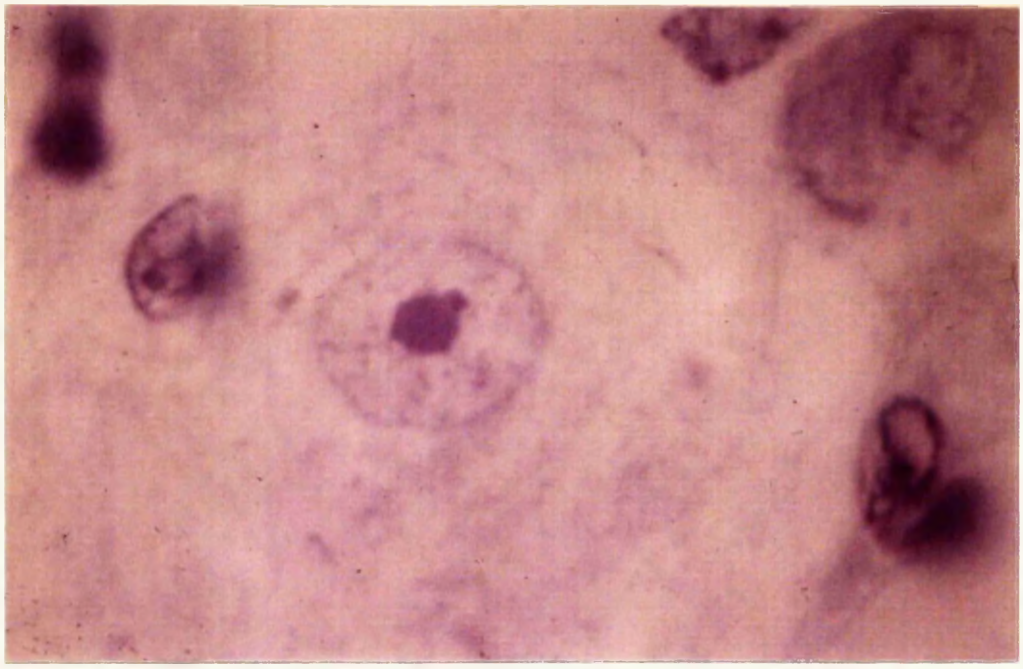


Fig. 5. Female Nerve Cell (Purkinje Cell) (X 800) Sex Chromatin Body in Contact with Nucleolus.

Fig. 6. Female Nerve Cell (Purkinje Cell of Lamb) (X 800) Double Nucleous and Sex Chromatin Body at Pepiphery of the Nucleus.



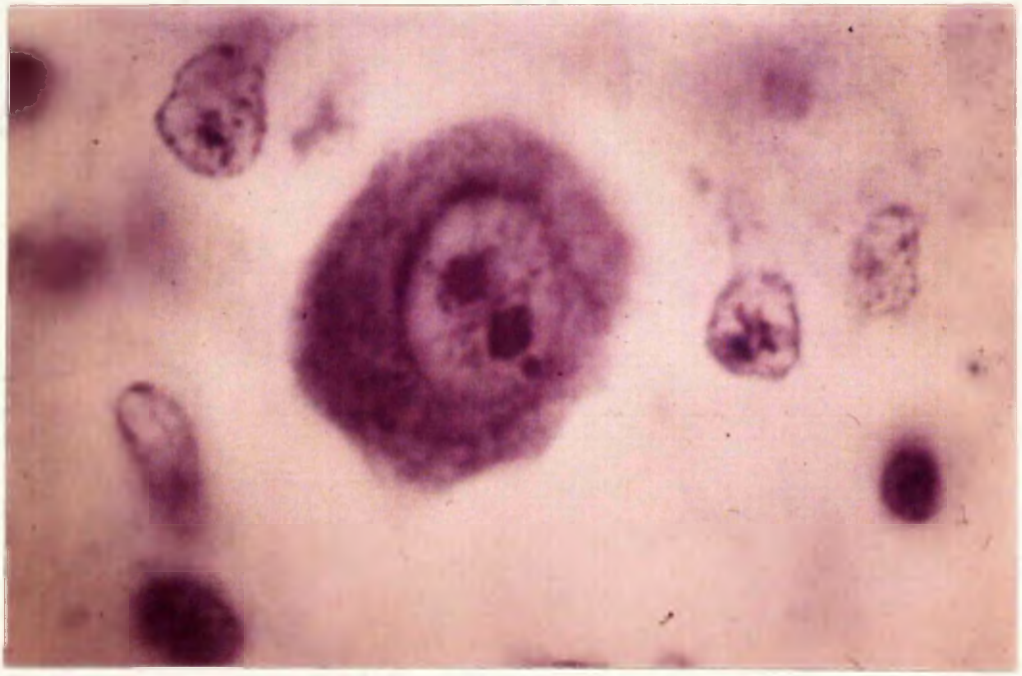
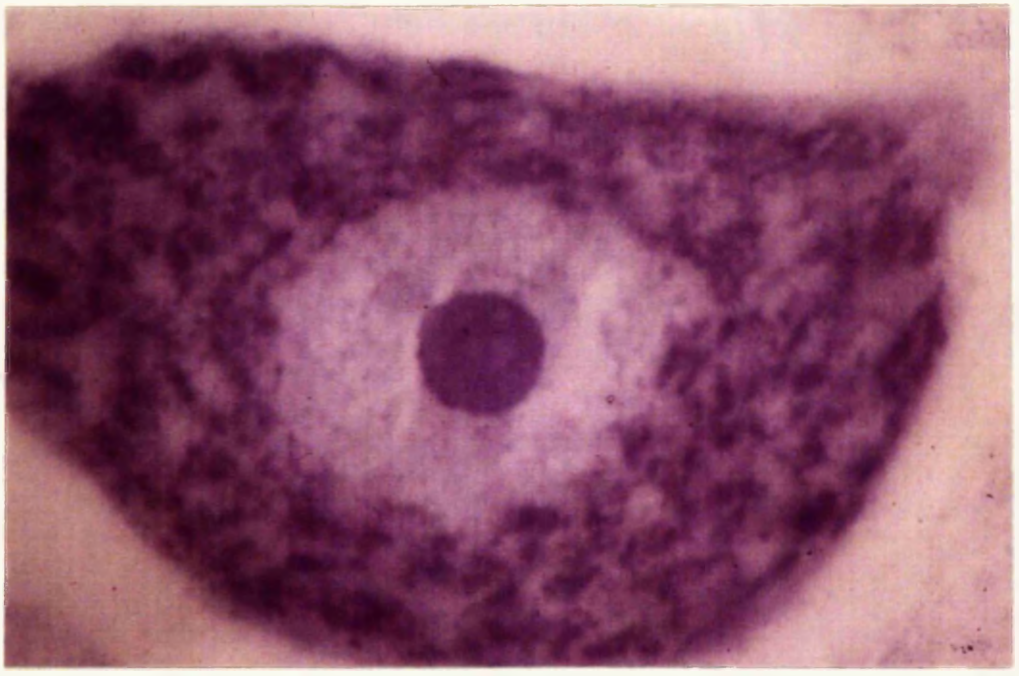
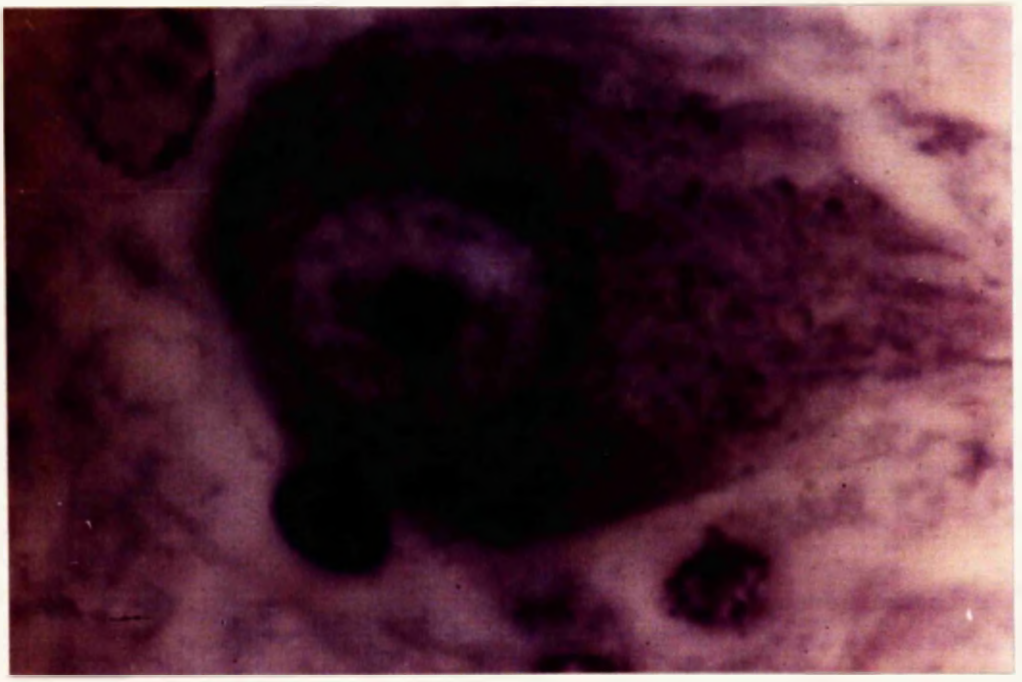


Fig. 7. Adult Male Nerve Cell (Spinal Cord)
No Sex Chromatin Body (X 1000).

Fig. 8. Adult Male Nerve Cell (Purkinje Cell)
No Sex Chromatin Body (X 1000).





percentage of cells in these positions showing sex chromatin in the four ewes studied, is in close agreement with those recorded in other members of the order artiodactyla.

As has been discussed already, it is extremely difficult to differentiate sex chromatin in other than nerve cells nuclei of the cloven hoofed animals. Two other tissues which were likely to be of value for the study of sex chromatin in the sheep however were considered. Amnion tissue in view of the possibility of antenatal sex determination and buccal cells because of their superficial nature and possible use as an adjunct to chromosome studies.

THE EXAMINATION OF AMNION CELLS FOR SEX CHROMATIN.

For the investigation of amnion cells two methods were considered, firstly the examination of free amnion cells from the amniotic fluid, and secondly cells of the amnio-chorion.

Free Amnion Cells.

Preparations of free amnion cells were made from both male and female foetal fluids by several methods, including:

(1) Smearing the centrifuge deposit of 10 ml.

of/

of amniotic fluid, resuspended in calf serum on to slides smeared with egg albumen and subsequently dried in air.

(2) Fixing smears of the centrifuge deposit in Papanicoloau's fixative.(see appendix).

Slides prepared in the above manner were stained in Cresyl echt violet by the same procedure as nerve tissue except that the period in stain was increased to five minutes.

The results were disappointing for two reasons. Firstly, the small number of fixed cells suitable for viewing, and secondly those cells suitable for study showed multiple nuclear chromocentres in both male and female preparations. The conclusion was the same as that of Sachs and Danon (1956), namely, it is not possible to differentiate the sex of sheep foetuses by studying cells from amniotic fluid.

Amniotic Membranes.

A technique was developed involving the careful dissection of the embryonic membranes overlying the amniotic sac and foetus, down to the last membrane. Suitable preparations from these were made and cells were found showing a peripheral chromocentre in female membranes.

Figs. 9 to 11 with descriptions, show the details of this technique. Slides prepared by this method were carefully fixed in Papanicoloau's fixative in Coplin jars. Fixation was for at least twenty-four hours, but sections in fixative for as long as three weeks were equally satisfactory for staining and nuclear observation. The staining procedure followed was the same for nerve tissue, except that five minutes was given each section in cresyl echt violet. Following staining, excess membrane was trimmed from the slide edge and the section mounted in D.P.X.

Results.

It was found in the sections prepared and viewed under oil immersion at X1000, that by focussing up and down, three different layers of cells all showing a distinct nuclear morphology were present. The nuclei of the innermost layer of cells were round and invariably contained 2-3 pale staining chromocentres of which on occasion, one was situated at the periphery. Cells of this layer were the same in both sexes.

Adjacent to this layer was another layer of cells with rounded nuclei, in which a distinct peripheral/

peripheral chromocentre could be seen in female preparations in a high proportion of cases, Fig. 13. This was not seen in male preparations (Fig.12). Both male and female nuclei contained other smaller non-specific chromatin particles. The third layer of cells seen had elongated nuclei. No distinctive chromocentre could be seen in slides from either sex.

As a difference was observed between sexes in these preparations, it was decided to test preparations from a number of membranes, the identity of which was masked from the examiner to see if this sex difference was diagnostically reliable.

Over a period of several days, fresh uteri were collected from the Glasgow Slaughter House at approximately 8 a.m. each morning. These were brought directly to the laboratory and preparations were made before 1 p.m. each afternoon, in order to ensure a minimum of decomposition. Seventy-five uteri were dissected and preparations made by the method described above. These were identified for sex from the foetus, and the identity concealed by plastic tape from the examiner. Measurements of weight and length (nose to tail tip) were taken on most foetuses.

In/

Fig. 9 shows the uterine wall and chorio-allantois carefully dissected away exposing the thin amniotic layer of cells lying directly over the amniotic sac containing the foetus.

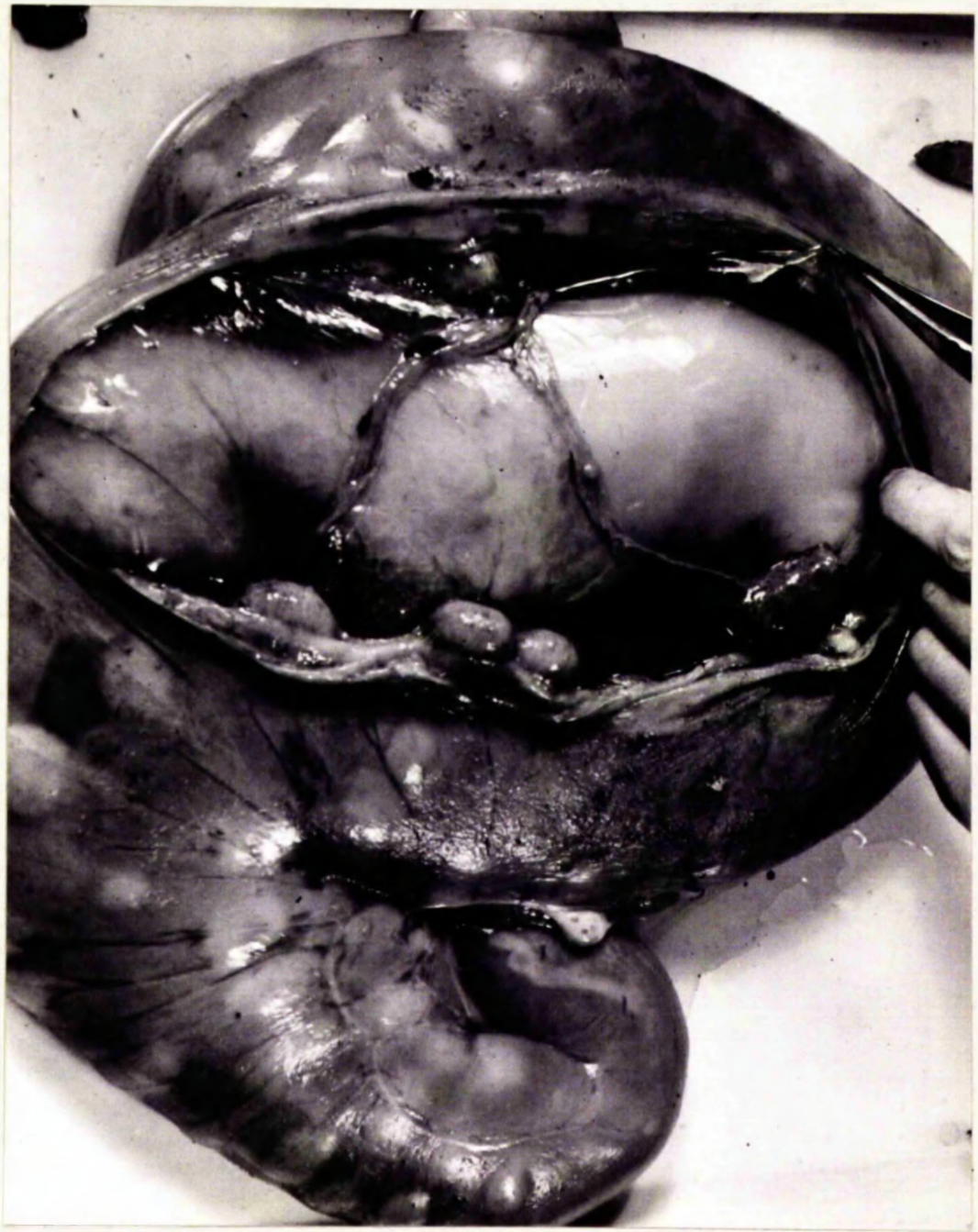


Fig. 10. shows the amniotic membrane extended and ready for stabbing prior to inserting the microscope slide.

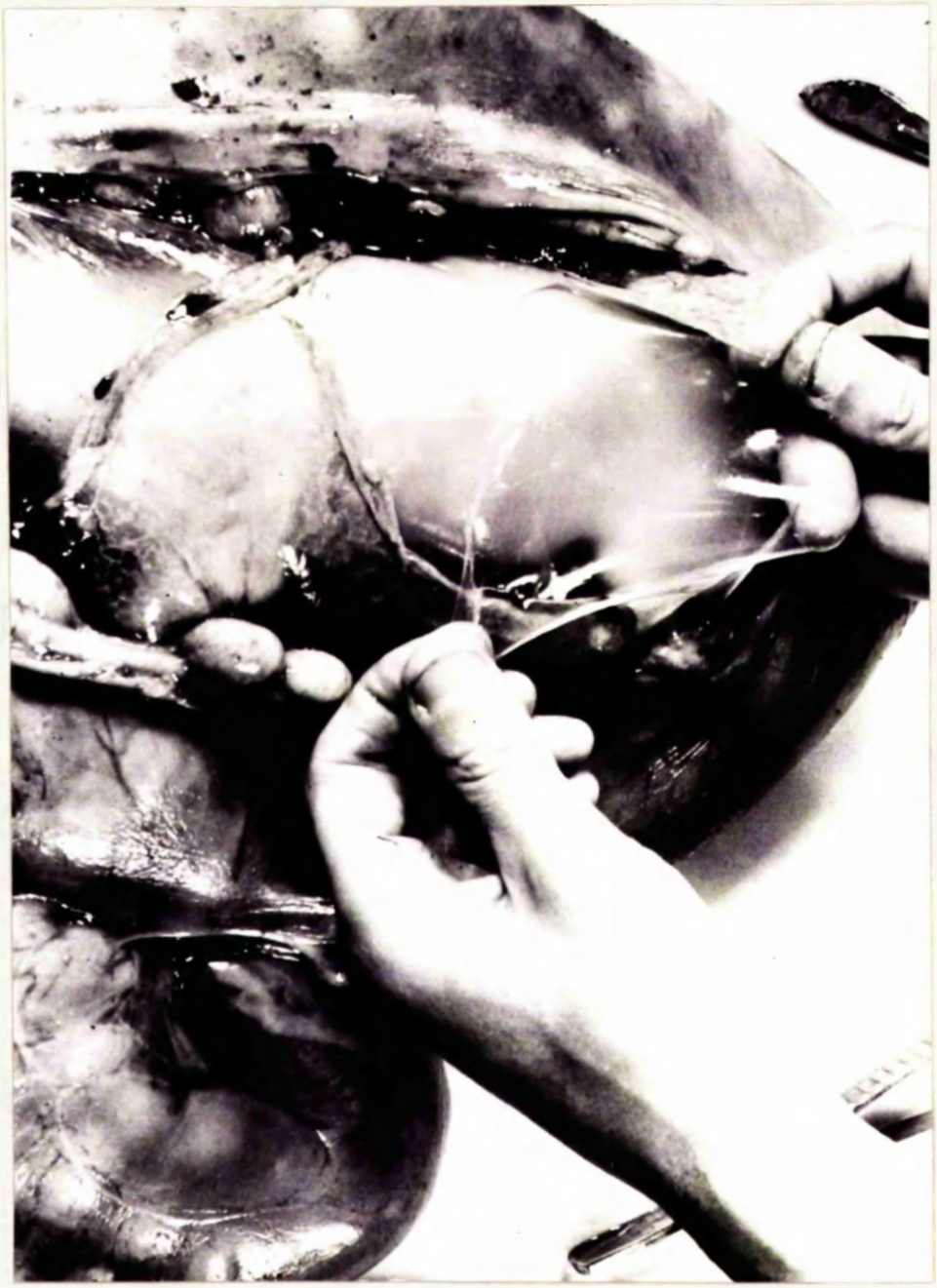


Fig. 11. shows slide pushed firmly against amnion to "stretch" the membrane over its surface prior to cutting.

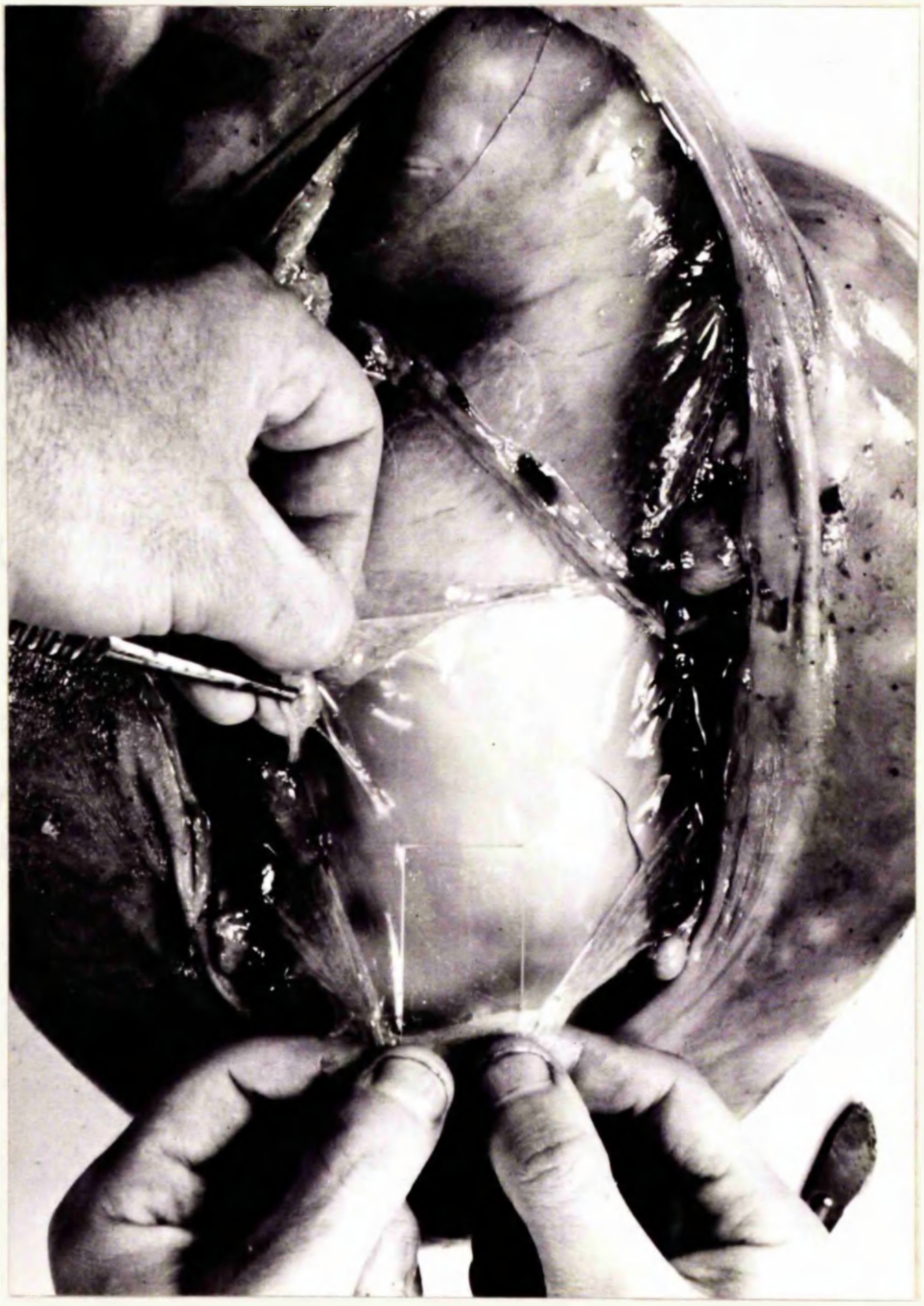
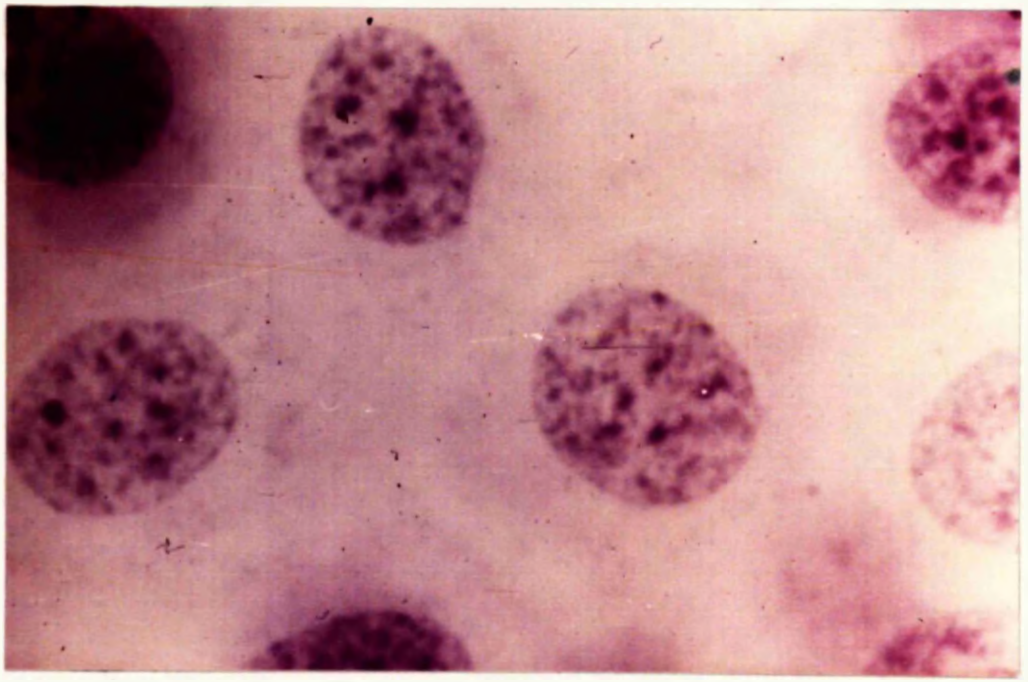
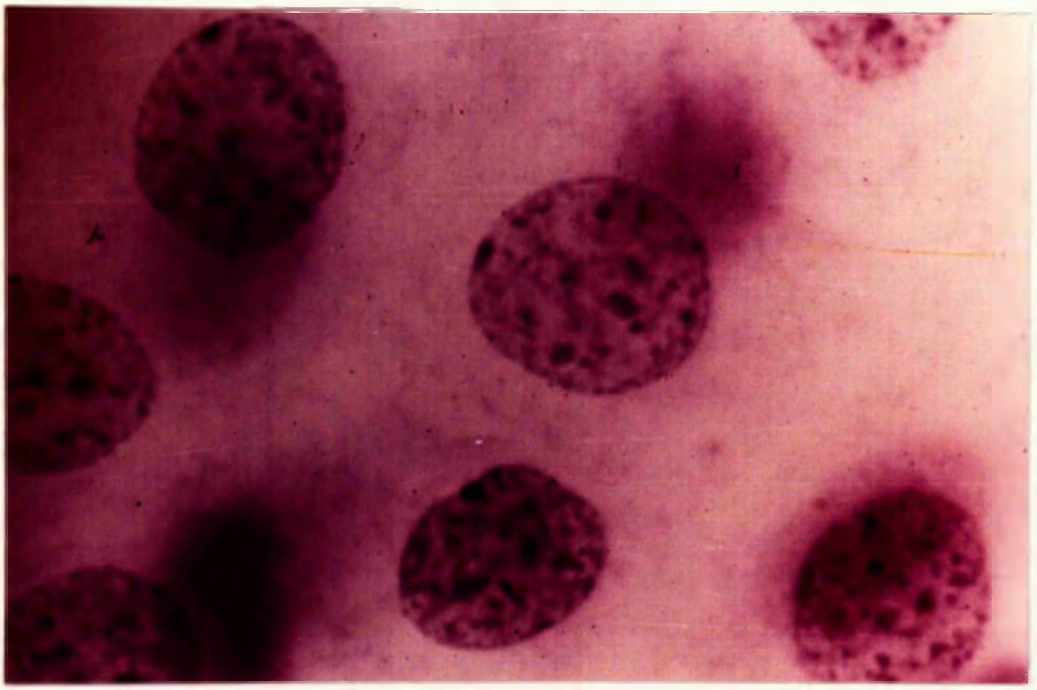


Fig. 12. Male Amnion Cells (X 1000)
No Peripheral Chromocentres
in Nuclei.

Fig. 13. Female Amnion Cells (X 1000)
Distinct Peripheral Chromocentres
in Nuclei of 4 Central Cells.





In each slide examined, approximately one hundred cells were scored either as having or not having the large peripheral chromocentre. In all a total of 5,000 cells were examined.

Observations were made on the four clearest cells nearest the centre of the microscope field. Of the seventy-five sections prepared, fifty were used in this examination. Twenty-five were of inferior quality, due to incomplete dissection of membrane layers during preparation.

The results of the trial can be seen in Table 2.

Discussion of Results.

The above results show that in suitable amnion preparations, sex dimorphism between sexes can be shown on one layer of amnio-chorion. This observation in the sheep is similar to that of Moller and Neimann-Sorenson (1956) in the bovine amnio-chorion, except that these authors did not stress the size of the peripheral chromocentre described. They also recorded a chromocentre in male cells. No chromocentre was seen in male preparations of the sheep comparable to that seen in female preparations. It can be assumed, therefore, that the chromocentre described represents the sex chromatin/

MICROSCOPIC SEX DIAGNOSIS FROM
(Scottish Blackface Ewe,

TABLE 2.

	Observed Sex.	Length of Foetus (ins.)	Weight of Foetus (g)	Incidence of Peripheral Sex Chromatin.	Microscopic Diagnosis.
1	♀	5.25	50	50	♀
4	♀	5.25	50	47	♀
6	♀	13	510	49	♀
7	♂	11.5	370	0	♂
8	♂	15	765	0	♂
12	♀	10.75	295	60	♀
13	♂	8.5	140	0	♂
14	♂	12.5	-	0	♂
17	♀	21.5	2155	52	♀
19	♀	-	-	45	♀
20	♀	-	-	47	♀
21	♂	12.5	-	0	♂
22	♂	14.5	850	0	♂
23	♂	4	20	0	♂
26	♀	-	-	55	♀
27	♀	-	-	45	♀
28	♀	-	-	52	♀
29	♂	-	-	0	♂
30	♀	-	-	45	♀
31	♂	-	-	0	♂
32	♂	12	-	0	♂
33	♂	12	-	0	♂
34	♀	10	-	55	♀
35	♀	14.5	-	61	♀
36	♂	8.5	140	0	♂

AMNION PREPARATIONS OF THE SHEEP
Cheviot a nd Suffolk Rams).

	Observed Sex.	Length of Foetus (ins.)	Weight of Foetus (g)	Incidence of Peripheral Sex Chromatin.	Microscopic Diagnosis.
37	♂	14.5	850	0	♂
38	♂	11.5	370	0	♂
39	♀	-	-	51	♀
40	♀	15.25	835	-	♀
41	♀	7.75	-	47	♀
42	♂	17.5	1310	0	♂
45	♀	8.75	170	44	♀
49	♂	11.5	370	0	♂
50	♀	10.75	295	24	♀
51	♂	14.5	850	0	♂
52	♀	21.5	2155	66	♀
54	♂	11.5	370	0	♂
56	♀	14.5	-	60	♀
57	♂	-	-	0	♂
59	♀	13	510	42	♀
61	♂	15	750	0	♂
63	♂	-	-	0	♂
64	♂	-	-	0	♂
66	♀	13	510	55	♀
67	♀	-	-	61	♀
69	♂	11.5	355	0	♂
72	♂	4	20	0	♂
73	♀	15.25	835	49	♀
74	♀	12.5	505	50	♀
75	♀	12.5	505	53	♀

chromatin body, as seen in nerve cells of other sheep, and as has been described in somatic cells of other species. The smallest female foetus examined in this study weighed 50 gms., but sex chromatin is recorded at a much earlier stage of development than this, (Austin and Amorosi 1957 and Park 1957). It is likely, therefore, that sex differentiation by nuclear sexing would be possible at least as soon as the foetal membranes have developed.

The demonstration of a sex difference as shown by the presence of sex chromatin in one tissue other than nerve tissues in the sheep raises the question of whether other tissues within the body may show this if examined.

The Examination of Buccal Cells for Sex Chromatin.

As reported by Struck (1961) the results of nuclear sexing of buccal cells of the sheep were inconclusive. In view of the fact that sex chromatin had been shown in one other than nerve tissue, and also since the introduction of a differential nuclear sex stain by Guard (1959) it was decided to reinvestigate nuclear sexing in these/

these cells.

Method.

Preparations were made by hard scraping of the buccal mucosa on the inside of the cheek, with a metal spatula. It was necessary to draw blood in order to gain sufficient cells with which to make smears. The smeared slides were then fixed rapidly in:

(a) Papanicolaou's fixative for the slides to be stained with cresyl echt violet.

(b) 95% ethyl alcohol for the slides to be stained by the method of Guard (1959).

Fixation was usually for a period of twenty-four hours, the slides being stored in Coplin jars.

Cresyl Echt Violet.

Slides stained by cresyl echt violet were put through a similar routine to the amnion preparations except that the time required to obtain good staining of the nuclear chromocentres was fifteen minutes.

Biebrich Scarlet - Fast Green (BS-FG), Guard (1959).

The routine followed for the use of this stain was described by Guard's technique No.1, in which the slides were stained as follows:

(1) 70% aocohol.

(2) Biebrich Scarlet, 2 minutes (see appendix)

(3) /

(3) Rinse in 50% alcohol.

(4) Differentiate in Fast Green F.C.F. (see appendix) 1 - ½ hours.

(5) During the differentiation, the slides were viewed at hourly intervals to check the green counterstaining.

Due to the special nature of this technique a brief explanation is pertinent. Biebrich scarlet is an amphoteric dye with basic groups, and in the presence of phosphotungstic acid shows a strong affinity for nuclear chromatin. In order to displace it in the differentiating process, another amphoteric dye with stronger basic groups is needed. This is fast green F.C.F., in the presence of phosphomolybdic acid. Guard observed that in vaginal cells, this differentiating process took several hours and that when the entire nucleus was green, the only structure that still retained the Biebrich scarlet was the sex chromatin. Guard further claimed that this process of differentiation could easily be controlled to affect the red staining of the sex chromatin only.

Staining by Guard's method failed to leave on any occasion, any undifferentiated red chromocentres as Guard was able to demonstrate in vaginal cell nuclei/

nuclei in women (representative of sex chromatin). Instead, a similar effect was obtained to that with cresyl echt violet stain, in that all the chromocentres in the nuclei stained a dark green against a pale green nucleoplasm and even paler cytoplasm when present. This technique was, therefore, of no value in differentiating the multiple chromocentres of the buccal cells of the sheep.

Results and Discussion.

The procedure for microscopic examination of buccal smears was similar to that of nerve tissue. Smears from two animals of each sex were involved and each suitable cell nucleus was drawn and major chromocentres marked as accurately as possible. A special note was made of any peripheral chromocentres.

Several factors limited the use of this test. Firstly, the number of suitable cells for counting in all preparations even after repeated efforts had been made, was very low, due to the very high proportion of crenated and poorly staining cells. A comparison was made with dog buccal mucosa, which like human preparations gave a high percentage of suitable cells for examination. After many preparations had been examined, it was possible to count and draw only two hundred/

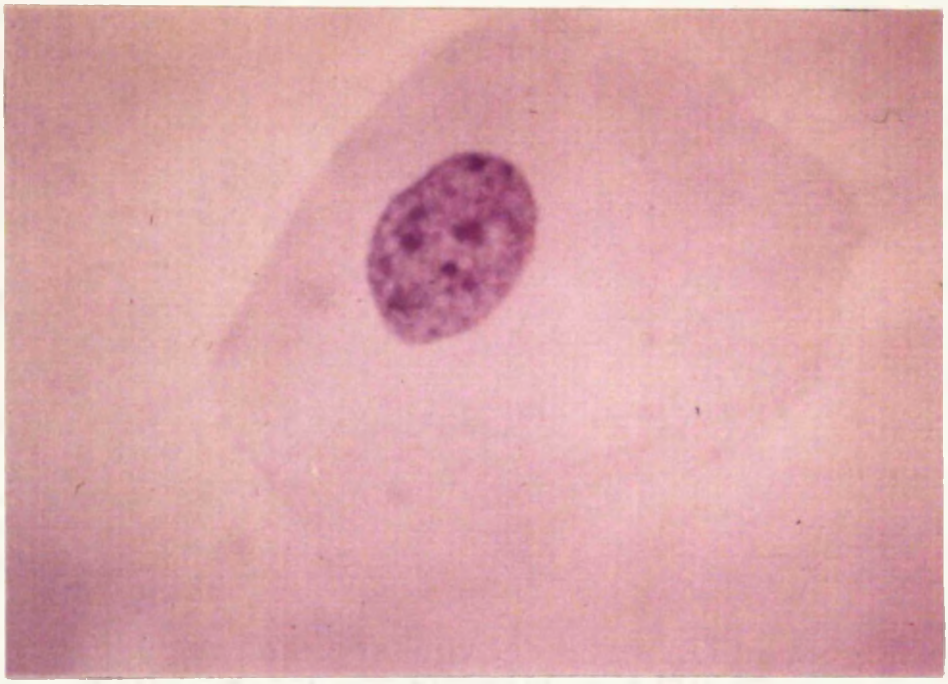
hundred and forty-two suitably stained male cells and four hundred and twenty female cells which displayed a satisfactorily recordable nuclear morphology. These cells, in both sexes showed coarse chromatin granules in the nucleus, varying from two to ten in number and showing no apparent difference between sexes.

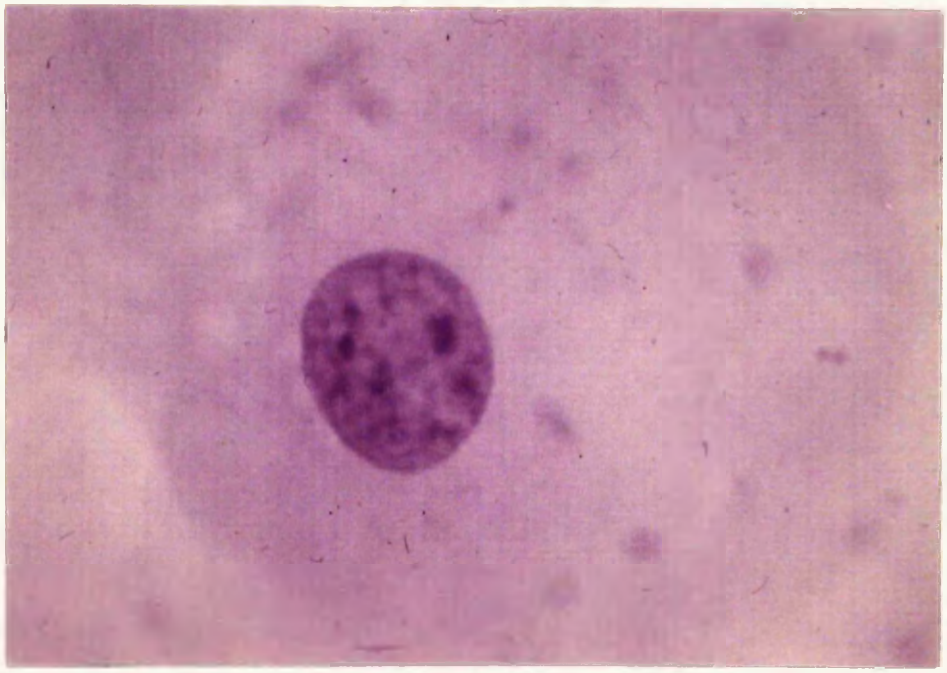
A peripheral chromocentre was present in 11.5% of male cells and 18% of female cells examined. In human buccal smear preparations (Barr and Carr, 1962), the percentage of female nuclei showing peripheral sex chromatin may be as low as 25%, but this is compensated by the fact that male cells show critical absence of any mass which could be mistaken for sex chromatin. The presence, therefore, of peripheral chromatin in both sexes of buccal smears of the sheep eliminated the possibility of using the buccal test for nuclear testing. Figs. 14 and 15 show the nuclei of buccal cells of both sexes. Stained sections, H. and E. of the buccal epithelium of the sheep showed that in both sexes, these non specific chromocentres were present in all levels of cells. The poor staining of the nuclei in cells from buccal scrapings in the sheep is probably as suggested by Struck (1961) due to the highly keratinised nature of/

of this tissue. It is emphasised, however, that failure to demonstrate sexual dimorphism in buccal cell nuclei does not mean that the sex chromatin is absent, but rather that its differentiation is masked by the presence of the other non-specific chromocentres.

Fig. 14. Nucleus of Buccal Epithelial Cell of Ewe (X 1000). Multiple Chromocentres and No Sex Difference.

Fig. 15. Nucleus of Buccal Epithelial Cell of Ram (X 1000). Multiple Chromocentres and No Sex Difference.





PART II.

THE PREPARATION OF CHROMOSOME METAPHASES IN THE SHEEP BY LEUCOCYTE CULTURE.

PART II

THE PREPARATION OF CHROMOSOME METAPHASES By LEUCOCYTE CULTURE.

For the examination of the mitotic chromosomes of man and other animals, it is essential that preparations with both adequate quantity and quality of metaphase figures can be produced. It is now possible to produce such preparations by a number of culture methods, including fibroblast cultures of various tissues such as lung, (Tjio and Levan, 1956), skin (Harnden, 1960) and kidney (Antonio and Torlone 1964). The initial work on human chromosomes was mainly carried out using the short term bone marrow culture techniques developed by Ford, Jacobs and Lajtha, (1958).

The disadvantages of these techniques, namely the time required for fibroblast cultures and the inconvenience of collecting bone marrow material have been overcome by the culturing of leucocytes from peripheral blood. Fibroblast cultures are now mainly used in medical cytogenetics to provide additional information in, for example, the diagnosis of mosaicism and corroboration of new or unexpected/

unexpected karyotypes, or the determination of karyotypes of embryos, babies and cadavers (Harnden and Brunton, 1965).

Bone marrow preparations are used mainly to study the chromosomes of cases of leukaemia (Sandberg, Ishikara, Crosswhite and Hauschka, 1962).

The first method for making chromosome preparations of leucocytes cultured from peripheral blood was described by Moorhead, Nowell, Mellman, Battips and Hungerford (1960). This method combines Nowell's (1960a and 1960b) method for the cultivation of peripheral blood leucocytes, with the air drying technique of Rothfels and Siminovitch (1958). Since all other leucocyte culture techniques are based on this method a brief description is warranted. The technique involves, firstly the separation of a buffy coat of leucocytes from a sterile heparinised sample of whole blood. The separation of this buffy coat is made possible by the use of slow centrifugation and a haemagglutinating agent, bacto-phytohaemagglutinin.

The concentration of cells in the buffy coat is determined and it is then diluted with a suitable tissue culture medium to give a final concentration of/

of cells of 1.0 -- 1.2x10^6 per ml: of medium.

Under the stimulus of phytohaemagglutinin, Nowell (1960b), and following incubation at 37°, the leucocytes divide many times and are harvested at 70 - 72 hours. Harvesting is assisted by arresting the leucocytes in full metaphase, using the amitotic agent, colchicine.

These arrested metaphases are then subjected to hypotonic treatment, using one quarter strength Hank's balanced salt solution. This swells the cell by osmosis and the exposed chromosomes are fixed in one part acetic acid and three parts ethyl alcohol. Following fixation of the chromosomes, a few drops of the suspension, dropped on to a wet cooled slide and air dried produce good mitotic chromosome preparations which, following staining with acetic orcein, are ready for viewing.

The main objection to using this method for culture of leucocytes from sheep and cattle is the difficulty in obtaining a buffy coat separation. This difficulty has been reported by several workers including Ulbrich, Weinhold and Pfeiffer (1963), Biggers and McFeely (1963), and Basrur and Gilman (1964), and is mainly attributed to the ineffective-

ness/

ineffectiveness of the haemagglutinating property in phytohaemagglutinin on sheep and cattle blood. Rigas and Osgood (1955), claim that phytohaemagglutinin effectively agglutinates erythrocytes of man, horse, pig, dog, cat, rabbit, chicken and frog but do not mention sheep or cattle. Further, the red blood cells of cattle and sheep sediment very slowly and show little tendency to rouleaux formation. Human red cells on the other hand, have some tendency to spontaneous agglutination, which is greatly assisted by the use of phytohaemagglutinin.

Methods have been described for the separation of the buffy coat layer in cattle and sheep. These include, the use of longer and harder centrifugation than prescribed by the human technique (Crossley and Clarke 1962; Biggers and McFeely 1963 and Ulbrich, Weinhold and Pfeiffer 1963). There is however the possible danger that with hard centrifugation the intrinsic properties of the white cells will be damaged. Other methods for leucocyte separation include the use of substances which will cause a slower sedimentation of the white cells and assist in the formation of a buffy coat. For example,

the/

the use of gum acacia (Spear 1948) albumen (Vallee, Hughes and Gibson 1947; Nichols, Levan and Lawrence 1962), Dextran, (Nichols and Levan 1961), and Ficoll (Biggers and McFeely, 1963). These separation techniques have practical disadvantages, which are firstly, the yield of white cells is disappointingly small (McFeely, 1964, personal communication), and secondly the additional handling of the blood sample increases the risk of contamination. Thirdly, a point which is important in the handling of many samples is that such separating agents as ficoll, fibriogen and bovine albumen are very costly. (Albumen Sigma, £4-13-0. per 50ml). This makes their routine use unattractive.

To overcome the problem of buffy coat separation Arakake and Sparkes (1963), showed that by culturing heperanised whole blood satisfactory chromosome preparations could be obtained from very small inocular of blood. These workers pointed out that this technique eliminated the special separation technique for leucocytes and also minimised the loss of leucocytes where separation is difficult. Usually a smaller number of slides was prepared by this method than in human work. Ten to fifteen mitotic figures/

figures per 100 leucocytes was common, but the yield in the deer mouse was much lower, being one metaphase per 100 leucocytes. Other authors, namely Gropp (1963) and Chaudurs (1964), have commented favourably on this whole blood technique, each adding his own variation.

The first description of a whole blood culture method for cattle and sheep was given by Basrur and Gilman (1964). In this method 1ml. of whole blood was incubated with 9 ml. of Eagle's medium with calf serum and 0.5 ml. of phytohaemagglutinin. Basrur (1965, personal communication), claimed 90% culture success by this method and said that she used either bovine or ovine serum in her medium but stated, as did McFeely (1965, personal communication), that better results were obtained using sheep serum rather than calf serum in the culture of sheep leucocytes. This latter point was contrary to Ford (1965, personal communication), who found calf serum more satisfactory than sheep serum, when using T.C. 199. Ford's opinion was based on twelve successful cultures out of seventeen. He also stated that, using his method for the sheep, sufficient metaphases were accumulated after forty eight hours.

A/

A further publication by McFee, Banner and Murphree, (1965) described a whole blood culture method for sheep leucocytes which was based on that of Basrur and Gilman.

Initial difficulties experienced by the author in attempting to separate buffy coats from sheep blood for leucocyte culture made him use, and perfect, the whole blood technique. Work was commenced in this laboratory before the publications of Basrur and Gilman (1964), or McFee et al., (1965) but communication with these workers and also with McFeely and Ford, helped to improve the culture method used.

METHOD FOR MAKING CHROMOSOME PREPARATIONS OF LEUCOCYTES CULTURED FROM PERIPHERAL BLOOD OF THE SHEEP.

1). Culture Technique.

Fifteen to twenty ml. of venous blood was collected aseptically from the jugular vein into universal containers to which 1 ml. of heparin (see appendix) had been added, care being taken to ensure sufficient mixing. Following a routine white cell count, 1 ml. of whole blood was mixed with 8.0 ml. of T.C.199 (see appendix), and 1 ml. of homologous sheep serum in 100 ml. specially sterile cleaned/

cleaned and sterile/medical flats. (see appendix). The homologous serum was prepared by collecting a sterile blood sample the night before culturing was anticipated. This was allowed to clot in an incubator for thirty minutes and stored in a refrigator at 4°C. overnight. From 10 ml. of blood collected in this manner sufficient serum was usually obtained for four culture bottles.

To the blood and culture medium, 0.4 ml. of bacto-phytohaemagglutinin (see appendix) was added and the whole mixture was then stored in a refrigerator for several hours to synchronize the growth of the leucocytes. Culturing was carried out with the culture flasks in an upright position in a water jacketed incubator at 38° C. for seventy five hours.

2). Harvesting Technique.

After approximately 72 hours of incubation, 0.15 ml. of colcemid, (see appendix), was added to each culture bottle, to give a concentration of 0.2 µg/ perml. On the addition of colcemid, each culture bottle was carefully shaken and replaced in the incubator for a further 3 hours. At this time, the cultures were again carefully shaken to ensure/

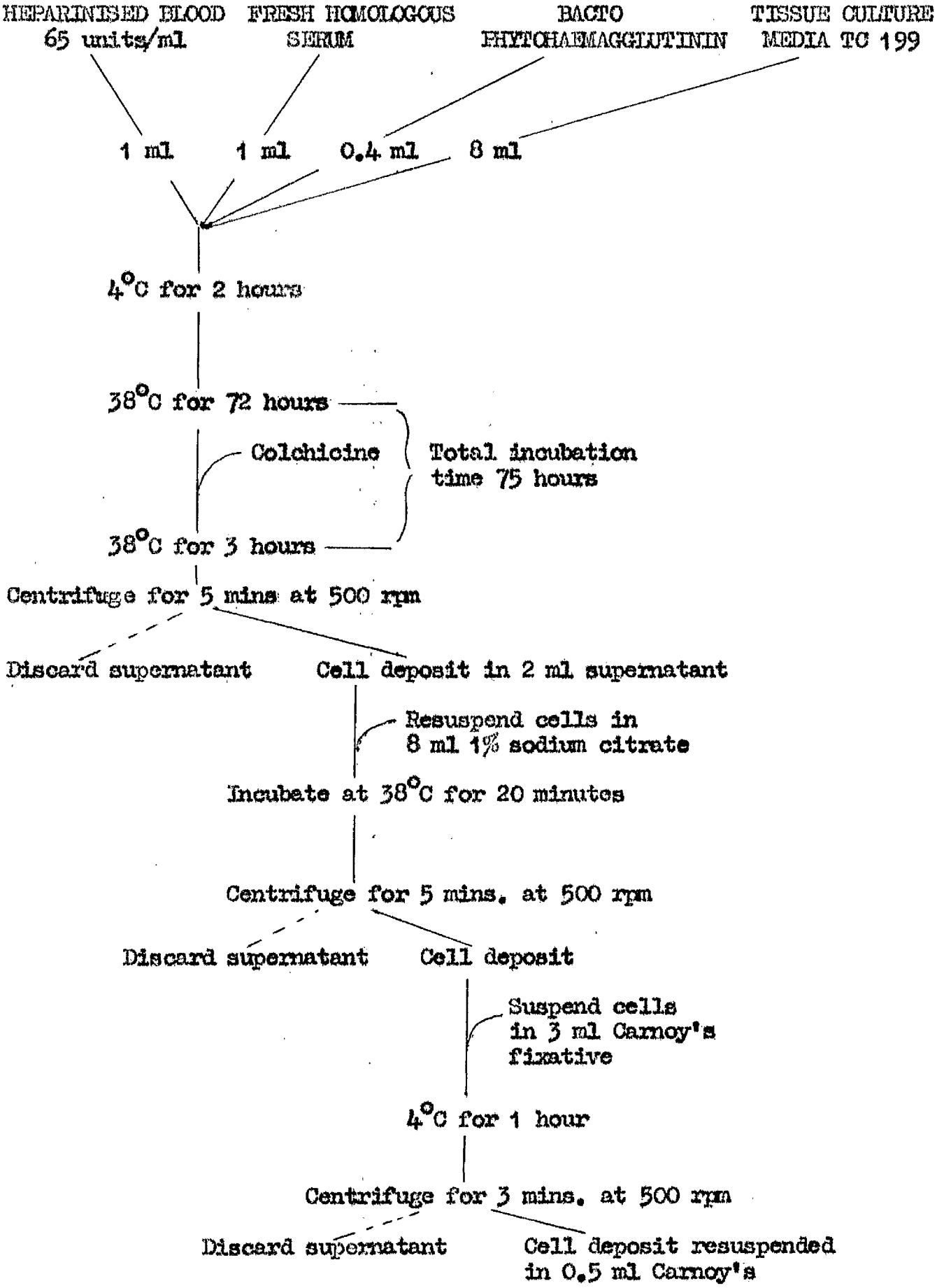
ensure that all clumps of cells were well dispersed and the contents transferred to 10 ml. centrifuge tubes. Each culture was centrifuged for five minutes at 500 R.P.M. in a six inch bench centrifuge and the top 8 ml. of supernatant fluid pipetted off and discarded. The cell deposit and remaining two ml. of supernatant were then carefully mixed with 8 ml. of a 1% sodium citrate solution, warmed to 38° C. by pipetting constantly with the tip of a pasteur pipette to ensure thorough fractionation of cell clumps. This process was repeated several times to each culture during the first five minutes of hypotonic treatment. The hypotonic treatment was continued for exactly twenty minutes in a water bath at 38° C. After this centrifugation of each sample at 500 R.P.M. for five minutes was followed by careful removal of the supernatant liquid without disturbing the button of cells deposited at the bottom of the tube. The button of cells containing the chromosome metaphases was then fixed by adding 3 ml. of a freshly prepared and cooled mixture of one part acetic acid to three parts ethyl alcohol, (Carnoy's fixative). The button of cells was then carefully broken up by repeated pipetting, again/

again using only the tip of the pipette. This cell suspension was then allowed to stand for 30-60 minutes in a refrigator to ensure complete fixation of chromosome material. After this it was again centrifuged for two minutes at 500 R.P.M., the supernatent removed and the cells resuspended in 1 ml. of fresh fixative. After fifteen minutes this process was repeated, using 0.5 ml. of fixative to make the final cell suspension.

Chromosome preparations were made by dropping 2-3 drops of the cell suspension from eighteen inches on to a moist, cooled and carefully cleaned slide, (see appendix) held with a slope of 15° to the bench. The slide was then dried by warming carefully over a Bunsen flame, overheating being avoided, and finally allowed to dry completely on a warm plate at 37° Centrigrade. Prepared slides were then stained using 2% acetic orcein, (see appendix), by the following procedure.

(1) Acetic orcein stain .... 1 hour.
(2) 50% Acetic acid ..... Dip.
(3) 95% Ethyl alcohol ..... 1-2 minutes.
(4) 95% Ethyl alcohol ..... 1-2 minutes
(5) Absolute ethyl alcohol . Dip
(6)/

Fig. 16. Diagram of Culture Technique
For the Sheep.


HEPARINISED BLOOD
65 units/ml
FRESH HOMOLOGOUS SERUM
BACTO PHYTOHAEMAGGLUTININ
TISSUE CULTURE MEDIA TC 199
1 ml
1 ml
0.4 ml
8 ml
4°C for 2 hours
38°C for 72 hours
Colchicine
Total incubation time 75 hours
38°C for 3 hours
Centrifuge for 5 mins at 500 rpm
Discard supernatant
Cell deposit in 2 ml supernatant
Resuspend cells in 8 ml 1% sodium citrate
Incubate at 38°C for 20 minutes
Centrifuge for 5 mins. at 500 rpm
Discard supernatant
Cell deposit
Suspend cells in 3 ml Carnoy's fixative
4°C for 1 hour
Centrifuge for 3 mins. at 500 rpm
Discard supernatant
Cell deposit resuspended in 0.5 ml Carnoy's

(6) Xylol, two changes, at least ten minutes each.

(7) Mounted with D.P.X. (see appendix).

Fig. 16 demonstrates the culture method diagrammatically.

DISCUSSION of OBSERVATIONS and MODIFICATIONS of CULTURE METHOD.

Media. Two media were used in these culture series, T.C. 199 and Waymouth's medium, (see appendix). Both media supported good growth of sheep leucocytes, provided other factors were adequate. Both media gave satisfactory results when used in conjunction with serum. The majority of cultures in this series was however grown in T.C.199.

Heparinisation of Blood.

Experience showed that the quantity of heparin used in the collection of blood for culture was important. Initially human blood bottles containing 100 units of heparin (see appendix), were used. These were designed to collect a 10 ml. sample of blood. Cultures using blood collected in this quantity of heparin invariably clotted to such an extent that it was very difficult/

difficult to break up the very large clumps of cells. A series of cultures using 10 ml. of blood collected in these bottles, and 10 ml. of blood collected in 500 units (½ ml.) of heparin and 1,000 units (1 ml.) of heparin, showed that this clumping was overcome in the latter cultures.

This problem was not encountered once a minimum quantity of 500 units (½ ml.) of heparin was used to 10 ml. of blood collected.

Concentration of Leucocytes.

Cultures containing white cell concentrations from 3.4×10^5 per ml. of culture medium to 1.8×10^6 per ml. of culture medium grew under equal conditions.

Also as good a yield of metaphases was obtained from low cell concentrations as from high cell concentrations.

The Addition of Serum to Medium.

Growth was obtained in cultures using either freshly prepared calf serum or freshly prepared homologous sheep serum. Calf serum was decanted from the freshly clotted blood of new-born calves, and inactivated by heating for thirty minutes in a water bath at 56°C. It was found, however, that/

that sheep serum collected and prepared as described above, produced cultures in which the yield of metaphases was higher. This was in agreement with Basrur (1964, personal communication) and McFeely (1965, personal communication), McFee (1965, personal communication), stated that he had not used homologous sheep serum.

Serum concentrations of 40%, 30%, 20%, 10% and 0% were used in two trial culture series. At 40% there was no growth, at 30% growth was poor, while equally good growth results were obtained from concentrations of 20% and 10%. The final choice of 10% serum was made firstly, because it proved adequate and secondly because the addition of more required the collection of larger blood samples. Occasionally growth was found in cultures to which no serum was added.

Plasma collected by centrifugation and used in place of serum resulted in poorer growth.

Concentration of Phytohaemagglutinin.

Growth was obtained using as little as 0.2 ml. of phytohaemagglutinin (see appendix), per 10 ml. culture. In a trial using 0.2, 0.3, 0.4 and 0.5 ml. per 10 ml. of culture in three series, each of two cultures,/

cultures, the best result was obtained by using 0.4-0.5 ml. This agrees with the findings of Basrur and Gilman (1964), and Ford (personal communication).

Incubation Time.

In order to find the time at which the highest number of metaphases was yielded, ten cultures were prepared as described, incubated together and harvested at intervals of twenty four hours over five days, two cultures being harvested each day. Each culture was assessed by estimating the degree of mitotic activity seen in prepared slides. It was found that at 24 hours, very few mitoses were present, but at 48 hours and 72 hours, enough mitoses were present for scoring routine karyotypes. At 96 hours and 120 hours, cultures became exhausted and large numbers of clumped and crenated cells were seen. A few metaphases were however, present.

By varying the harvesting time around 72 hours over a number of cultures, it was found that the best mitotic yields were in cultures allowed to grow to approximately 72 hours before adding colchine. Although metaphases were present in recordable/

recordable numbers at 48 hours as described by Ford (personal communication), culturing for 72 hours gave a higher number of scorable mitoses.

Amitotic Agent.

By varying the quantities of colcemid it was shown that 0.2 ml. of a solution of 100 μg per ml. in a 10 ml. culture gave good metaphase arrest by three hours without undue chromosome contraction. Higher concentrations reduced the number of scorable metaphases by inducing greater chromosome contraction and chromatid separation.

Hypotonic Treatment.

To produce chromosome separation two hypotonic solutions, a one in ten Hank's Balanced Salt Solution and 1% sodium citrate solution were used. Each produced the desired result but a preference for sodium citrate developed because it improved the staining of the chromosomes, resulting in clearer photographic enlargements. Sodium citrate was not used until a large part of the work was completed. It was found however, that in preparations in which it had been used more secondary constrictions were seen. A further discussion of this is made under Part 3.

The/

The optimum time for hypotonic treatment was found by varying the time of exposure from 10 minutes to 30 minutes at intervals of five minutes over two series of cultures. Twenty five minutes of hypotonic treatment, including spinning, was found to give the best spread metaphases.

Summary.

This culture method and harvesting procedure was used on 110 culture series for sheep and six for cattle, (a series being from 4-8 culture bottles). Of the last 25 series cultured, a satisfactory growth was recorded on 22 occasions. One of the cultures which failed was in a faulty incubator which unaccountably rose to 40°C. during one night. Many variations were made at the two stages, the culturing stage and the harvesting stage which form two quite distinct sides to obtaining good chromosome preparations.

This technique as described, has proved quite satisfactory for the examination of the mitotic chromosomes of a number of sheep and several cattle on a basis similar to that of man. Little explanation however, can be offered for the variations in yield of metaphases as seen from culture to culture.

PART III.

THE KARYOTYPE OF THE SHEEP.

PART III.

THE KARYOTYPE OF THE SHEEP.

Historical Review.

In a broad sense, the term karyotype is used to describe the chromosomal constitution of a cell, either of an individual or of a species (Dorland's Illustrated Medical Dictionary). In medical cytogenetics, the term now emphasis two aspects of nuclear structure, namely chromosome number and chromosome morphology. Of the published information on the karyotype of the sheep, very little is known other than that the bulk of workers are in agreement that the diploid chromosome number is 2n=54. The Y chromosome can be defined and latterly the X chromosome has been suggested as being the largest of the acrocentric chromosome (McFee et al., 1965). Further, the karyotype falls into two groups for the autosomes, six large metacentric or sub-metacentric chromosomes and 46 acrocentric chromosomes.

While people have published work on the Chromosomes of the sheep, there is nothing approaching the detail which is accorded the human chromosome complement. Wodsedalek (1922 and 1929), studying spermatogonial/

spermatogonial material gave the diploid chromosome number as thirty three for the male with an XO sex chromosome complement and thirty four for the female with an XX sex chromosomes complement. The same author in 1929, studied four breeds of sheep, two horned, namely the Dorset and the Rambouillet and two unhorned, the Southdown and the Lincoln. He claimed that the ram did in fact have a very small Y chromosome but that in the two horned breeds, it was larger than the polled breeds and that it possessed a clearly noticeable modification. From this observation, he made the remarkable claim that the variation in the Y chromosome was associated with the presence or absence of horns and that this fact threw considerable light on the subject of sex linked inheritance. The following year Shivago (1930) studying the amnion cells, described the diploid number of chromosomes correctly at 54 and the male as having an XY sex chromosome complement. Krallinger (1931) studying spermatogonial tissue of Merino sheep, while giving the male sex chromosomes the XY designation, put the diploid complement at 50-60. A similar picture was given by Bruce (1934 and 1935) and again studying spermatogonial metaphases/

metaphases of Merino Sheep.

Butarin, (1933-34) and (1935), using an Asiatic species of wild sheep, the Arkar, reported on two occasions chromosome numbers from spermatogonial cells of 54-56 on the first report and 52-54 from the same stages in the fat rumped sheep. In his second report he recorded 60 as the diploid chromosome complex for the Arkar and the fat rumped sheep cross and hybrid, between these. Butarin, from these findings suggested that different breeds of sheep may have had different chromosome numbers.

Novikov (1935) found the diploid chromosome number to be 60 in spermatogonial cells of hybrid European Mouflon (Ovis Musimon) and the domestic sheep Ovis Aries. Both Novikov and Butarin gave the male an XY sex chromosome complement.

Pchakadze (1936) suggested the haploid cell complement as 30, again working on spermatagonial tissue. Berry (1938) in a comparative study on the chromosomes of the sheepand goat and its hybrids using amnion cells, reported the diploid chromosome number of the sheep as 54 and in 1941 confirmed this in another study in the spermatogonial tissue of the Rambouillet/

Rambouillet sheep. He also made comments on the morphology of individual chromosomes, having examined the chromosomal arrangement in 65 spermatogonial cells. He classified the chromosomes into V's, long rods, short rods, bent rods and a sphere. The V's corresponded to the six large metacentric chromosomes. The sphere was the description given to the small Y chromosome and the X was concluded from the examination of sex bivalents as being one of the medium sized acrocentric chromosomes. The photomicrograph arrangements of the chromosomes in Karyotypes given by Berry are remarkable considering the tissue he was using and in fact closely approximate present-day karyotypes of the sheep.

Ahmed (1940) using Leicester rams in a study of the structure and behaviour of the chromosomes of the sheep during mitosis and meiosis, again confirmed the diploid complement as 54. He described the X as being large and having a median centromere and the Y as smaller with a subterminal centromere. He also classified the autosomes into four groups in respect of position of the centromere, four having submedian, fifteen subterminal and six almost terminal centromeres, and one pair had a

near/

near median centromere. Ahmed's findings in the sex chromosomes were not in agreement with Berry and in the light of present-day studies it is apparent that he incorrectly described the morphology of both sex chromosomes.

In 1943, a very eminent mammalian chromosome worker, Sajiro Makino, studying the relationship of the chromosome complexes of the goat and the sheep, again confirmed the sheep number as 54. He, like Berry, made some of the first pertinent comments on the morphology of the chromosomes. Three large pairs, he described as V shaped and atelometic and twenty three pairs as rod shaped and telometic. He apparently described the sex chromosomes correctly. The X is the largest of the telometic chromosomes and the Y is easily recognisable by its minute size compared with the rest of the chromosome complement.

Using lung tissue for culture from two embryos, Melander (1959), in discussing the mitotic chromosomes of various cavicorn animals including the bull, the bison and the sheep again gave the Karakul sheep the diploid number of 54 and described three pairs of large autosomes with medially inserted centromeres/

centromeres which collaborates Makino's description. He could not identify the X chromosome, but described the Y as being very small and having a median centromere. Melander also discussed the significance of the large autosomes in the sheep, describing them as phylogenetically new, having arisen by some kind of fusion between chromosomes with a terminal centromere, according to Robertson's law. He also made an attempt at drawing an idiogram from the examination of 118 chromosomes of two rams and recorded frequency of arm sizes of the chromosomes and concluded that the chromosome length ranges from 8-1 micra.

Two Spanish publications by Gimenez Martin and Lopez-Saez (1962) on the metaphase preparations of the chromosomes of several animals again confirmed the diploid number of the sheep as 54. They identified the Y chromosome and suggested that the X was the largest of the acrocentric chromosomes. No karyotyping was performed by these workers. Yet a further worker, using bone marrow squash preparations, namely Borland (1964), confirmed the diploid number at 54 in several breeds of sheep including Merino, Cheviot, Southdown, Border Leicester, Romney Marsh, Dorset Horn and Ryeland.

He/

He identified the Y chromosome as being a small "dot like" chromosome, the X, he suggested, was one of the smaller acrocentric chromosomes. Borland also like previous workers, described six large metacentric and forty eight acrocentric chromosomes in the ewe. He made the first attempt at a rudimentary idiogram suggesting on arm measurements alone, that the chromosomes fell into four main groups A, B, C and D numbering:

Group A. 1 - 3, large metacentrics.
Group B. 4 -11, Acrocentric.
Group C. 12 -17, Acrocentric.
Group D. 18 -26, Acrocentric.
Sex chromosomes.

The most recent paper on the chromosomes of the sheep by McFee et al., (1965) showed clearly the advantages of following the human workers in this field, in that the chromosome karyotypes prepared by these workers using the blood culture techniques were well ahead of previous workers. The X chromosome was fairly clearly identified as the largest of the acrocentric chromosomes; the Y chromosome was described as probably submetacentric. Satellited chromosomes were not encountered and no mention/

mention was made of secondary constrictions. These workers claimed to have counted the chromosomes in a hundred metaphases, but they did not mention the number of photographic karyotypes examined, and upon which they drew such conclusions as the X chromosome being 1 - 1.15 times longer than the next pair of acrocentric chromosomes.

The following table 3 summarises the findings of the morphological characteristics of the chromosomes of the sheep to date and the breeds of sheep so far examined.

TABLE 3. SUMMARY OF HISTOLOGICAL FINDINGS BY WORKERS ON SHEEP C

INVESTIGATOR	DATE	BREEDS EXAMINED	MATERIAL USED	DIPLOID Chromosome Number.	SEX CHROMOSOME Complex.	
					1922	1929
WODSEDALEK, J.E.	1922	Southdown Dorset Horn Rambouillet	Spermatogonial tissue.	33 ♂ 34 ♀	XO ♂	XY ♂
	1929	Lincoln			XX ♀	XX ♀
SHIVAGO, P.I.	1930	Merino	Amnion Cells	54	XY ♂	
KRALLINGER, H.F.	1931	Merino	Spermatogonial Tissue.	50-60	XY ♂	
MEUCE, H.P.	1934	Merino	Spermatogonial Tissue	60	XY ♂	
SUTARIN, N.S.	1933-34	Askhar Fat rumped sheep.	Spermatogonial Tissue	54-56	XY ♂	
			" "	52-54	XY ♂	
	1935	X & Hybrids of above	" "	60	XY ♂	
NOVIKOV, I.I.	1935	European Mouflon X Merino	" "	60	XY ♂	
PCHAKADZE, G.H.	1936	Georgia Fat-tailed.	Spermatogyte Tissue	Haploid 30	-	
BERRY, R.O.	1938	Merino X Goat Sheep	Amnion Cells	54	-	
	1941	Rambouillet Rams	Spermatogonial Tissue	54	XY ♂	
AHMED, I.A.	1940	Leicester	Spermatogonial	54	XY ♂	
MAKINO, S.	1943	Merino Karakul Corriedale	Testis	54	XY ♂	
MELANDER, Y.	1959	Karakul	Embryo Tissue	54	XY ♂	
GIMENEZ MARTIN, G. & LOPEZ-SAEZ, J.F.	1962	-	Bone Marrow Squashes.	54	XY ♂	
BORLAND, R.A.	1964	Merino, Cheviot, Southdown, Border Leic. Romney Marsh, Dorset Horn, Ryeland.	Bone Marrow Squashes.	54	XY ♂	
McFEE, A.F.		Suffolk and	Leucocyte	54	XY ♂	
BANNER, M.W.	1965	Crosses	Culture.		XX ♀	
MURPHREE, R.L.						

Chromosome Counts, Karyotypes and Idiogram Construction.

For the study of the chromosomes of the sheep to be of use in investigation of possible chromosomal abnormalities, more basic information is required both about chromosome number and the morphological characteristics of individual chromosomes. For this reason, the steps followed in this study were in accordance with current human chromosome methodology. Three aspects were considerd, chromosome counts, karyotype morphology and idiogram construction.

Chromosome Counts.

The diploid chromosome number is characteristic for all somatic cells of normal members of a given species of animals. The chromosome number of the species, however, has to be distinguished both from the chromosome number of an individual, and from the chromosome count of a given cell.

As well as the hypodiploid chromosome number encountered in for example, XO Turner's syndrome and hyperdiploid chromosome number of XXY Klinefelter's syndrome, other variations in chromosome counts do occur. When the chromosomes of/

of a number of metaphase preparations, made from cultured cells are counted, there is considerable variation from the accepted diploid chromosome number of the normal individual of the species under examination. This is largely due to chromosome loss through breakage of cells during preparation. Court-Brown, Jacobs and Doll (1960) showed that in human bone marrow preparations, the chromosome count from a number of such counted metaphases is negatively skewed around the modal number of fortyssix, eighty five per cent of cells showing a count of forty six chromosomes and twelve per cent, less than forty six chromosomes.

In human metaphase preparations from leucocyte cultures, the percentage of modal cells is much higher, being ninety four per cent (Court-Brown, Harnden, Jacobs, MacLean and Mantle 1964) while Boyd (1965), from counts of over four thousand metaphases of one hundred patients recorded 94.5% of cells with the modal number forty six.

Various factors have been shown to affect the percentage of modal cells in chromosome counts from leucocyte cultures. For example, exposure to radiation and radiomimetic drugs cause an increased proportion/

proportion of non-modal cells (Jacobs 1965).

Non-modal cells in chromosome counts from leucocytes cultures have also been shown to be associated with the aging of man (Jacobs, Court Brown and Doll 1961), (Jacobs, Brunton, Court Brown, 1964; and Jacobs, Brunton, Court Brown, Doll and Goldstein 1963). In the last paper evidence was given also suggesting a difference between men and women, both in the manner in which the proportion of non-modal (aneuploid) cells changed with age and also as to the chromosomes affected. In men, it was the Y chromosome and in women it was presumed to be the X chromosome.

This last point becomes important when the criteria for the establishment of sex chromosome mosaicism are to be satisfied.

In some cases of human chromosome abnormality, variations in chromosome number have been found in counts made from metaphases of the same tissue. Such variations are due to chromosome mosaicism, which infers cell lines containing diploid and hyperdiploid chromosome counts, as in XY/XXY Klinefelter's syndrome (Sandberg 1961), or diploid and hypodiploid chromosome counts as in XO/XY mosaicism (Jacobs, Harnden/

Harnden, Buckton, Court Brown, King, McBride, MacGregor and MacLean 1961). Even mosaics involving three cell lines have been reported XY/XXY/XXYY, (MacLean et al., 1962).

Therefore, with the examination of some sexually abnormal sheep, in which mosaicism was likely to be encountered as part of this study, it was necessary to find what was the variation from the modal number of chromosomes from normal sheep.

Method.

All chromosome counts were made using an M20 Wild microscope. Slides were viewed under low power X 10 using X 6 wide field eye pieces. When a suitable metaphase was seen, it was studied under an oil immersion lens at X 1000. Chromosome metaphases which were well spread and in which there were a minimum of overlapping chromosomes, were counted. Once a decision had been reached to count the chromosomes in a metaphase, the final number was recorded irrespective of the result. All metaphases were counted twice, three or four times until the same total was recorded on two successive counts. Metaphases presenting ambiguities for counting, were tentatively recorded until a photographic karyotype/

karyotype could be made and a final decision reached on the chromosome number. With the exception of one sheep of unrecorded age, the twenty two animals, thirteen male and nine female were all under two years of age.

The results of counts on 1,832 metaphases from these sheep is shown in table 4 and individual animals with breed and sex in Tables 5 and 6.

Discussion of Results.

Using this system of counting, the percentage of cells showing the modal number of fifty four chromosomes from 1,832 counted cells of the sheep is 87.44. Although six cells with fifty five chromosomes were included in the table, in fact, only one could be clearly classified as a true aneuploid, four others were technically doubtful even after photographic karyotyping and one cell was in the vicinity of another metaphase from which it could have acquired an additional chromosome. Some polyploid cells could be counted and usually contained the tetraploid chromosome number of one hundred and eight, others that were difficult to count were merely recorded as polyploid cells. To compare the results of this method with comparative human counts,

cells/

TABLE 4.

CHROMOSOME COUNTS FROM 22 NORMAL EWES and RAMS.

(LEUCOCYTE CULTURE)

Chromosome Number	50	51	52	53	54	55	56	Poly-ploid	Total
Number of Metaphases Counted	14	12	39	133	1602	6*	o	25	1832
% of Metaphases Counted	0.76	0.66	2.13	7.25	87.44	0.32	o	1.36	

* Only one cell a genuine aneuploid. One was undecided - near to another metaphase. 4 cells were technically doubtful, could be artefact.

TABLE 5.

CHROMOSOME COUNTS OF MALE SHEEP.

Sheep	Breed	Distribution of Metaphases According to Chromosome Number														Total Metaphases Counted.
		50		51		52		53		54		55		Polyploid		
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
N 165	Suffolk							9		79						88
									10.23		89.77					
C 165	Suffolk	2		1		2		9		73				1		88
			2.27		1.14		2.27		10.23		82.94				1.14	
A 64	Suffolk	2		1		4		12		83						102
			1.96		0.98		3.92		11.76		81.37					
J 65	Cheviot					1		5		88				1		95
							1.05		5.26		92.63					
	Soay	2		1		1				12						16
			12.50		6.25		6.25				75.00					
H 165	Dorset			1		4		8		72				1		86
	Horn X				1.16		4.65		9.30		83.72				1.16	
26013	S.B.F.					1		10		44						55
							1.82		18.18		80.0					
26012	S.B.F.					4		5		76						85
							4.71		5.88		89.41					

26019	S.B.F.					1		2		28						31
							3.23		6.45		90.34					
T 360	S.B.F.	1		1		3		4		64		1		2		76
			1.32		1.32		3.95		84.21		84.21		1.32		2.63	
S 345	S.B.F.			1		2		1		55		1		2		62
					1.61		3.23		1.61		88.71		1.61		3.23	
26015	S.B.F.					1		1		34						36
							2.78		2.78		94.44					
H.I.	S.B.F.					1		1		8						10
							10.0		10.0		80.0					
TOTAL		7		6		25		67		716		2		7		830

TABLE 6

CHROMOSOME COUNTS OF FEMALE SHEEP

Sheep	Breed	Distribution of Metaphases According to Chromosome Number														Total Metaphases Counted
		50		51		52		53		54		55		Polyploid		
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
A 15	S.B.F.			1		4		10		75						90
					1.11		4.44		11.11		83.33					
117	S.B.F.					2		8		156		1		7		174
							1.15		4.60		89.65		0.57		4.02	
22	S.B.F.	5		3		4		7		187				3		209
			2.39		1.44		1.91		3.35		89.47				1.44	
E/1	S.B.F.							1		9						10
									10.00		90.00					
88	Grey-Face							5		65		1		1		72
									6.94		90.28		1.39		1.39	
53	Grey-Face					1		8		55				2		66
							1.52		12.12		83.33				3.03	
86	Grey-Face					1		10		63				2		76
							1.32		13.16		82.89				2.63	
33	Grey-Face	2		2		2		9		162		1		1		179
			1.12		1.12		1.12		5.02		90.50		0.56		0.56	
18	Grey-Face							8		115		1		2		126
									6.35		91.27		0.79		1.58	
		7		6		14		66		887		4		18		1,002

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cells containing less than fifty three chromosomes were deleted and the modal cells again expressed as a percentage of the total cells, which in this case was 1,714, and polyploid cells were not included. Table 7.

By the comparison of the results of Table 7 with the results of Boyd (1965), Table 8, it can be seen that the modal count rises to 92.1% of cells counted. Considering the sheep has eight more chromosomes than man, this would suggest that both the method of recording and the quality of metaphases examined was near comparable to human method.

However, for purposes of consistency the counts on abnormal animals were recorded down to cells with fifty chromosomes. The percentage of cells with the modal number fifty four was taken as 87.44% for comparative purposes. From the results of this work, it can further be seen, Tables 5&6 and Table I5, part 4, that the chromosome number of 2 n = 54, is confirmed for five additional breeds of sheep, including the Scottish Black Face, Scottish Black Face x Border Leicester (Grey Face), Clun Forest, Welsh Mountain and Soay. The latter sheep is of interest as it is considered to be one of the oldest known/

known existing pure breed lines. Additional confirmation to already reported chromosome numbers for Cheviot, Border Leicester and Suffolk breeds of sheep is also given.

TABLE 8.

HUMAN CHROMOSOME COUNTS (BOYD 1965).

	45	46	47	TOTAL CELLS
Bone Marrow	10%	84%	6%	223
100 recent patients (leucocyte)	5	94.5	0.5	4348
All patients	6	93	1	8022

TABLE 7

CHROMOSOME COUNTS - 22 NORMAL EWES and RAMS.

No. of Chromosomes	53	54	55	TOTAL.
No. of Metaphases	133	1602	6	1714
% of Metaphases	7.5	92.1	0.4	100

KARYOTYPE MORPHOLOGY.

As pointed out, the term karyotype originally included the characteristics of the chromosomes of a species both numerically and morphologically. This term is now used in medical cytogenetics to describe the systemised array of chromosomes of a cell, prepared either by drawing or by photography, (Denver Report, 1960). The Denver Report further suggested that the term idiogram, which is sometimes used in the same sense as the term karyotype, be used solely to refer to the diagrammatic representation of a karyotype. An idiogram for any given species is based on the measurements of the individual chromosomes in several or many cells, and is in fact an "average" karyotype.

The reason for arranging into karyotypes the mitotic chromosome preparations used in cytogenetics is for the purpose of chromosome identification. Again, as an aid to chromosome identification, the Denver Report (1960) and the Report of the London Conference (1963) suggested that several salient points be followed in karyotype preparation. These include, the arrangement of chromosomes in descending order of size, the alignment of the centromeres,/

centromeres, the point of attachment of the chromosome to the spindle (Darlington, 1936) and latterly the recognition of additional chromosome markers such as secondary constrictions. These will be discussed in detail below.

The differences in length between chromosomes is one of the main factors used in identification. Apart from the innate variation in length between individual chromosomes in a given karyotype, the conception of relative difference in size depends largely on the type of mitotic preparation viewed. In improved leucocyte culture techniques, provided early mitotic metaphases are selected for viewing in which the chromosomes are not unduly contracted, chromosome length can be of value in identification (Ferguson-Smith, 1964). With high concentrations and prolonged colchicine treatment, the chromatid arms of chromosomes become very contracted and length differences are not so easily perceived.

A further most useful landmark for the identification of chromosomes is the centromere position. The centromere divides chromosomes into two parts, referred to as arms. On the suggestion of White (1945), the position of the centromere designates/

designates a chromosome as being either metacentric or acrocentric. If the centromere divides the chromosome arms equally, it is referred to as metacentric. If on the other hand, the centromere is near the end, and the long and short arms are of very unequal length, the chromosome is termed acrocentric. Metacentric and acrocentric are now accepted terminology in medical cytogenetics (Harnden, 1962). These have largely replaced the older terms telocentric and atelocentric, the former denoting a chromosome with a terminal centromere and the latter non-terminal. The term telocentric is still used to describe a chromosome with a terminal centromere, but the existence of such chromosomes is apparently doubtful, although several investigators have reported species which contain telocentric chromosomes, Mattsson, (1962) and Eid, (1963).

Levan, Fredga and Sandberg (1965) do however make the point that the theoretical possibilities of existence, do not affect morphological observation and that a chromosome with no visible short arms should be termed telocentric. A fourth term submetacentric chromosome is frequently used to describe/

describe one having its centromere between the acrocentric and metacentric positions. Criticism has arisen of the accuracy of the terms metacentric and acrocentric, by Levan et al., (1965) who suggest six different categories of chromosome identification according to centromere position. In the case of the sheep, and indeed other members of the same taxon, namely cattle and the goat, there is little chance as will be seen, of identifying individual chromosomes on centromere position alone, as is the case in man. A change from accepted nomenclature would therefore appear pointless.

Chromosomes within a prepared karyotype are usually numbered in pairs or lettered in groups, either system being acceptable, although the Denver Report suggested numbering human chromosomes. Patau (1961) criticised the use of numbers in identifying human chromosomes. He suggested that these chromosomes could not be more clearly identified than into seven lettered groups. If length and centromere position were the only anticipated means of identification, Patau's criticism may be valid. However, with the possibility of using other morphological features, such as secondary constrictions (Ferguson-Smith et al., 1962) and autoradiography patters (Gilbert, Muldal,/

Muldal, Lajtha and Rowley 1962) for identification of chromosomes the use of numbers in karyotyping has advantages. This advantage becomes more obvious with a karyotype such as the sheep which under a group lettering system could only be divided into two groups; Group A, metacentric and Group B, acrocentric chromosomes, sex chromosomes excepted.

In such a homogeneous karyotype, with this large group of acrocentric chromosomes, a number signifies a relative position in the series, while reference to a member of group B., does not indicate relative position. A member of group B., could mean any chromosome in this group, the largest of them being several times larger than the smallest members of the group.

Secondary Constrictions.

In contrast to the centromere, the primary constriction, other constricted regions not associated with spindle attachment do occur in chromosomes of several species. These have been shown to be valuable as additional chromosome markers in the Rhesus monkey (Rothfels and Siminovitch 1958) and by several workers in the human field. In the latter field, Ferguson-Smith et al., (1962) showed secondary constrictions to occur/

occur most frequently in all the acrocentric chromosomes of the karyotype. In these, a secondary constriction in the short arm separates a terminal mass, the satellite, from the rest of the chromosome arm. These workers also showed that secondary constrictions occur at specific sites on other chromosomes although less frequently than the former. The regularity of these findings is in close association with Wald and Turner (1964), who also showed that constrictions rarely occur on both homologues in the same preparation.

The structural nature and functional significance of secondary constrictions is still in doubt. Darlington and La Cour (1941) suggested that they are areas of a chromosome in which there is a reduced amount of nucleic acid (negatively heteropynotic). Woodward and Swift (1964) however, did not find reduced nucleic acid in these areas and suggested that the negative heteropyknosis seen by Darlington and La Cour, was a local uncoiling effect due to the cold treatment used in their preparations. Autoradiographic studies of human chromosome have shown that secondary constrictions correspond to regions which are later replicating (Schmid/

(Schmid 1963).

Although the term primary constriction is used for the centromere, the nature of primary and secondary constrictions does not appear to be entirely similar. Saksela and Moorehead (1962) showed that these regions responded differently to experimental fixatives and flame drying. They suggested that secondary constrictions may be heterochromatic.

A further property of secondary constrictions is that there is apparently no significant variability in frequencies of homologues with or without constrictions between individuals (Palmer and Funderburk 1965). This is of considerable importance if they are to be valuable in chromosome identification.

Secondary constrictions on the chromosomes of domestic animals have been described only in the cat, as occurring on Chromosome I, Group E., (Chu, Thuline and Norby 1964). Odd reference is made to single cases occurring in karyotypes of some wild animals such as elk deer, (Nes, Amrud and Tondevold 1965).

In the karyotype of the sheep, in which the identification/

identification of individual autosomes is difficult, the possibility of finding additional markers such as secondary constrictions has an added importance. Further, as suggested by Palmer and Funderburk (1965), secondary constrictions may assist in delimination of smaller chromosomal aberrations than can be shown by present techniques.

CHROMOSOME ASSOCIATIONS and ABERRATIONS.

Two types of chromosomal associations have been demonstrated in human mitotic chromosome preparations - association between the five pairs of satellite acrocentric chromosomes, and associations of the satellited chromosomes to sites on non-satellited chromosomes, where secondary constrictions have been demonstrated (Ferguson-Smith and Handmaker, 1961, 1963).

The association of the five pairs of satellited acrocentric chromosomes has been shown by these authors to be of a specific nature, and concerned with nucleolus formation. Confirmatory evidence of this was given from terminal nucleoli seen in chromosome bivalents of meiotic preparations, which were interpreted as being the five pairs of satellited/

satellited chromosomes (Ferguson-Smith 1964).

In man the hypothesis has been put forward that the association between acrocentric chromosomes and sites on other non-satellited chromosomes, where secondary constrictions have been demonstrated, are near to nucleolar-organiser regions (Ferguson-Smith and Handmaker, 1963).

Nucleolar-organiser regions are regions near which nucleoli arise, and have been shown in several species to be associated with secondary constrictions (Heitz, 1931 and b, and McLintock 1934).

The association of chromosomes is not only important in helping chromosome identification, but also because of their possible involvement in chromosome aberration.

The main autosomal aberrations in the chromosomes of man have been found to be connected with satellited chromosomes, for example trisomy of Chromosome 21-22 (Lejeune, Gautier and Turpin 1959). In the hamster, Hsu and Somers, (1961) noted that chromatid breaks occurred close to regions where secondary constrictions existed. This added evidence to the suggestion of Ferguson-Smith and Handmaker, (2963) that the deletion of Chromosome/

Chromosome 21, to produce the Philadelphia Chromosome (Nowell and Hungerford, 1960) coincided with the secondary constriction on its longarm.

KARYOTYPE PREPARATION.

Method.

The construction of an idiogram and the examination of karyotype are complementary. It is not possible to construct a complete idiogram without the examination of a number of karyotypes, and the full significance of the karyotype does not become apparent without the idiogram. In this study at least five photographic karyotypes were made from each animal, but in cases of very good preparations, more karyotypes were prepared. In the abnormal animals this was extended even further to ensure that coverage was as complete as possible. This included the preparation of karyotypes of suspected aneuploid cells (hyperdiploid) in order to differentiate additional chromosomes from chromosome fragments.

As stated previously, suitable metaphases for photographing were collected during routine counting examination of slides. As far as possible only metaphases/

metaphases in which the individual chromosomes were reasonably well separated were photographed. Overlapping of chromosomes obviously reduced vision of morphological features and further made enlargement more difficult.

All photographs were taken under a Wild M20 microscope with photographic attachment and camera. For photography, the wide field eye pieces were exchanged for one ordinary X 10 eye piece and X 10 photographic eye piece with focussing sight and frame. A Balzer's green filter was placed over the built-in light source for photography to improve chromosome definition. A Leitz exposure meter, interchangeable with one eye piece was used to make the exposure reading. All photographs were timed accordingly, and made on Ilford micro-neg. panchromatic film.

Developed film was used to make enlargements for karyotype analysis. For this purpose a "Gnome" Kodak enlarger was used and the enlarging head was set at such a predetermined position as to give a final photographic enlargement of three and a half thousand. This position was found by photographing a calibrated stage micrometer and then/

then using this negative in the enlarger to find the position to give a magnification of three and a half thousand. This was achieved by adjusting the enlarger head so that the distance measured between the middle of two micrometer lines from the negative scale 1000 microns was 3.5 cms.

Three or four prints were made of each photographed metaphase at this enlargement on a suitably graded printing paper. This aspect of the work required experience. Soft negatives requiring harder paper to give comparable contrast with normal negative printed on normal paper. These were developed in D 163 developer for a period of one and half to two minutes and then placed momentarily in a stop solution of 2% acetic acid. Fixation was for ten minutes to half an hour. Developer was freshly prepared from a stock solution before each batch of prints was made. The developing process was completed by washing for several hours in a tank with circulating water. Prints were finally glazed and sorted ready for karyotyping.

With an ademco hot iron, ademco dry mount paper was pressed on to the back of each photo-

graphic/

photographic enlargement of a metaphase. Each individual chromosome was then carefully cut out, checking that none were missed and none duplicated. These were placed on sheets of white card measuring 10" by 12" with ruled lines at 1½", 1½", 1¼" and 1¼". The chromosomes were then arranged by visual appraisal in paired homologues in descending order of size with the centromere, as nearly as could be judged, placed on the line. In the acrocentric group of chromosomes it was usually very difficult to determine a centromere position, even though what appeared to be small arms projected from the terminal end. When these were visible they were placed above the marking line.

What was tentatively considered to be the X chromosome was placed at the beginning of the large acrocentric series of chromosomes. The Y chromosome in male preparations fell into its obvious position at the end of the whole series.

Thus a routine karyotype consisted of:-

Row 1. Three pairs of metacentric chromosomes and four pairs of acrocentric chromosomes in a female or three pairs of metacentric chromosomes and one X, and three pairs of acrocentric/

acrocentric chromosomes in the male.

Row 2. Seven pairs of acrocentric chromosomes.

Row 3. Seven pairs of acrocentric chromosomes.

Row 4. Six pairs of acrocentric chromosomes in the female or six pairs of acrocentric chromosomes plus the Y, in the male.

The prepared karyotype was then mounted by heating in a hot press, an uncut photograph of the metaphase attached to it and the whole labelled and filed for reference in idiogram construction. In the course of this study, approximately three hundred and twenty photographic karyotypes were prepared from twenty seven different sheep.

MORPHOLOGICAL FEATURES OF THE SHEEP CHROMOSOMES.

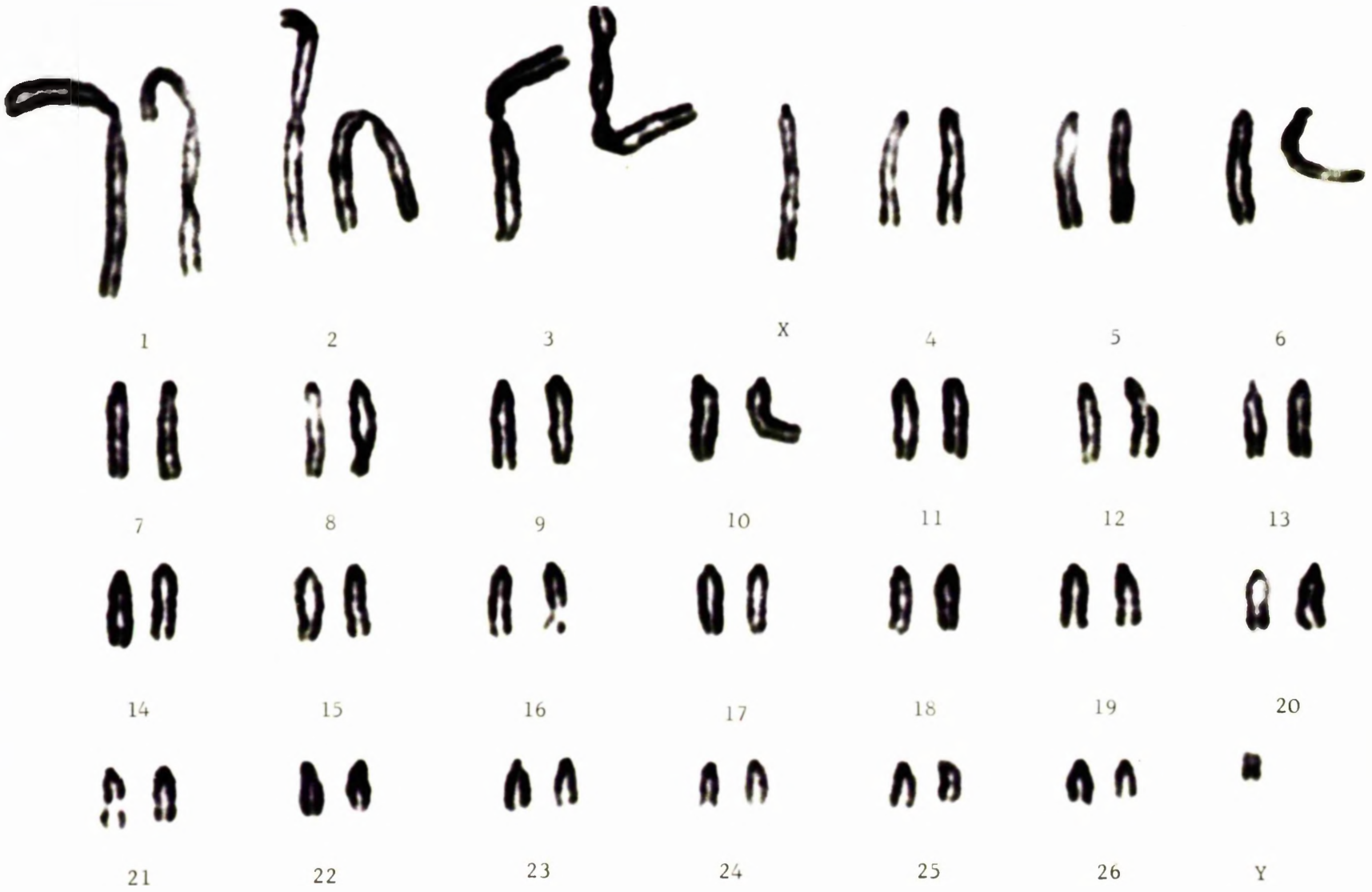
The main morphological findings of the examination of the sheep chromosome are featured in the series of photographs included in the following pages. Several points are demonstrated. In Fig. 17 a typical male karyotype is shown with the small submetacentric Y chromosome clearly distinguishable at the end of the chromosome series. Also shown is the additional long arm length of the X chromosome over the next largest pair of acrocentric chromosomes.

The/

The advantage of this distinction is less obvious when more contracted metaphases are examined. Metaphases which have had prolonged colchicine treatment, while still suitable for counting, do not permit easy identification of the X chromosomes (Fig. 18). Apart from the extra length of the long arms of the X chromosomes it has quite distinct short arms, which are generally more apparent than those of other members of the acrocentric group of chromosomes. Even in the more contracted chromosome preparations, the size of the short arms of the X chromosome often assist in its identification. This latter feature is shown clearly in Fig. 19.

While the identification of the sex chromosomes of the sheep, particularly the Y chromosome was found to be possible, autosomal identification was shown to be very difficult. The pairing of the six large metacentric chromosomes can usually be carried out with reasonable visual accuracy but the pairing of the forty six acrocentric chromosomes is approximate, no degree of accuracy being suggested on length measurements alone.

Fig. 17. KARYOTYPE of the RAM. (X 3500).


1
2
3
X
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25
26
Y

Fig. 18. KARYOTYPE of the EWE. (X 3500).

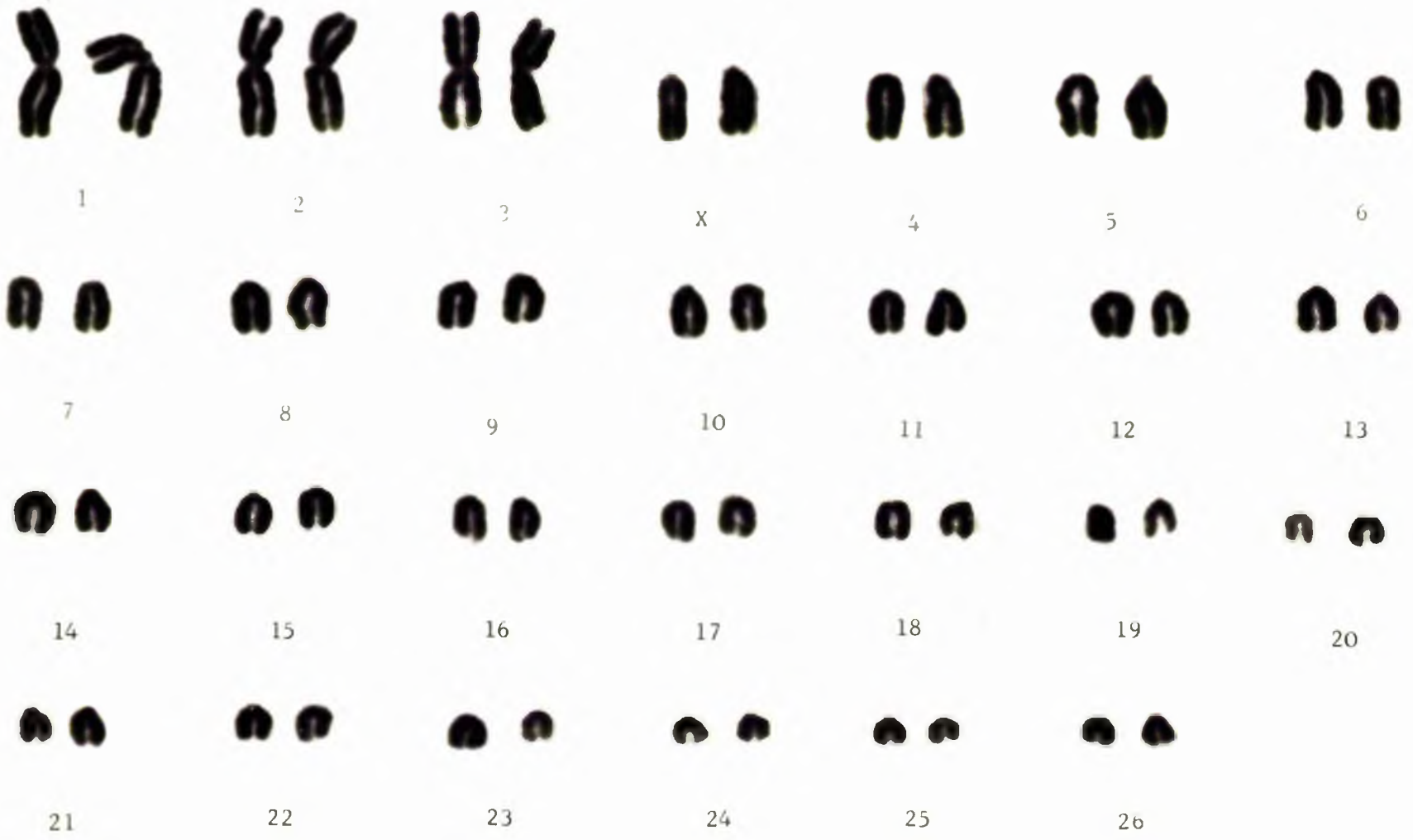
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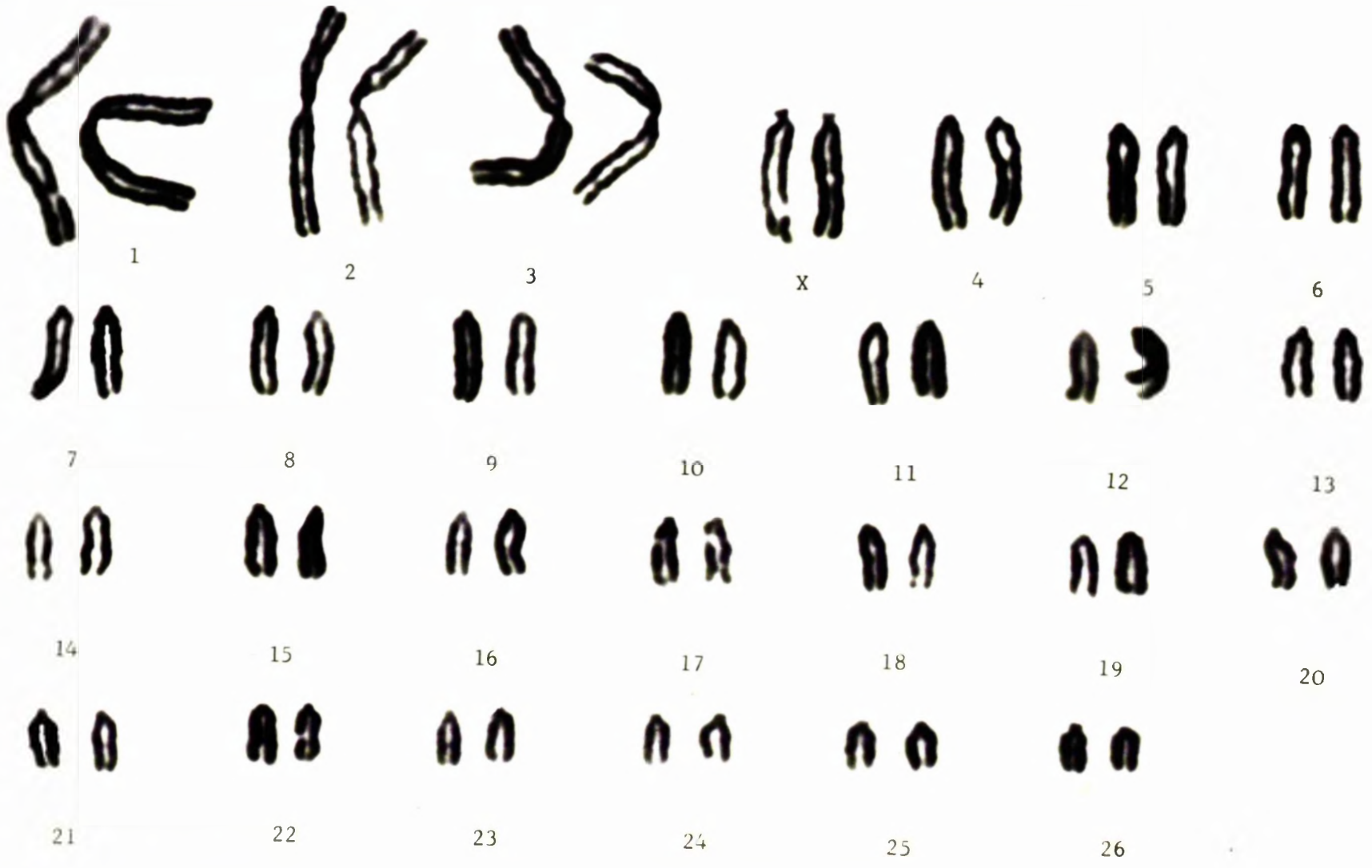
Fig. 19. KARYOTYPE of the EWE. (X 3500).

Note: short arms on both X chromosomes.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THE IDIOGRAM OF THE SHEEP.

It can be seen from the nature of the sheep's karyotype that the identification of individual chromosomes, particularly the acrocentric group, is extremely difficult so that in order to extend present knowledge every recordable morphological feature becomes important. The construction of an idiogram purely on length measurements would do little to add to the present limited means of identification. It is however essential that measurements are made and an idiogram constructed in order to form a foundation on which to add additional morphological features. In this study, the general principles of idiogram construction followed were those of the Denver Report (1960). The main difference in the construction of idiograms of different species by different workers is not the method of measurement followed so much as the number of cells measured. In this there is considerable variation. Rothfels and Siminovitch (1958) produced an excellent idiogram of the Rhesus monkey measuring the chromosomes of ten cells, five of each sex. Likewise, Levan and Hsu (1959) produced on the same number of cells a human idiogram which agrees closely with other workers. No advantage/

advantage could be seen in measuring the chromosomes of very large numbers of cells unless for special reasons such as the comparison of cell strains (Levan, Hsu and Stich, 1962) or checking the sources of chromosome measurement variation (Hsu and Zenzes, 1964).

METHOD.

The method of construction largely followed was that of Rothfels and Siminovitch (1958) and in the addition of secondary constrictions Ferguson-Smith et al. (1962).

Care was taken to select metaphases that were evenly spread and in which a minimum of chromosome overlapping had occurred. No great stress was placed on degree of chromosome contraction since all measurements were to be reduced to a relative association. Very long early metaphases and very short metaphase preparations were discarded. Ten suitable metaphases for measurement were chosen, five male and five female, from seven different animals, three ewes and four rams. In each cell measured a corresponding photographic karyotype with assigned chromosome numbers was prepared.

Measurements were made by tracing photographic negatives held in a film carrier and projected on to white/

white drawing paper pinned to a wall. Care was taken to ensure that the projector was focussed on the drawing paper so that an imaginary line drawn through the light source film and lenses met the paper at 90°. The projector was placed at a measured distance in front of the screen gauged by using a projected micrometer scale, so that the chromosomes were enlarged to approximately X 10,000.

In tracing the chromosomes, each chromatid was drawn separately. To avoid ambiguity over centromere position of the large metacentric chromosomes, a cross was placed where the centre of the non-staining centromere was judged to be. The individual chromatid arms were then retraced from end to centromere for each chromosome using a map marker. Measurements were recorded to the nearest half millimetre. For each karyotype traced, the following data was then finally recorded (Table 9) forming the primary data from which the final relative relationships between chromosomes was calculated. In the five male karyotypes, the Y chromosome was not included in the total chromosome length, but instead the X chromosome measurement was included twice, bringing the male and female measurements/

measurements to a common level. It was realised that this may lead to some small error in X chromosome measurement, since one very often appeared longer than its homologue.

The Y chromosome was finally measured and compared with the total chromosome length, as estimated by this procedure.

From the primary data for each chromosome the lengths of both chromatids were averaged (Table 10) including both arms for the six metacentric chromosomes. Finally the measurements for the paired homologues for each metaphase were averaged. Thus, the mean data for each metaphase consisted of measurements of the haploid set of twenty-seven measurements. Three of the twenty-seven measurements were subdivided into two measurements corresponding to the short and long arms of the metacentric chromosomes. No rearrangement of chromosomes was made in the karotype following measurements as was done by Rothfels and Siminovitch (1958). With only few exceptions among the small acrocentric chromosomes the measurements were of a descending order. It was further considered that the changing of pairs did not affect the total chromosome length and the differentiation of individual members of the large acrocentric/

acrocentric group of chromosomes obviously cannot be concluded on length measurement alone.

For each measured metaphase the length of each chromosome was expressed as a percentage per thousand parts of the total chromosome length. (Calculated by adding the data in the final column at Table 10).

$$\frac{X \times 1,000}{\text{Total chromosome length}}$$

The results of these calculations for each of the 10 metaphases are shown in Table 11, and the average results used in idiogram construction in Table 12.

For the three large metacentric chromosomes and the Y chromosome, the position of the centromere relative to the total chromosome length was determined by comparison of the length of the long arm with the latter using the following calculation.

$$\frac{\text{Long Arm Measurement} \times 1,000}{\text{Total Chromosome Length.}}$$

the result being known as the centromeric position.

This modification of the Denver Report centromeric index made direct measurement for drawing the idiogram simpler. See Table 13.

The final diagnostic parameter, again for the metacentric chromosomes and the Y chromosome only, was/

TABLE 9.

EXAMPLE OF PRIMARY DATA RECORDED FROM CHROMOSOME MEASUREMENTS OF ONE EWE (EWE 18).

		Long Arm	Short Arm	Total Chromosome Length
1	1	6.70	4.80	11.50
	2	6.25	4.70	10.95
2	1	5.70	5.05	10.75
	2	5.50	5.05	10.55
3	1	6.00	4.75	10.75
	2	5.70	4.50	10.20
4	1	5.75	4.65	10.40
	2	5.85	4.80	10.65
5	1	5.10	4.55	9.95
	2	6.20	4.30	10.50
6	1	5.30	5.85	11.15
	2	5.00	4.40	9.40
X	1			5.60
	2			5.75
X	1			5.75
	2			5.70
7	1			5.50
	2			5.55
8	1			5.60
	2			5.25
9	1			5.00
	2			4.80
10	1			4.70
	2			4.65
11	1			4.40
	2			4.90
12	1			4.20
	2			4.30
13	1			4.30
	2			4.40
14	1			4.05
	2			4.05
15	1			4.15
	2			3.90
16	1			3.70
	2			3.80
17	1			4.10
	2			3.85
18	1			3.85
	2			3.65
19	1			3.30
	2			3.40
20	1			3.65
	2			3.40
21	1			3.40
	2			3.40
22	1			3.80
	2			3.35
23	1			3.50
	2			3.75
24	1			3.30
	2			3.30
25	1			3.30
	2			3.20
26	1			3.10
	2			3.10
27	1			3.10
	2			2.95
28	1			3.10
	2			3.00
29	1			3.20
	2			3.00
30	1			3.10
	2			3.05

		Total Chromosome Length
31	1	3.40
	2	3.15
32	1	2.95
	2	2.90
33	1	2.90
	2	2.90
34	1	2.85
	2	3.00
35	1	2.80
	2	2.80
36	1	2.80
	2	2.70
37	1	2.75
	2	2.30
38	1	2.60
	2	2.90
39	1	2.60
	2	2.60
40	1	2.70
	2	2.70
41	1	2.50
	2	2.45
42	1	2.40
	2	2.50
43	1	2.50
	2	2.40
44	1	2.60
	2	2.25
45	1	2.50
	2	2.30
46	1	2.40
	2	2.35
47	1	2.30
	2	2.20
48	1	2.25
	2	2.20
49	1	2.30
	2	2.30
50	1	2.25
	2	2.30
51	1	2.20
	2	2.15
52	1	2.20
	2	2.30

TABLE 10.

EXAMPLE OF MEAN PRIMARY DATA OF TABLE 9. (EVE 18).

		Mean Length of Long Arm.	Mean Length of Short Arm.	Mean Total Lengths	Mean Length of Homologues
1	1	6.475	4.75	11.225	10.9375
	2	5.6	5.05	10.65	
2	3	5.85	4.625	10.475	10.5
	4	5.80	4.725	10.525	
3	5	5.65	4.425	10.075	10.175
	6	5.15	5.125	10.275	
X	7			5.675	5.7
	8			5.725	
4	9			5.525	5.475
	10			5.425	
5	11			4.9	4.7875
	12			4.675	
6	13			4.65	4.45
	14			4.25	
7	15			4.35	4.2
	16			4.05	
8	17			4.025	3.8825
	18			3.75	
9	19			3.975	3.8625
	20			3.75	
10	21			3.35	3.4375
	22			3.525	
11	23			3.4	3.4825
	24			3.575	
12	25			3.625	3.4625
	26			3.3	
13	27			3.25	3.175
	28			3.1	
14	29			3.025	3.0375
	30			3.05	
15	31			3.1	3.08725
	32			3.075	

		Mean Total Lengths	Mean Lengths of Homologues
16	33	3.275	3.1
	34	2.925	
17	35	2.9	2.9125
	36	2.925	
18	37	2.8	2.775
	38	2.75	
19	39	2.525	2.6375
	40	2.75	
20	41	2.6	2.65
	42	2.7	
21	43	2.475	2.4625
	44	2.45	
22	45	2.45	2.4375
	46	2.425	
23	47	2.4	2.3875
	48	2.375	
24	49	2.25	2.2375
	50	2.225	
25	51	2.3	2.2875
	52	2.275	
26	53	2.175	2.2125
	54	2.25	

TABLE 11.

MEAN RELATIVE LENGTHS OF HOMOLOGUOS CHROMOSOMES FROM

Chromosome	117 ♀	18 ♀	33 ♀	18 ♀	33 ♀	J65 ♂
1	109.062	97.873	108.566	96.960	99.0	111.400
2	93.017	93.958	99.673	87.229	91.526	91.942
3	83.568	91.050	95.142	79.560	86.697	87.564
X	49.314	51.006	52.857	49.338	51.627	49.619
4	46.925	48.992	46.061	45.904	44.383	43.539
5	44.885	42.840	43.628	41.830	42.313	42.687
6	43.802	39.820	38.090	40.295	40.474	40.255
7	38.329	37.583	38.342	40.180	41.279	38.552
8	35.927	34.742	35.825	36.403	36.334	35.877
9	34.352	34.563	32.805	34.571	34.725	31.742
10	31.990	30.76	32.637	33.770	33.573	29.796
11	32.581	31.163	30.623	32.511	31.965	32.228
12	31.104	30.984	30.456	31.939	29.780	31.620
13	29.628	28.411	27.351	31.480	29.550	29.918
14	28.151	27.181	26.093	31.435	28.400	29.674
15	27.954	27.626	26.428	29.076	28.400	27.364
16	25.690	27.740	25.002	26.901	26.216	25.904
17	25.001	26.062	24.331	26.901	25.641	26.585
18	25.789	24.832	23.660	26.558	25.871	26.512
19	22.639	23.601	23.324	24.726	24.951	21.891
20	21.261	23.713	21.982	25.986	25.006	21.891
21	21.556	22.035	22.233	23.696	22.307	21.891
22	22.245	21.812	21.394	22.551	22.077	22.864
23	20.375	21.364	20.555	21.979	20.812	22.621
24	18.210	20.022	19.549	20.033	19.432	20.801
25	19.686	20.470	17.032	19.346	19.202	17.513
26	16.930	19.798	16.360	18.888	18.397	17.628
Y						10.702

10 KARYOTYPES EXPRESSED AS LENGTH OF CHROMOSOME X 1000 / T. C. L.

26012 ♂	26012 ♂	N165 ♂	26013 ♂	MEAN
103.088	99.273	100.956	99.08	102.526
95.659	87.037	90.296	88.04	91.838
77.462	80.919	85.396	78.981	84.634
52.420	48.020	51.458	50.389	50.605
47.245	45.134	44.230	44.728	45.717
45.743	43.518	40.799	41.755	43.000
42.237	43.288	40.309	40.481	40.905
38.147	38.670	37.736	37.650	38.647
36.227	38.555	34.795	37.226	36.191
36.728	34.630	33.815	35.810	34.374
33.306	33.707	32.100	35.669	32.731
32.387	32.321	32.835	31.847	32.036
30.217	30.590	32.100	31.423	31.021
30.134	31.167	30.752	30.715	29.910
29.382	29.551	30.507	30.149	29.052
27.963	29.089	27.689	29.866	28.146
26.878	27.242	29.282	27.742	26.860
26.210	28.050	28.424	26.752	26.396
24.040	25.049	25.484	26.184	25.298
23.122	22.856	25.974	24.345	23.743
21.703	24.241	24.749	23.779	23.437
21.703	23.202	22.421	22.505	22.355
20.701	21.817	21.318	23.779	22.055
20.868	21.355	20.706	21.231	21.187
18.447	21.701	18.623	20.382	19.72
19.115	19.970	18.743	19.674	19.075
18.197	19.047	18.5	19.816	18.356
11.018	12.005	12.252	8.492	10.894

TABLE 12.

MEAN % TCL (HAPLOID) EXPRESSED AS $\frac{\text{CHROMOSOME LENGTH} \times 1000}{\text{TCL}}$

FOR 5 MALE and 5 FEMALE METAPHASES.

	MEAN % OF T.C.L.		
Chromosome	5 ♂	5 ♀	5 ♂ + 5 ♀
1	102.76	102.29	102.53
2	90.60	93.08	91.84
3	82.06	87.20	84.63
X	50.38	50.83	50.60
4	44.98	46.46	45.72
5	42.90	43.10	43.00
6	41.31	40.50	40.91
7	38.15	39.14	38.65
8	36.54	35.85	36.19
9	34.55	34.20	34.37
10	32.92	32.55	32.73
11	32.32	31.77	32.05
12	31.20	30.85	31.02
13	30.54	29.28	29.91
14	29.85	28.25	29.05
15	28.39	27.90	28.15
16	27.41	26.31	26.86
17	27.20	25.59	26.40
18	25.45	25.34	25.40
19	23.65	23.85	23.75
20	23.27	23.60	23.44
21	22.34	22.37	22.35
22	22.10	22.02	22.06
23	21.36	21.02	21.19
24	19.99	19.45	19.72
25	19.00	19.15	19.08
26	18.64	18.08	18.36
Y	10.89		10.89

TABLE 13

CENTROMERIC POSITION EXPRESSED AS $\frac{\text{LONG ARM} \times 1000}{\text{TCL}}$ FOR METACENTRIC CHROMOSOMES.

(BASED ON 10 METAPHASES)

Chromosome	117 ♀	18 ♀	33 ♀	18 ♀	33 ♀	J65 ♂	26012 ♂	26012c ♂	M165 ♂	26013 ♂	MEAN
1	56.696	54.026	61.163	52.315	54.617	60.565	57.679	55.870	54.153	56.476	56.356
2	49.806	52.124	57.639	48.880	48.522	48.890	53.172	49.597	49.865	48.408	50.590
3	44.196	48.321	55.206	42.699	47.833	44.147	42.905	40.633	45.670	42.038	45.365
Y						5.838	6.678	6.464	6.861	4.529	6.074

TABLE 14.

ARM RATIO EXPRESSED AS $\frac{\text{LONG ARM LENGTH}}{\text{SHORT ARM LENGTH}}$ FOR METACENTRIC CHROMOSOMES.

(BASED ON 10 METAPHASES)

Chromosome	117 ♀	18 ♀	33 ♀	18 ♀	33 ♀	J65 ♂	26012 ♂	26012 ♂	M165 ♂	26013 ♂	MEAN
1	1.083	1.232	1.290	1.172	1.231	1.191	1.270	1.287	1.157	1.326	1.224
2	1.153	1.246	1.371	1.275	1.128	1.136	1.252	1.264	1.219	1.224	1.227
3	1.123	1.131	1.382	1.158	1.231	1.017	1.242	1.009	1.151	1.138	1.158
Y						1.200	1.539	1.167	1.273	1.143	1.264

was the arm ratio expressed as,

$$\frac{\text{Length of Long Arm}}{\text{Length of Short Arm}},$$

the results of which are shown in Table 14.

Before discussion of these results, it is pertinent that other morphological features be viewed so that the suggested idiogram can be considered as whole.

THE ADDITION OF SECONDARY CONSTRICTIONS TO THE IDIOGRAM

Three hundred and twenty photographic karyotypes were prepared in order to estimate whether sufficient secondary constrictions were present in any one chromosome, to help identification. These included the better metaphases viewed. No selection was made for the presence of secondary constrictions in these karyotypes. Photographic karyotypes do not always show constrictions to advantage but the bias was in favour of a low estimate rather than a high one. Further, except for the metacentric chromosomes it was difficult to assign a constriction to a given acrocentric chromosome without karyotyping.

Any euchromatic gap on either chromosome chromatid, or both, was accepted as a constriction. If the chromatids showed non-alignment a constriction

was/

was not recorded. Non-alignment of the chromatids at a euchromatic gap was taken as breakage unless clear strands of chromatin could be seen bridging the gap. These criteria for recognition of secondary constrictions are similar to those of Ferguson-Smith et al., (1962), and Oppenheim and Fishbein (1965).

A number of accurate sketch idiograms were duplicated, from the average measurements recorded in Table 12. The arms of each chromosome on these were divided into three parts from the centromere out as paracentric, middle and terminal thirds, as a guide to positioning. Each of the karyotypes was then examined and, as a constriction was noted, its relative position was carefully estimated and marked on to the sketch idiogram. So that, for each karyotype with a chromosome showing a constriction, there was a corresponding sketch idiogram. Using this method it was then a simple procedure to sort out recorded data for each chromosome and also to measure the position of each recorded constriction. The measurement was taken directly with a millimeter ruler from the centromere outwards.

The chromosome measurements obtained from the average of the ten karyotypes as show in Table 12 were/

were then used to draw up the final idiogram using a suitable scale, (Fig. 20). Lines drawn to scale and measured from the sketch idiograms were then added to the "ghost" set of chromosomes to represent the constrictions recorded. Finally, a number placed beside each line which represented a secondary constriction indicated the number of constrictions at this point on the three hundred and twenty karyotypes examined. If the constriction were very clear in one chromatid and not clear in the other this was indicated by the bracketed figure.

Examples of the more commonly occurring secondary constrictions are given in the following series of photographs Fig. 21 - Fig. 42.

DISCUSSION OF THE SHEEP IDIOGRAM AND KARYOTYPE.

While showing that there are clearly measurable differences between chromosome lengths in the sheep, the idiogram also emphasises the already obvious fact of how close are these differences, (Table I2 and Fig. 20).

The accuracy of visual estimation of chromosome length and position as shown in the karyotype, proved to be low at positions below Chromosome 7 in/

Fig. 20 THE IDIOGRAM of the SHEEP.

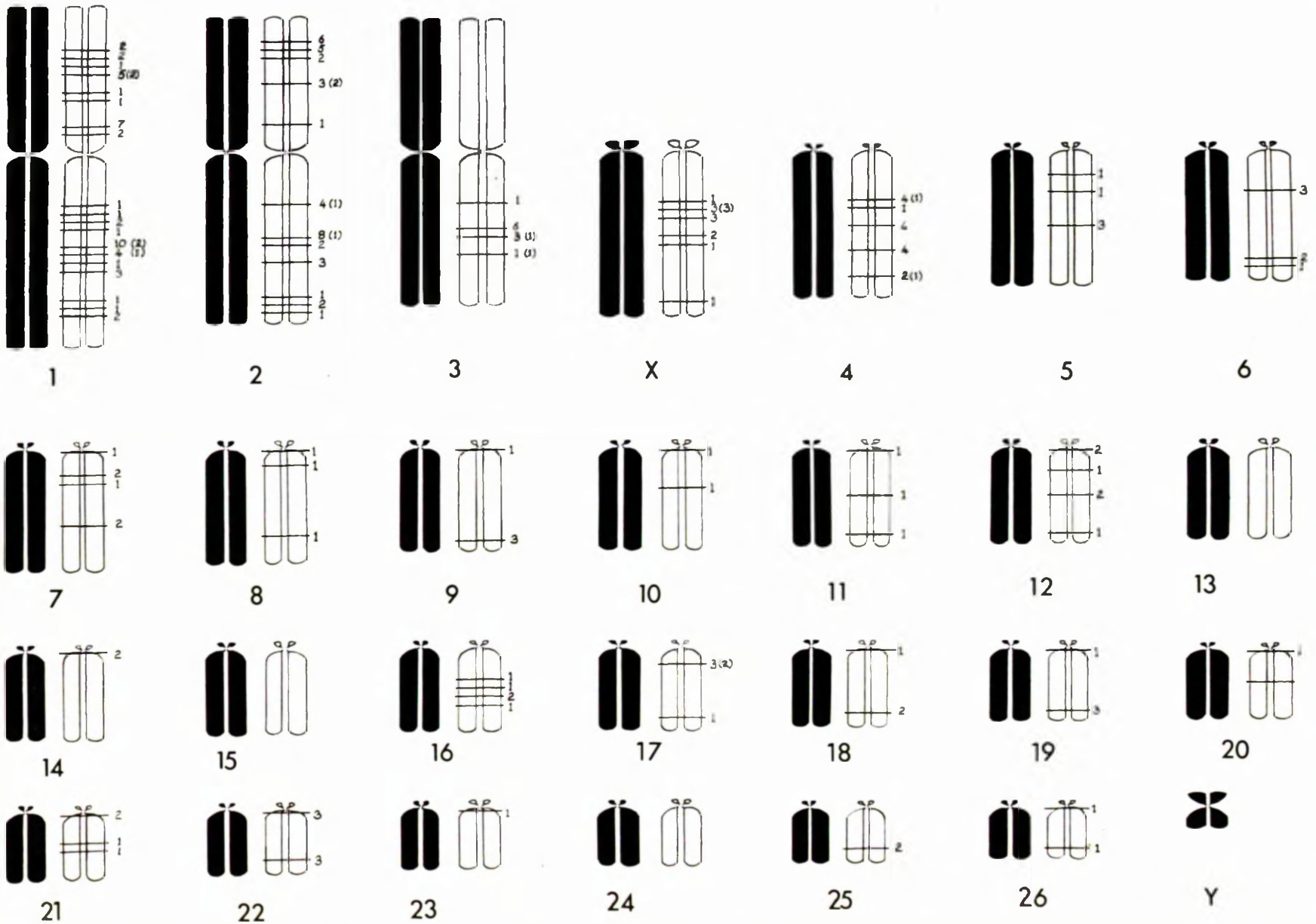

1
2
3
X
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
Y

Fig. 21.

Fig. 22.

Figs. 21, 22. Secondary Constrictions on Chromosome I at the middle/terminal position of the Short Arms. (X 3500).

1 2 3 X

1 2 3 X

Fig. 23. Paracentric Secondary Constrictions on Short Arms of Chromosome I. (X 3500)

Fig. 24. Terminal Long Arm Secondary Constriction on Chromosome I. (X 3500).

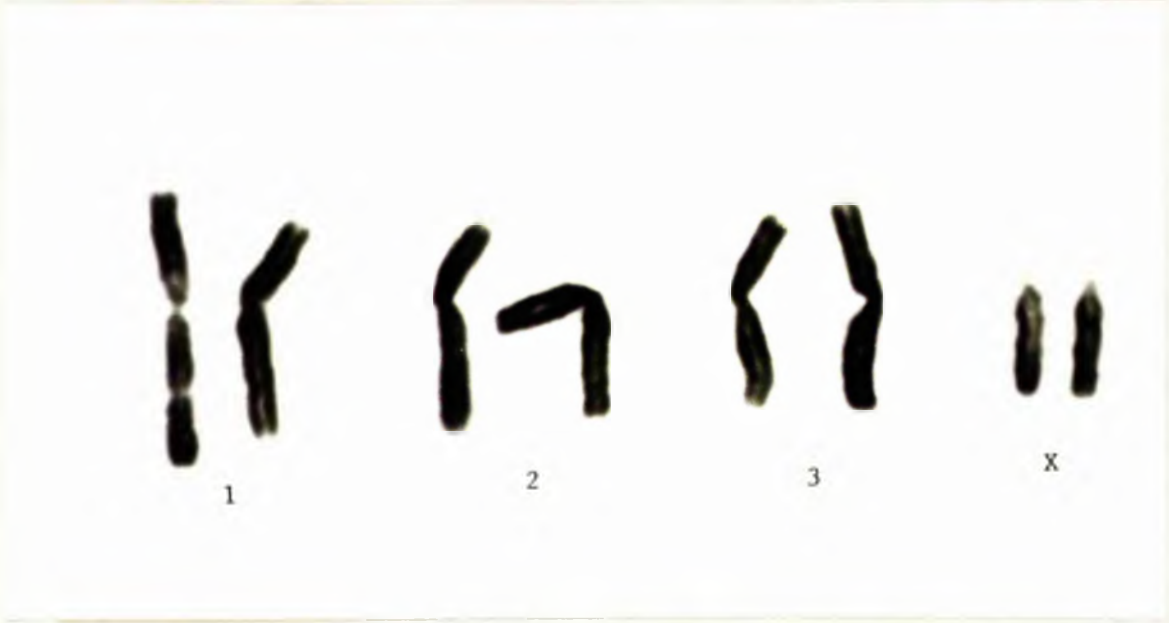

1
2
3
X


1
2
3
X

Fig. 25.

Fig. 26.

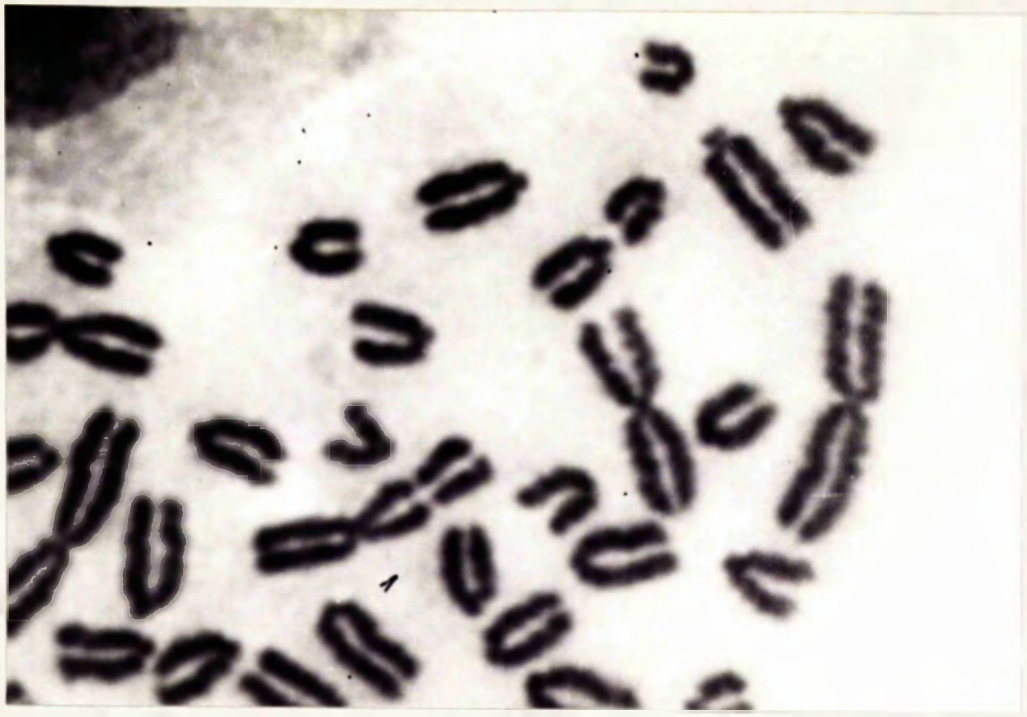
Figs. 25, 26. Middle Long Arm Secondary Constrictions on Chromosome I. (X 3500).


1
2
3
X


1
2
3
X

Fig. 27. Secondary Constriction. Lond Arm Chromosome I. (X 3500)

Fig. 28. Secondary Constriction. Short Arm Chromosome I. (X 3500).



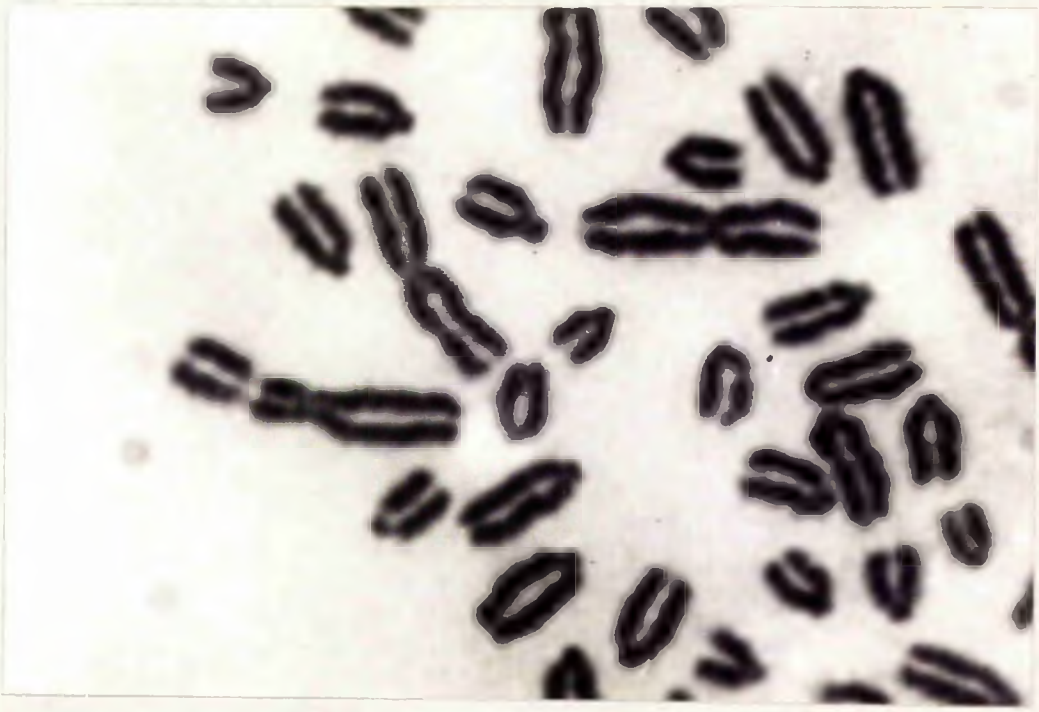


Fig. 29. Paracentric/Middle Short Arm Secondary Constriction on Chromosome 2. (X 3500).

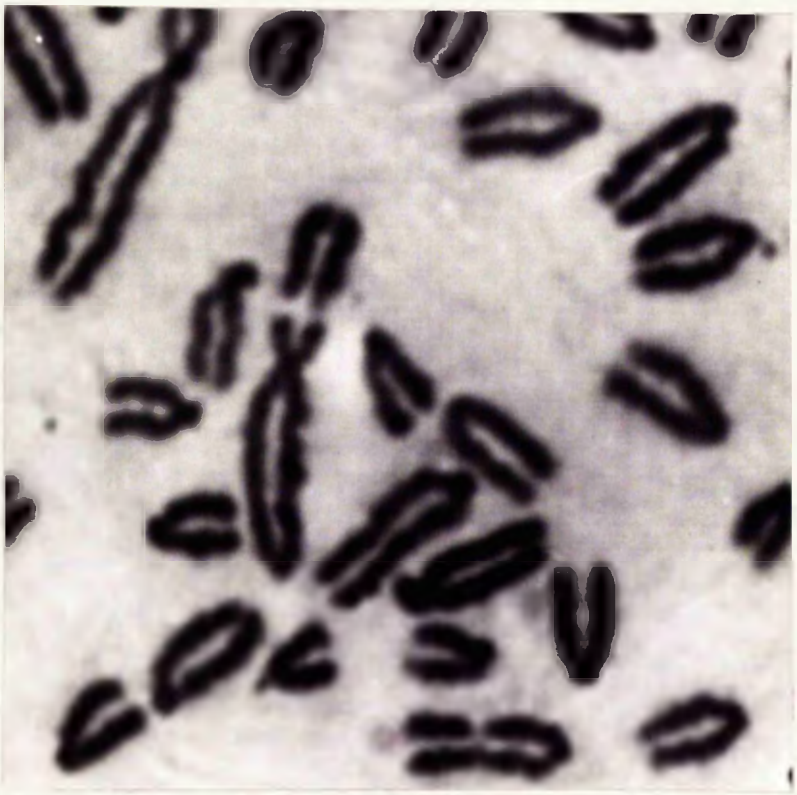


Fig. 30. Terminal Short Arm Secondary Constrictions on Both Homologues of Chromosome 2. (X 3500).

Fig. 31. Terminal Short Arm Secondary Constriction on Chromosome 2 also constriction on one X Chromosome and Both Arms of Chromosome 7. (X 3500).


1
2
3
X

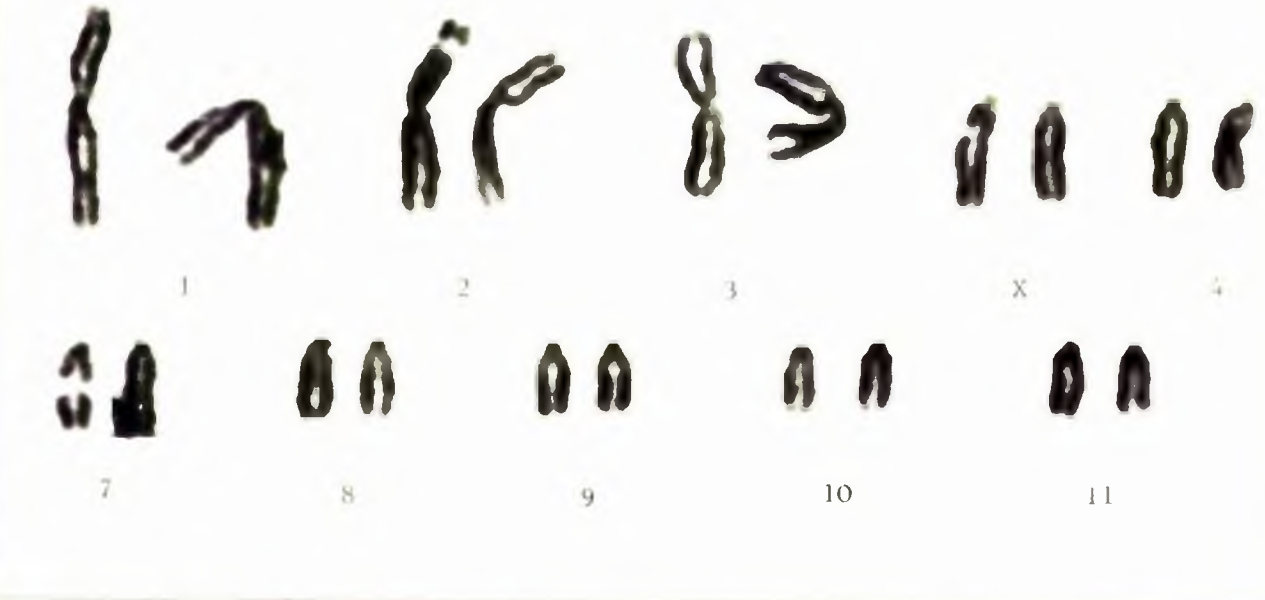
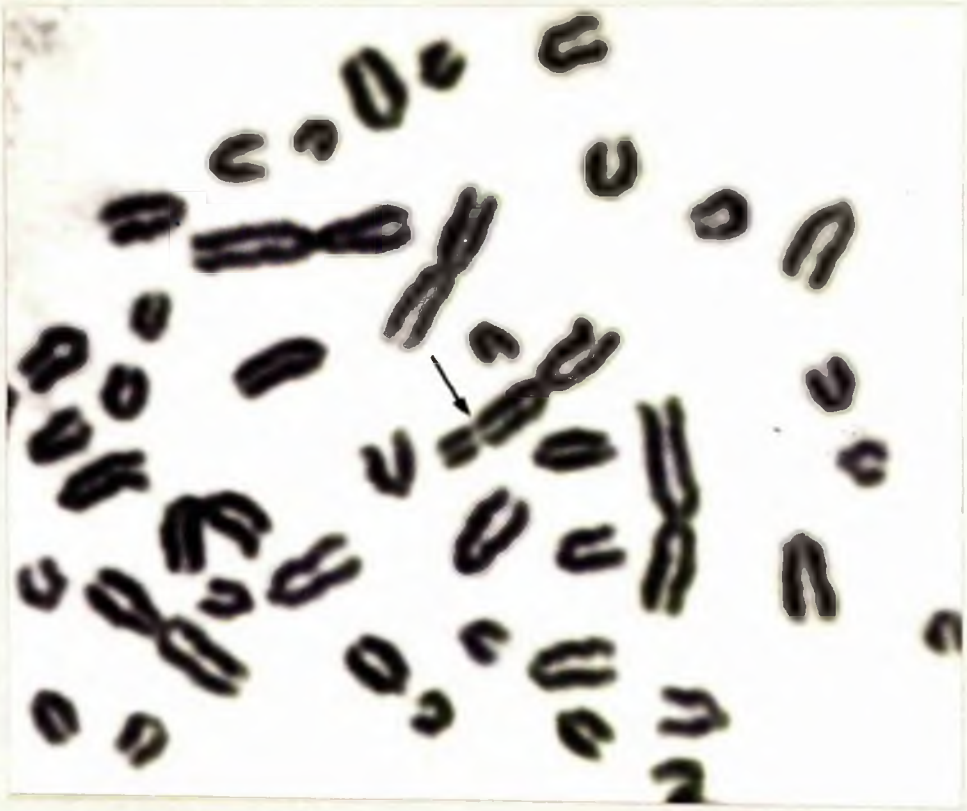

1
2
3
X
4
7
8
9
10
11

Fig. 32. Secondary Constrictions. Long Arm Chromosome 2. (X 3500).

Fig. 33. Secondary Constrictions. Long Arm Chromosome 2. (X 3500).



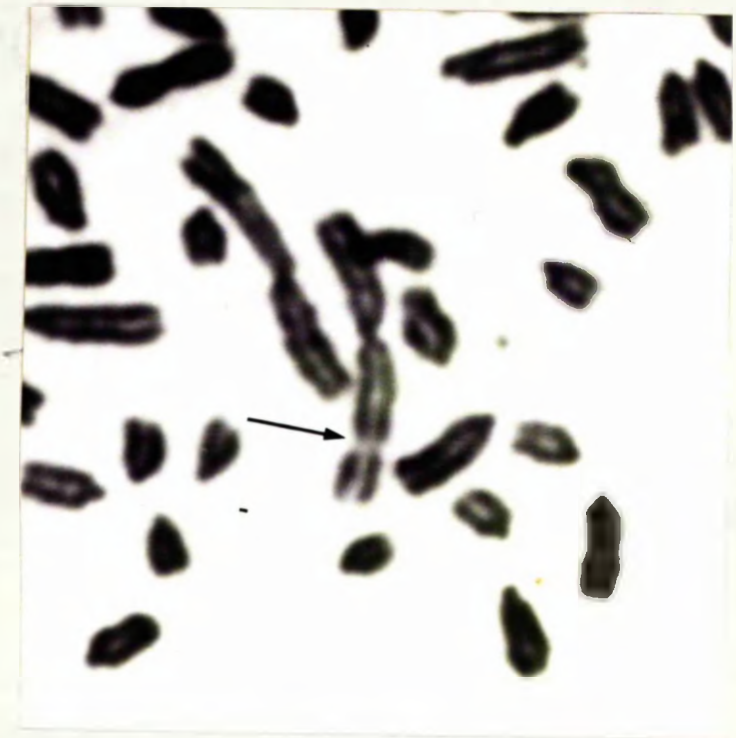


Fig. 34. Middle Long Arm Secondary Constriction on Chromosome 3. (X 3500)

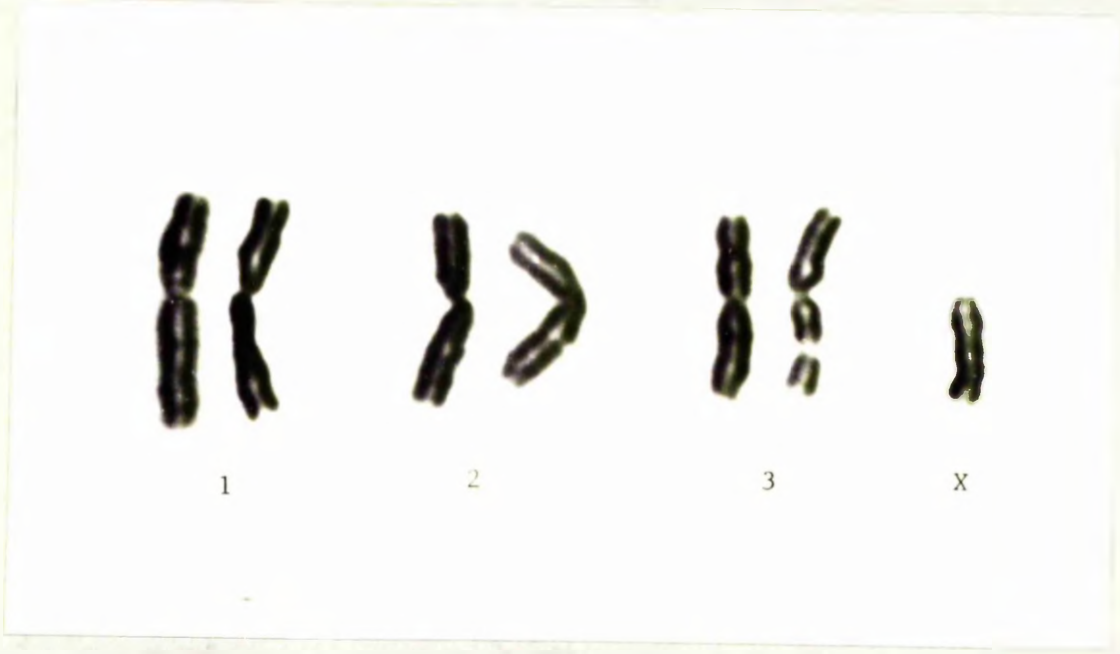
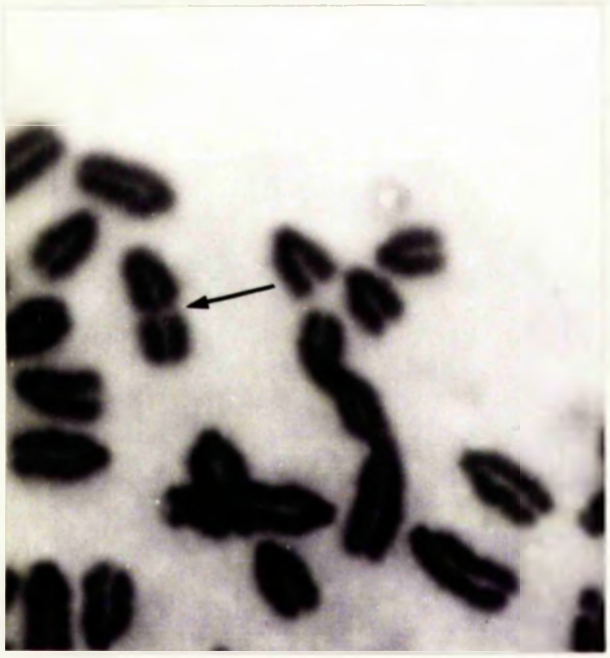

1
2
3
X

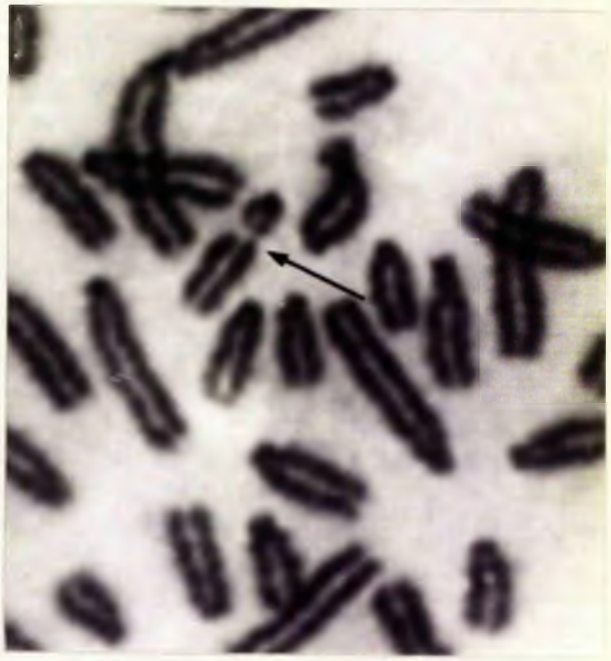
Fig. 35.

Fig. 36

Fig. 37.

Typical Secondary Constrictions on Various Acrocentric Chromosomes (X 3500).





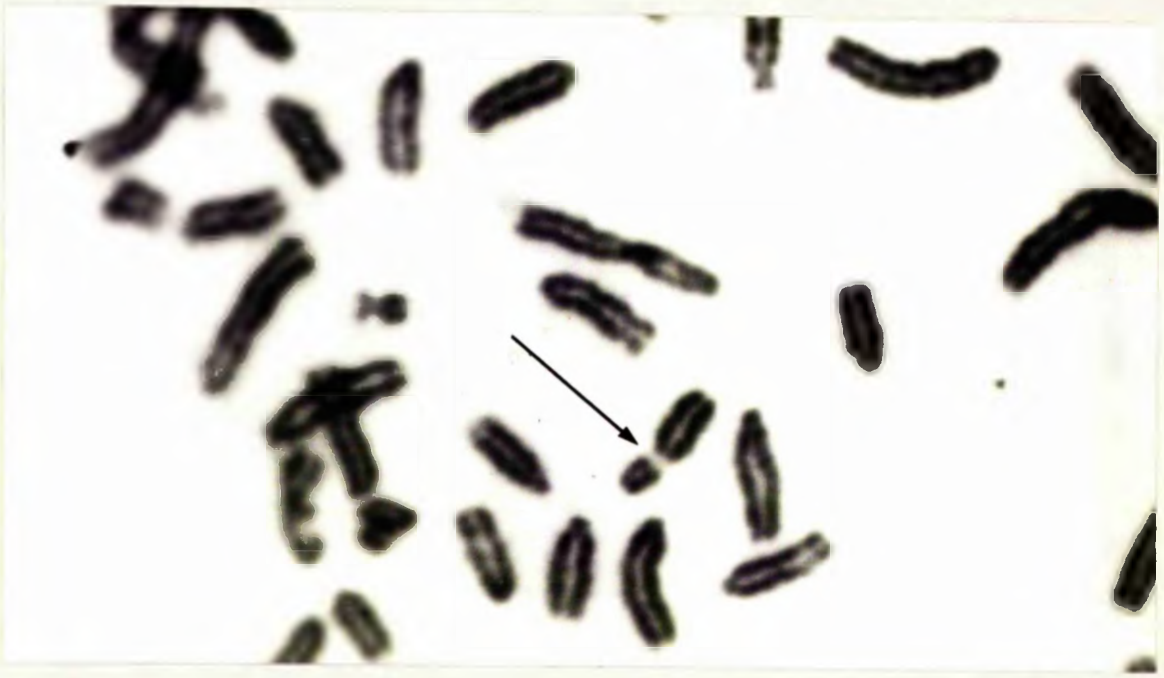


Fig. 38.

Fig. 39.

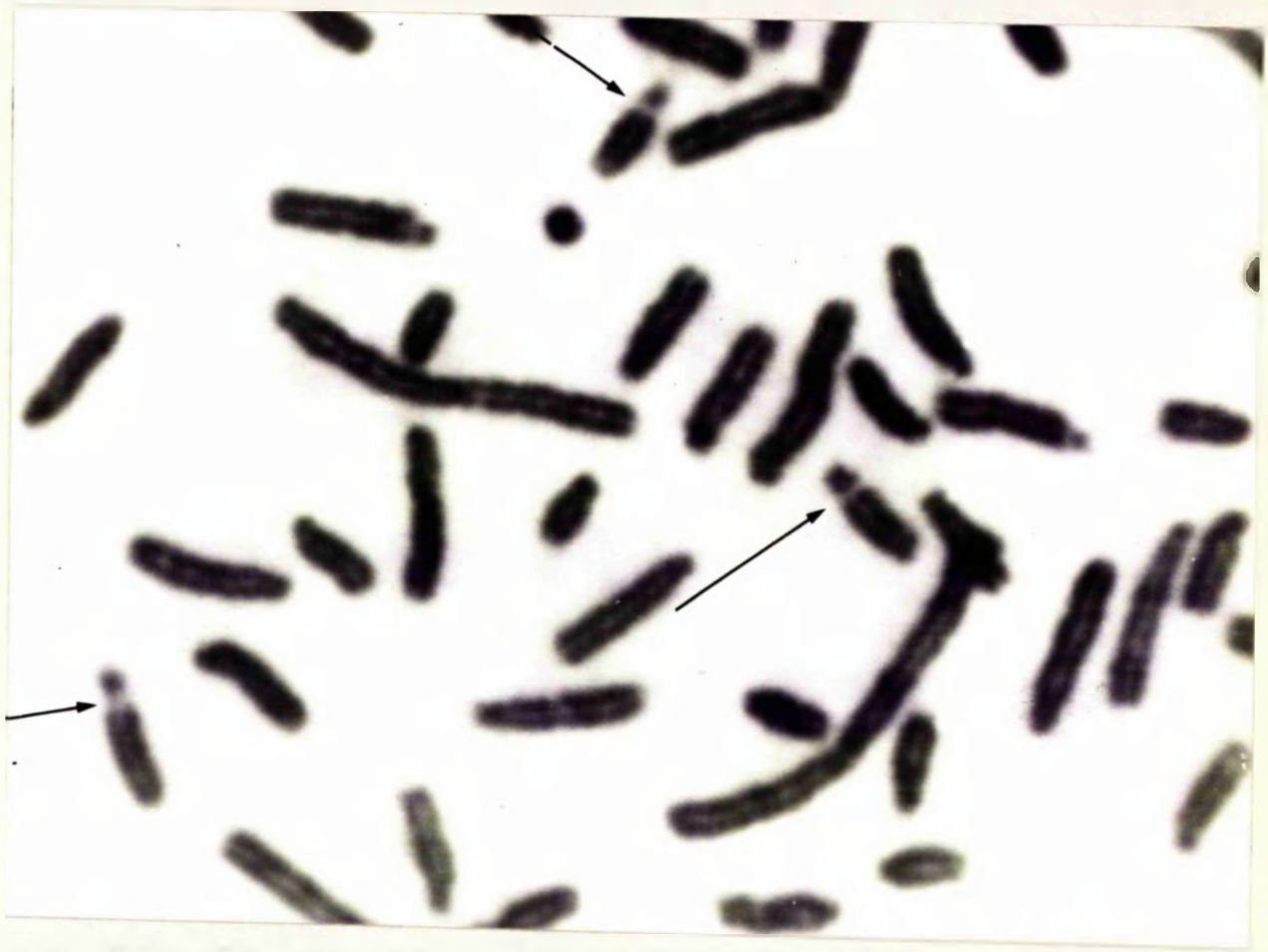
Two Clear Secondary Constrictions on the One Chromosome in Two Metaphases. (Acrocentric Chromosome). (X 3500).

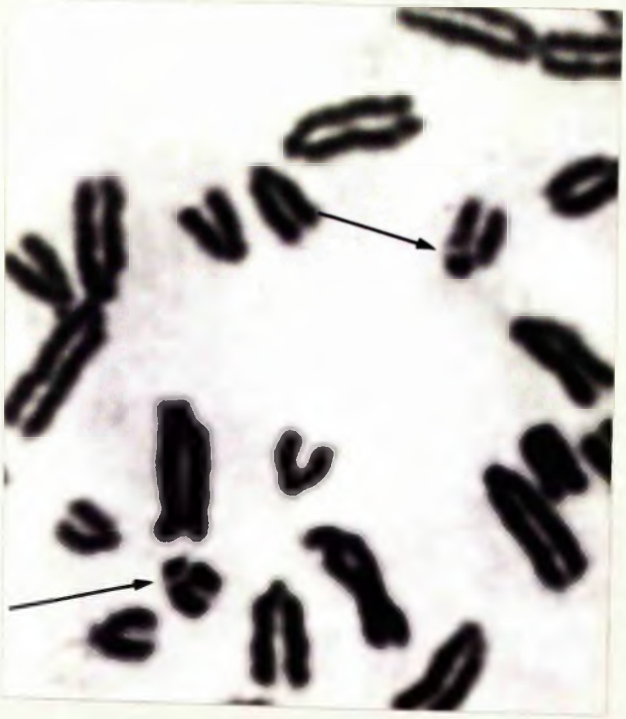
Fig. 40.

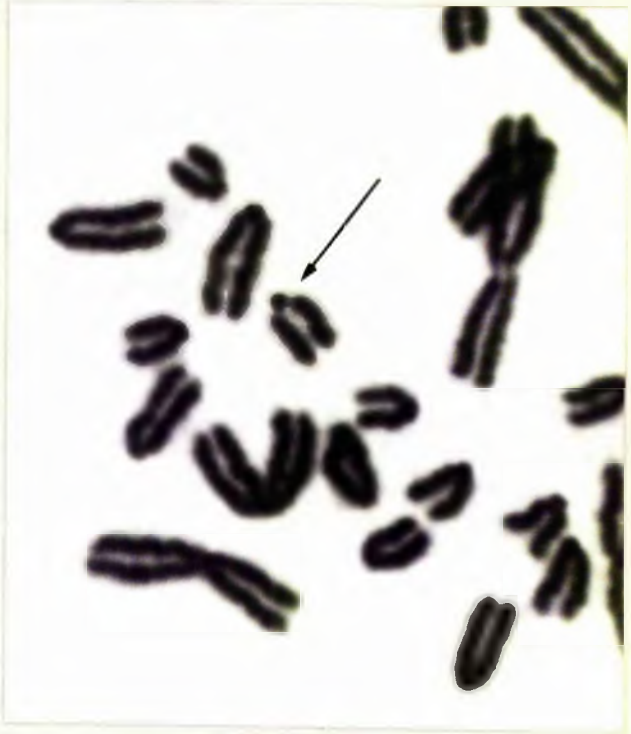
Fig. 41.

Fig. 42.

Examples of Presumed Secondary Constrictions in the Paracentric Position of Acrocentric Chromosomes Typical of the "Rabbit's ear" effect (Hsu, Billen and Levan, 1961). (X 3500).







in the ten karyotypes measured for the idiogram. Seventy four measurements were misplaced out of a possible total of two hundred and seventy, (Table 11). This large number of misplacements by measurement does not necessarily indicate the futility of karyotyping and numbering of these unidentifiable chromosomes, as has been suggested by Patau (1961) and Patau (1965).

However, although there were a large number of misplacements, the maximum misplacement was two chromosomal places in 2 cases and one place in 25 cases. Therefore there is probably no gross misplacement of chromosomes so that additional makers, i.e. secondary constrictions, if shown regularly, are bound to occur within the same region of the karyotype. This has been the experience of workers on other species. For example, Chromosomes 13 and 21 present similar problems in man, as do the acrocentric chromosomes of sheep, but Ferguson-Smith *et al.*, (1962) and Palmer and Funderburk (1965), have shown a secondary constriction on the long arm of each of these chromosomes in addition to the satellites on the short arms. A similar example may be quoted of the "rabbit's ear" chromosome/

chromosome found in the mouse karyotype (Levan, Hsu and Stick, (1962), and Chromosomes 9 and 10 of the Rhesus monkey (Rothfels and Siminovitch, 1962).

The idiogram measurements verified the visual impression gained from viewing karyotypes, that the metacentric chromosomes can usually be identified by length in good preparations. This was found to be most evident in differentiating Chromosome I from the other two pairs., The difference in length was not always so obvious between Chromosomes II and III, but centromere position which is more central in Chromosome III (Table 14) proved useful for identification. A feature of the metacentric chromosomes, particularly Chromosome I, was the frequent dissimilarity in length between homologoues, (Figs. 17 and 26). This anomaly is reported in other species also (Ford, 1962) and may, on occasion, be exaggerated by the position of chromosomes in metaphase preparations, being further increased during chromosome preparation (Patau, 1965).

The average ratio found between the length of the X chromosomes, from idiogram measurements, and the

the next pair of acrocentric chromosomes was 1.1 - 1.0. This figure is lower than that quoted by McFee et al.,(1965) who did not state, however, the number of chromosomes measured. The range of this ratio over ten measurements was 1.16 - 1 to 1.04 - 1. This difference was again analogous to the impression gained by viewing a large number of metaphases in this study. The consistent length difference and the usually more obvious short arms seen on the X chromosome made its identification possible in good metaphases.

Secondary Constrictions.

From so homogeneous a karyotype as that of the sheep, small points of difference between chromosomes are of great importance for purposes of identification. For this reason great care was taken to record all demonstrable secondary constrictions. Of the constrictions recorded there was some variation both in position and morphology. In some cases they appeared as understained areas, "negative heteropyknosis"(White, 1954) with no great separation of opposing ends of the chromatids (Figs. 21 and 22). On other occasions they appeared as wide non-staining areas of "euchromatin" (White, 1954). Sometimes thin/

thin strands of chromatid ends (Fig. 28). This variation is similar to their expression as recorded by others (Rothfels and Simonovitch, 1958; Ferguson-Smith et. al., 1962).

A total of one hundred and ninety-seven clear constrictions other than centromeres, were recorded from three hundred and twenty karyotypes. The reason for this comparatively small number of constrictions may be associated with the fact that the main part of this work was done using a dilute Hank's Balanced Salt Solution for hypotonic treatment. Very few secondary constrictions were recorded during this period. Latterly when a change was made to sodium citrate solution there was an increase in the number of constrictions seen and further their demarcation was more distinct. Figs. 27, 28, & 32.

It can be seen that the majority of these constrictions are found in autosomes 1-4 and the X chromosome. Although the incidence of secondary constrictions is low when all the chromosomes alone are taken, 5.05% of the above chromosomes show secondary constrictions. This percentage, however, is split between several positions on each of these chromosomes, at which secondary constrictions may occur.

Accepting the fact that it is possible for the same constriction to be seen over a variable position within each chromosome, depending on the state of the metaphase at fixation, it was found that the recorded constrictions on the three metacentric chromosomes tended to occur regularly in the following positions.

Chromosome 1.

On the short arm there were two areas where constrictions occurred. Ten were recorded near the middle-terminal region (9-12 mm.) and nine were recorded at the paracentric (2-3 mm.) region. On the long arm, on twenty-two occasions, a constriction was recorded near the mid-arm region, 6-14 mm. range, and a very distinct constriction was also recorded at the terminal end of the long arm on four occasions.

Chromosome 2.

Two sites where regular constrictions occurred were on the short arm where there were thirteen constrictions at the 11-13 mm. position and on the long arm, a mid-arm constriction occurring thirteen times at the 10-13 mm. position. Again as in Chromosome 1, a clear constriction was shown at the terminal/

terminal end of the long arm, but only on four occasions.

Chromosome 3.

Nine very clear constrictions were seen at the 9-10 mm. stage in the mid long arm position. Two other constrictions were recorded on either side of this region and may in fact have been representative of the same constriction.

The occurrence of a number of recordable constrictions of the six large metacentric chromosomes would suggest that in the sheep these are the main nucleolar organiser chromosomes.

X Chromosome.

There were insufficient constrictions on any given region of the long arms of the X chromosome to form a pattern as in the metacentric chromosomes. It is possible, however, that the seven constrictions recorded at the 6-8 mm. position may indicate a region where constrictions could be expected to occur. (Fig.20).

Chromosome 4.

Fifteen constrictions recorded on this chromosome appeared to fall into two positions, at the 6-9 mm. range near the middle long arm position and/

and at the 12-15 mm. terminal position. Two examples of what was assumed to be chromosome 4-5 showed these constrictions occurring concurrently. (Figs. 38 and 39).

Other Chromosomes.

The various constrictions recorded on the rest of the acrocentric group did not suggest any means of differentiating this difficult splay of chromosomes. Two types of constrictions were noted, those which occurred as definite secondary constrictions on the long arms of the acrocentric chromosomes, e.g. Figs. 35 - 39, and those which showed a characteristic constriction on the paracentric area of the chromosome, similar to the "rabbit ear" effect reported by Hsu, Billen and Levan (1961). This latterconstriction was recorded on more than twenty occasions (Figs. 40 - 42). Whether this constriction is, as these authors suggest, a combination of a secondary constriction and heteropyknotic area, or a secondary constriction very close to a centromere, or in fact the centromere itself could not be decided. The fact that this type of constriction was seen at various stages of metaphase and that the short arms though very small never appeared/

appeared separated, while the long arms were separate, would suggest that Hsu, Billen and Levan (1961) were correct.

The centromere position was never very clearly seen in either metacentric or acrocentric chromosomes of the sheep, certainly never with such distinction as this contriction.

CHROMOSOME ASSOCIATIONS.

No feature comparable to the satellites seen in human acrocentric chromosomes was recorded, but what were interpreted as associations between acrocentric chromosomes could be seen (Fig. 44-48). Evidence was also presented suggesting that associations may occur between acrocentric and metacentric chromosomes. (Fig. 43 and 44).

There were, however, in the material examined too few examples upon which to draw any correlation between associations and secondary constrictions. The presence of associations of acrocentric chromosomes with regions where secondary constrictions had been recorded, particularly in the large metacentric chromosomes would have been useful confirmation of the sites of these constrictions. The absence of visible satellites/

Fig. 43. Metacentric Chromosome with a Paracentric Secondary Constriction and Association of Small Acrocentric Chromosome. (X 3500).

Note: In Figs. 43, 44 and 45, spreading of Chromosomes is not complete, and the dark staining background (Fig. 43) suggests that some adhesive material is still retained. This may have helped these delicate associations to persist.

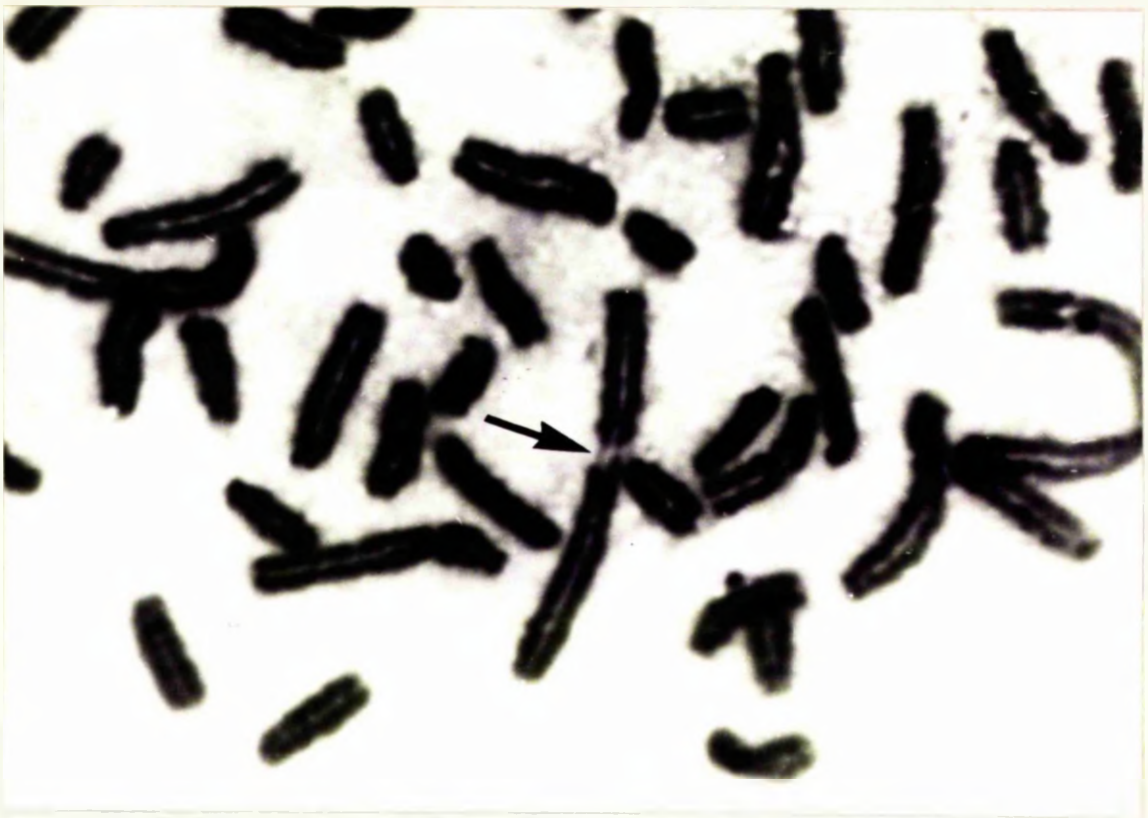
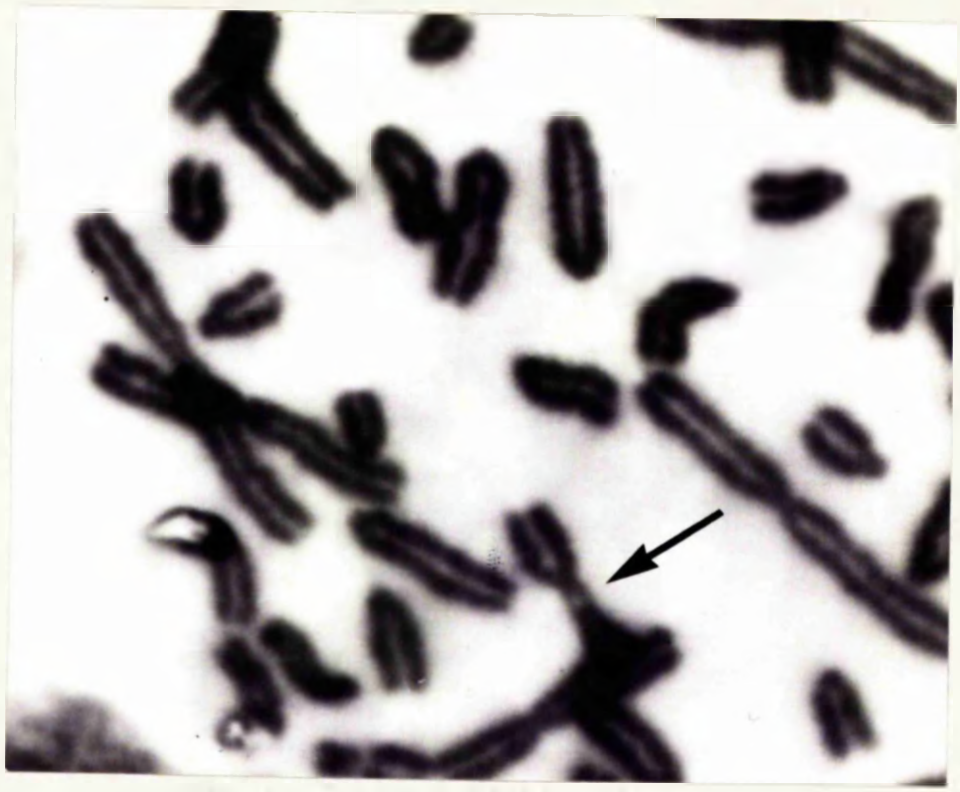


Fig. 44.

Fig. 45.

Figs. 44 and 45. Association of Acrocentric Chromosomes as seen in Several Metaphases. (Heavy arrows.

Fig. 45. Secondary Constrictions are present on two Acrocentric Chromosomes (Light arrows). (X 3500).



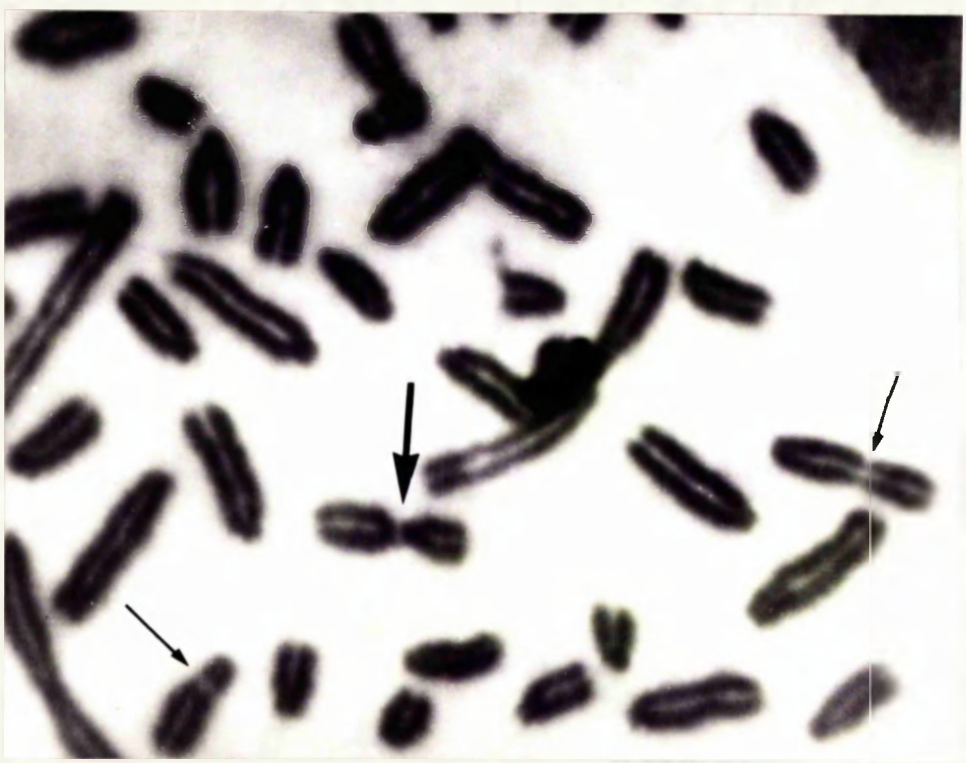


Fig. 46. Association of 2 Acrocentric Chromosomes. (X 3500).

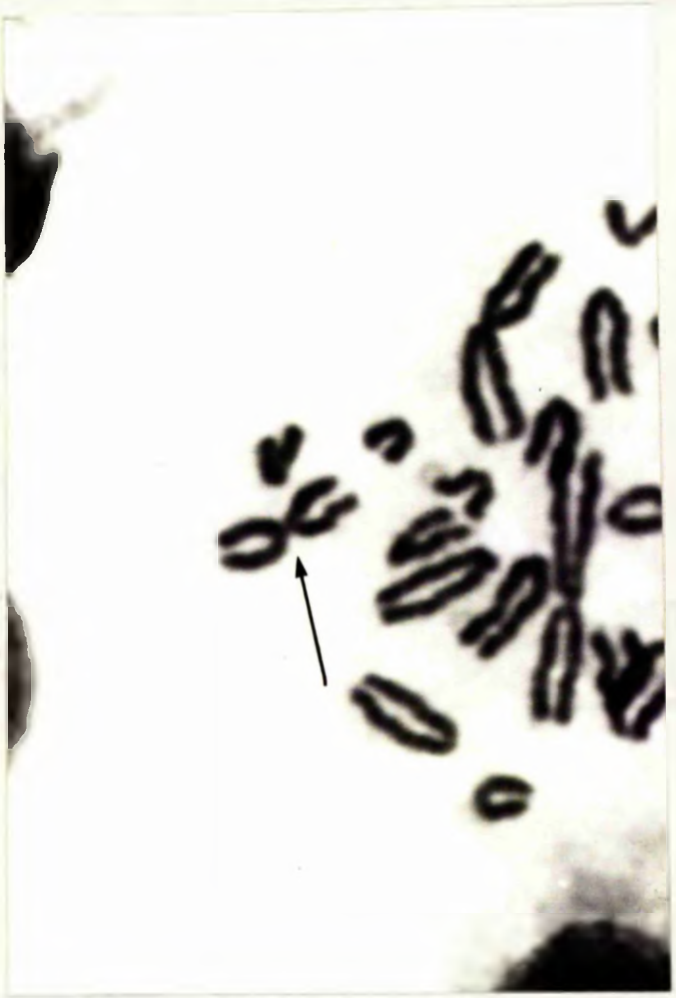
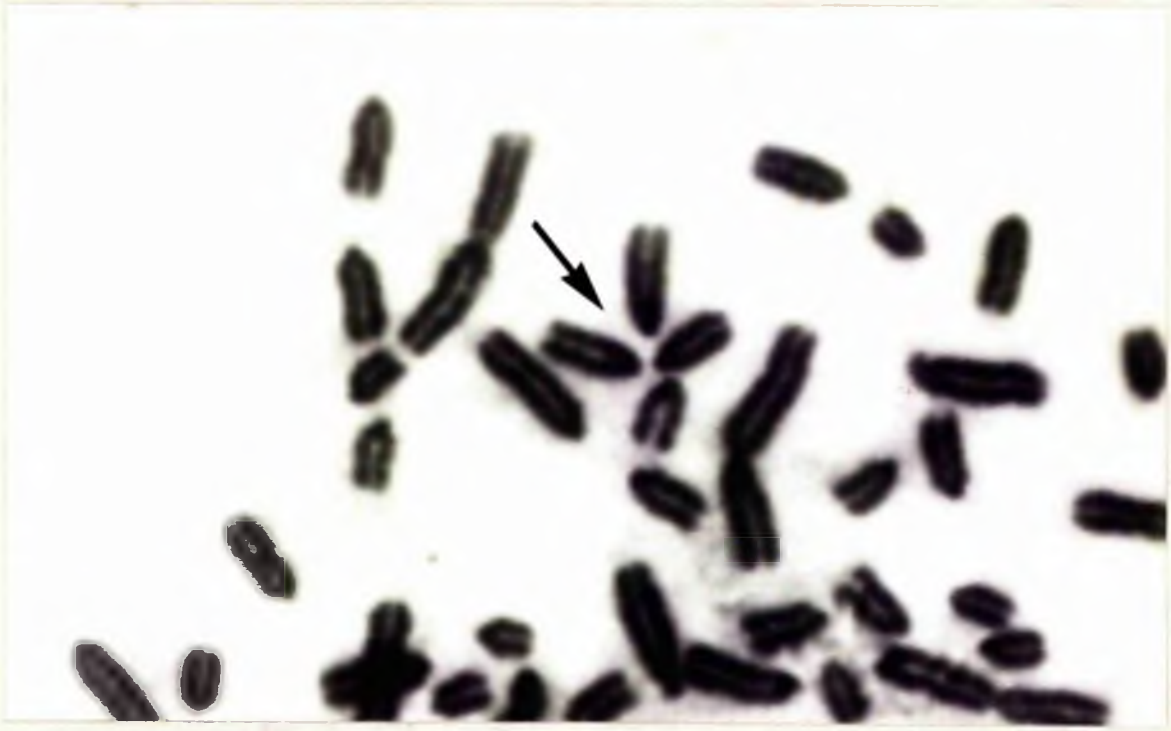
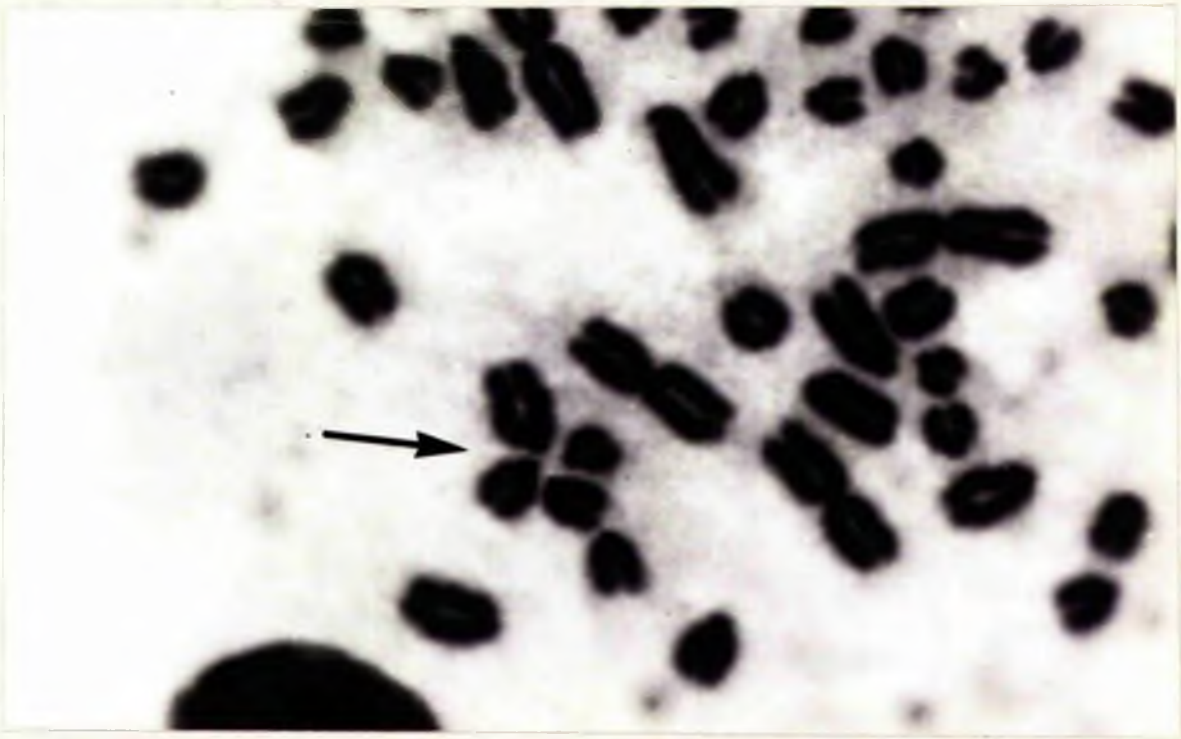


Fig. 47.

Fig. 48.

Fig. 47 and 48. Associations of Groups of Acrocentric Chromosomes. (X 3500).





satellites and numbers of demonstrable associations comparable to that found in man, does not preclude the possibility that associations occur in sheep. As pointed out by Ferguson-Smith and Handmaker (1963) excessive hypotonic treatment appeared to reduce the incidence of associations. It is suggested that the satellite associations in the sheep may be of a more complex and delicate nature than in man, and may not withstand current technical manipulations.

It must also be stressed that while the chromosome arrangements recorded in the following photographs may suggest that associations do occur in the sheep it was not possible to demonstrate, except in some of these few examples, any visible strands of chromatin joining possible associations.

CONCLUSIONS AND FUTURE OUTLOOK.

Using a modification of the human leucocyte culture technique, the following conclusions were reached on the identification of individual chromosomes in the karyotype of the sheep. In the ewe, the karyotype can be divided into two main groups of chromosomes six being metacentric and forty-eight acrocentric while the karyotype of the ram has six metacentric and forty-seven acrocentric chromosomes and one submetacentric chromosome./

The Y chromosome, as well as being submetacentric, is the smallest in the karyotype and in most metaphases has the appearance of a four-leafed clover.

The X chromosome was found to be the largest of the acrocentric chromosomes. This was shown by its greater length measurements in both male and female metaphases, by comparison with the next largest pair of acrocentric chromosomes. It was also recognised by its larger short arms.

Both karyotype observations and idiogram measurements showed that the six large metacentric chromosomes could be differentiated on length measurements, particularly Chromosome 1, which was more obviously longer than Chromosomes 2 and 3. The length difference between Chromosomes 2 and 3 was not always so distinct, but differentiation was assisted by the more centrally placed centromere of Chromosome 3.

The separation of other chromosomes in the karyotype, on length alone is, however, not possible. Although the number of secondary constrictions recorded was small by comparison with human chromosome work, sufficient numbers were seen to indicate their possible use in chromosome identification. This was particularly so for chromosomes 1, 2, 3, X and 4, on which

the/

the numbers of constrictions recorded at specific regions were sufficient to suggest a regular pattern.

The use of some of the various methods to accentuate these constrictions may be valuable in increasing the number of recordable secondary constrictions. Sasaki and Makino (1963), for example, showed that this could be achieved by using a calcium-free medium for tissue culture. Bromodeoxyuridine (B.U.D.R.), was used to similar effect by Hsu and Somers (1961), and Kabach, Saksela and Mellman (1964), while Palmer and Funderburk (1965) suggested that the concentration of mitotic accumulating agent may affect the occurrence of secondary constrictions.

These avenues of investigation, together with auto-radiographic studies using tritiated thymidine (Schmid 1963), may be valuable in future studies. In the latter field, information on the possible late labelling properties of the X chromosome in the sheep would be an advantage, particularly in view of the difficulties associated with differentiating sex chromatin in tissues of this animal.

The differentiation of the acrocentric chromosomes was not possible at this stage, either by using arm measurements or recording secondary constrictions. However,/

However, the fact that constrictions do occur on these chromosomes, does offer a possible opening for investigation.

Before further work is contemplated on chromosome associations in mitotic preparations a study of meiotic preparations from spermatocyte cells may be valuable. Such a study would help enumerate the sites of nucleolus formation and may help to indicate which chromosomes take part in associations in mitotic preparations.

PART IV.

A CYTOGENETICAL INVESTIGATION OF SOME INTERSEX SHEEP.

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Introduction.

Until recently studies on intersexuality have been based largely on morphological information. An intersex is described as an individual with any form of discrepancy of the sex structures of congenital origin, resulting in a mixture of male and female features. Usually when describing such intersexes, the terms hermaphrodite or male pseudo-hermaphrodite or female pseudo-hermaphrodite have been used, the choice of term being decided by the gonadal structure. When both ovarian and testicular structures are present an individual is called a true hermaphrodite. If, on the other hand, only one type of gonad is present together with ambiguous external genitalia the subject is termed a pseudo-hermaphrodite. With more recent information provided by endocrinologists, embryologists, geneticists and physiologists there is a renewed interest in the mechanisms causing intersexuality, not only in man but also in animals. (Hafez and Jainudeen, 1966).

It is beyond the scope of this study to consider all these aspects but some reference to the part played/

played by recent cytogenetical techniques in the study of intersexuality is relevant since both chromosome and sex chromotan studies have furthered the understanding of hermaphroditism. As well as chromosome studies in man, several workers have carried out chromosomal investigations of bovine intersexes. (Ohno, Trujillo, Stenius, Christian and Teplitz, 1962; Fechheimer, Herschler and Gilmore, 1963; Goodfellow et al., 1965; Kanagawa et al., 1965).

It is well known that the genetic sex of man and mammals is determined at fertilization and depends on the sex determining genes present in the chromosomes. If fertilization results in a zygote of XY constitution, the resulting animal will be a male. If, on the other hand, two X chromosomes are present the animal will be female. In the classical case of Drosophila, the Y chromosome makes no contribution to sex determination but is merely responsible for fertility (Bridges, 1916). In man, however, it is strongly sex determining and in its absence normal male differentiation is not possible (Ferguson-Smith, 1963). In view of the high degree of differentiation of the sex chromosomes seen in our farm animals, it is highly likely that the Y chromosome has a similar function/

function to that in man. This fact seen in the light of foetal castration experiments of Jost (1947) would suggest that in higher mammals, although the genetic sex may be determined by the presence or absence of the Y chromosome, the development of the male genitalia and sex characteristics is under the control of an organiser substance produced in the differentiating gonad. Jost showed that if the gonads of the male foetus were removed before the male duct system had differentiated, the development of these was in fact towards female genitalia.

The Embryology of Sex Differentiation.

Mammalian embryos initially have gonads which show no evidence of becoming either testes or ovaries. In their earliest form the gonads arise as ridge-like thickenings (gonadal ridges) on the ventro-mesial face of the mesonephros. Histologically they consist of a mesenchymal thickening covered by mesothelium. The cells of the gonad differentiate rapidly from the mesothelium at the same time becoming much larger. Some are conspicuously larger than others, these being the primordial germ cells of the gonad. There is considerable evidence that these cells are not formed *in situ*, but are derived from migrating cells

from/

from the yolk sac entoderm (Witchi, 1948). The proposed route of migration is by way of the splanchmic mesoderm, but Simon (1960) demonstrated in birds that many of these migrating germ cells enter the circulating blood and home on the gonadal ridges. Two routes of migration are, therefore, possible. If the goned is to develop into a testis the cells of the germinal epithelium grow into the underlying mesenchyme forming cord-like masses which eventually become the seminiferous tubules. In the female, the germ cells grow into the mesenchyme and eventually form ovarian follicles. The growth of the germ cells in the female, however, occurs in two stages, the cells in the second ingrowth forming what are known as the cords of Pflüger (Chapin, 1917).

The male sexual duct system with the exception of the urethra but including epididymis, ductus deferens and seminal vesicle is derived from the mesonephros. The prostate gland develops from the urethral epithelium. In the female, the oviducts, uterus and vagina are derived from the Mullerian duct system which arises beside, but separately from, the mesonephric ducts. During gonadal differentiation both tests and ovaries change their positions. The/

The ovaries, oviducts and uterus stretch the peritoneum into a mesentry-like structure which ultimately acts as the supporting ligaments for the organs. The testes on the other hand, slide close to the body wall beneath the peritoneum passing through the inguinal canal into the scrotal pouches. The descent of the testes is largely facilitated by the gubernaculum, a fold of peritoneum from the caudal end of the developing gonad which becomes fibrous and stretches down through the inguinal ring to the scrotal pouches, where it is ultimately attached. In the bovine, this descent has usually taken place by the 26 cm. embryo stage (Idllie, 1917).

The external genitalia of both sexes are derived from the genital tubercle, genital folds and genital swellings. In the male the genital tubercle elongates forming the penis, the genital folds fnrm the prepuce, while the genital swellings form the scrotum. In the female the genital tubercle becomes the clitoris and the genital folds and the genital swellings become the vulval lips.

Intersexuality in Man, Cattle and Sheep.

Very few cases have been recorded of true hermaphroditism in cattle and there are no reported cases/

cases of true hermaphroditism in sheep. Three cases were reported recently in Norwegian cattle by Nes (1966), but these would seem to be exceptional. In these cases testicular pseudo-hermaphroditism was observed in three offspring out of eight from one cow. The hermaphrodites had well developed udders, female external genitalia, rudimentary female and male genital ducts and intra-abdominal testes. The karyotype in these cases was XY, the same as a normal bull, as seen in skin cultures only. No sex chromatin studies were carried out.

However, in man there are a number of reported cases of true hermaphroditism.

Chromosome Studies on True Hermaphrodites.

Of twenty-five cases of true hermaphroditism studied by Grumbach and Barr (1958), nineteen were sex chromatin positive and six, sex chromatin negative. In 1959, the chromosomes of two human chromatin positive true hermaphrodites were found to be female XX, by Harnden and Armstrong; and Hungerford, Donnelly, Nowell and Beck. Many such cases have been described including three true hermaphrodite siblings all of XX chromosome constitution (Rosenberg, Clayton and Hsu, 1963).

This/

This latter case provided evidence in favour of the development of testicular material in the absence of the Y chromosome. In many of the cases of XX true hermaphroditism described in medical literature the numbers of mitoses examined were not large. In addition, insufficient tissues were examined to eliminate the possibility of mosaicism occurring, although this was apparently suspect in some of these (Ferguson-Smith, Johnston and Weinberg, 1960).

The first case of true hermaphroditism in man with an XX/XY sex chromosome complement was reported by Gartler, Waxman and Giblett (1962). In this case the existence of two cell lines was established by examination of tissue from skin, gonads and clitoris. On the basis of the findings in this case the authors postulated that it had arisen by double fertilisation and fusion of two egg nuclei. A similar case was reported by Josso, de Grouchy, Auvert, Nezelof, Jayle, Moullec, Frezal, de Casaubon and Lamy (1965), in which XX/XY chromosome mosaicism was shown in skin and leucocyte cultures. Serological studies on this patient revealed a double haptoglobin phenotype, suggesting that this mosaicism was due to double fertilisation of the ovum. An interesting true hermaphrodite/

hermaphrodite described by Brøgger and Aagenaes (1965), showed a female karyotype in tissue culture of bone marrow, skin, peripheral blood and both gonads. A second culture from one gonad, however, revealed a male karyotype XY as well as the female XX demonstrating how protracted may be the diagnosis of sex chromosome mosaicism. Further, this case strengthened the hypothesis that the Y chromosome contains genes which control the development of testicular structures in man. Two other types of mosaicism have been described in association with true hermaphroditism in man, namely an XX/XXY true hermaphrodite (Turpin, Le Jeune and Breton, 1962) and an XX/XXY/XXYY true hermaphrodite (Fraccaro, Taylor, Bodian and Newns, 1962).

The Freemartin Condition in Cattle.

While hermaphroditism as a result of single pregnancies in cattle is uncommon, animals showing signs of intersexuality from dizygous pregnancies are very common. Such animals are called freemartins. The precise derivation of the term freemartin is obscure. The word martin of English origin, and the Gaelic term mart both have the basic meaning of a cow, ox or speyed heifer. The term free is probably derived from the prefixes farrow or ferrow, which are old fashioned/

fashioned terms referring to a barren cow, or one not giving milk (Forbes, 1946). Whatever the original meaning of the word, nowadays it is the accepted term describing the sterile intersex animal, which is born co-twin to a bull. Many accounts of freemartinism have been recorded, one of the earliest English publications being that of Hunter (1779), who commented also on intersexes in other species. The incidence of freemartinism in cattle is high, approximately 87% of the female twins of mixed pregnancies being sterile (Lillie, 1917). (This figure is variously described by different authors, e.g. 91% by Gilmore (1949) and 92% by Arthur (1964).) Freemartins usually have the external genitalia of the female but the internal reproductive organs present a wide variation between male and female characteristics. The general body appearance is intermediate between male and female. The bull twin is invariably normal and fertile. Freemartinism is not only confined to twin pregnancies. In any mixed multiple pregnancies there is a risk of freemartinism developing in one or more female embryos even in the presence of a single male (Wallace, 1960); and author's personal experience).

The/

The first explanation of the freemartin condition was offered by Tandler & Keller (1911), but Lillie (1916 and 1917), presented a long and detailed explanation independently of these authors and has been the acknowledged authority on this subject for a number of years. He put forward the view that the sterile intersex born co-twin to a male was the result of the masculinisation effect of male hormones carried to the early female twin embryo by way of a placental anastomosis. Lillie based his deductions on several factors, firstly that hetero-sexual cattle twins are dizygotic, as supported by sex ratios in cattle, and secondly that in all of the cases he had observed that an anastomosis invariably occurred between placentae. Finally, from the observations of Chapin (1917), under Lillie's direction, histological examination of the developing gonads of both male and female embryos showed that the stage of differentiation in the male calf gonad was reached while the female calf gonads were still in the indifferent stage.

The precocious differentiation of the male gonad, according to Chapin (1917), was associated with the enlargement/

enlargement of interstitial cells. She suggested that these cells were responsible for the secretion of male hormones which crossed the placental anastomosis and modified the sexual development of the female twin. Chapin concluded that the high degree of variation of the reproductive system of the freemartin was indicative of the variability of the time at which the interstitial secretion of the male embryo may first be introduced into the circulation of the female embryo.

Convincing as Lillie's theory of the cause of freemartinism may appear, an alternative theory has been suggested. The basis of the alternative view is founded on the evidence of established mosaicism. Mosaicism of red cells in the circulating blood of twin adult cattle was first reported by Owen (1945), who concluded that this was due to the foetal interchange of embryonal cells ancestral to the erythrocytes. Further evidence of the transfer of embryonal cells came from homograft exchange tests between dizygous cattle twins (Anderson, Billingham, Lampkin and Medawa, 1951). Skin grafts between full siblings other than twins failed to survive, while grafts between dizygous twins were retained. This evidence/

evidence lead some workers to suggest that cells other than erythrocyte precursors may be exchanged and established within sexually opposite twins. The conclusion is that the freemartin condition may not be caused by a humoral agent as suggested by Lillie (1917), but may be a function of sex chromosome mosaicism (Fechheimer, Herschler and Gilmore, 1963). The first attempt to show this was made by Ohno, Trujillo, Sternius, Christian and Teplitz (1962). These workers, using the leucocyte culture technique, established the existence of blood cell chimaerism between bull twins and freemartin twins and then examined the gonads of both sets of animals for evidence of germ cell chimaerism. They found twice as many 2A XX cells as 2A XY cells in the testes of one twin bull, but no evidence of 2A XY cells in the freemartin gonads. They were unable to ascertain whether the 2A XY cells were germ cells. Four Japanese workers Kanagawa, Muramito, Kawata and Ishikawa (1965), claimed to have demonstrated both male and female XX and XY cell lines in lung and gonad culture of one freemartin calf. This claim was based on the demonstration of 13 XY cells out of 969 cells counted in tissues other than from leucocyte cultures.

Other/

Other attempts to vindicate the theory of chimaerism of germ cells as a cause of freemartinism have been made by Goodfellow, Strong and Stewart (1965). They suggested from evidence of sex chromosome mosaicism and histological examination of the gonad that the freemartin was a true hermaphrodite caused by virilisation of the embryonic gonadal ridge by male germ cells. These authors also claimed that the differing phenotypes of the two freemartins examined corresponded to the proportion of male cells as seen in the leucocyte preparations. More recent evidence for the germ cell theory has been produced by Ohno and Gropp (1965). From the examination of alternate serial sections of 12 mm. freemartin embryos they concluded that the alkaline phosphatase-positive cells found in the embryonic blood vessels of these sections were primordial germ cells which had wandered into the blood stream. These cells were claimed to be in the process of migration from the yolk sac to the developing gonadal ridges.

Much of the current evidence for germ cell chimaerism as a cause of freemartinism is open to criticism. Wislocki (1939) observed that in the marmoset monkey twin pregnancies occurred in 87.5% of/

of cases studied and that the female partner of heterosexual twins is never sterile despite regular anastomosis between twin placentae. Later, Benirshke, Anderson and Brownhill (1962) demonstrated bone marrow chimerism between heterosexual twin marmosets and probable germ cell chimerism in the male twins, which was analagous to the conditions found later in the bull co-twin to a freemartin (Benirshke and Brownhill, 1963). Similarly, cases of permanent blood chimerism have been reported in man (Dunsford, Bowley, Hutchison, Thompson, Sanger and Race, 1953; and Choun, Lewis and Bowman, 1963). In the first of these human cases the woman was normal and had given birth to one child so was clearly not a freemartin. An explanation for sterility not developing in man and marmoset monkeys under analagous conditions to the bovine has been proposed by Ryan, Benirshke and Smith (1961). From enzymic studies, they suggested that a difference exists between bovine and primate placentae in their respective abilities to aromatise androgens. Thus, in cattle foetuses higher levels of androgens may accumulate than in primate foetuses, resulting in the virilisation of the female co-twin to a male.

Finally, the foetal castration experiments of Jost (1947) on the rabbit would favour a humoral agent rather than a cellular agent as the modifying of the freemartin/

freemartin reproduction system. Jost found that the dissection of the gonads of the foetal rabbit at a critical stage of differentiation resulted in the genital ducts and external genitalia developing along female lines irrespective of the genetic sex of the embryo. Male differentiation is, therefore, dependent upon the production of an organiser substance by the gonad at a critical stage of development. Such an inference would tend to discount migrating germ cells from the yolk sac as being the modifiers of the female gonad.

Cytogenetical studies of the bovine freemartin can at present be summarised as having demonstrated sex chromosome chimerism in both co-twins. Germ cells chimerism has been demonstrated in the gonads of male calves but not freemartin calves other than in the Japanese report of Kanagawa et al., (1965) which unfortunately is lacking in details of method. In view of the short culture time of ninety-six hours, some white cells may have survived and produced the small number of male cells recorded by these workers.

Whatever future cytogenetical studies may reveal about the freemartin condition a comparison of some of the features of freemartinism and true human hermaphroditism/

hermaphroditism is necessary before the intersex sheep used in this study are discussed.

Chimerism and Mosaicism.

The presence of mixed male and female cell lines in blood cells only, as in the bovine freemartin and marmoset monkey, is referred to as blood cell chimerism. However, when the mixed cell lines are found in more than one tissue, or in one tissue other than blood cells, ~~this is referred to as mosaicism.~~ cell mosaicism is usually suspected. In the true hermaphrodite, mosaicism has been diagnosed on several occasions (Gartler et al., 1962, Josso et al., 1965).

In some cases, however, referred to as mosaics, mosaicism has been shown in one tissue only (Brogger and Aagenaes, 1965). While it can be assumed that a chimaera is an individual with two or more cell lines, one of which has been derived from a totally different individual, a chromosome mosaic is an individual whose body contains two or more different lines of cells which have arisen within the same individual. As well as naturally occurring chimaeras such as the freemartin and marmoset, artificial chimaerism can be produced in laboratory animals and men by transplanting haemopoietic tissue (Woodruff, Fox, Buckton and Jacobs, 1962). The/

The difference between natural and artificial chimaeras, would appear to be that the former are permanent and the latter temporary.

Mechanism of Mosaic Formation.

While the mechanism of placental anastomosis and foetal transfer of blood cells between opposite sexed twins is the accepted cause of blood cell chimaerism, the formation of mosaicism is more complicated. Two possible mechanisms have been suggested (Bain and Scott 1965). These include either an exceptional sequence of early mitotic errors in an XY zygote or a dispermic conception. The first must be extremely rare indeed. One human hermaphrodite however has been reported in which there was probably non-disjunction of an XY zygote at the first mitotic division, followed by non-disjunction of the XO cell line, and non-viability of the cell with no sex chromosome (Forteza, Bouilla, Bageuna, Monmeneu, Hallis and Zaragoza 1963). The second mechanism, namely a dispermic conception has been suggested on several occasions (Bain and Scott 1965). For such a process to take place either two fertilized ova may fuse or the egg nucleus and its polar body may fuse after fertilization or a mature egg may under go mitotic/

mitotic division before fertilization. Evidence for fusion of fertilised ova has been offered by Tarkowski (1961), who showed that a single mouse chimaera may develop from two eggs which fuse as late as the eight cell stage. The evidence for dispermic conception and fusion of two ova makes the distinction between chimaerism and mosaicism even more difficult (Bain and Scott 1965); since such an individual falls into the definition of a chimaera, in that two cell lines are derived from different individual zygotes. In view of such intricacies in definition, the term blood cell chimaera used in connection with the freemartin condition has even greater significance.

The Diagnosis of Chimerism and Mosaicism.

There are several ways of recognising blood cell chimaerism. These include differential red cell grouping, as described by Owen (1945) in cattle and Dunsford et al., (1953) in man. It is pointed out however, that this method of detecting chimaerism requires more evidence than that of the blood group picture alone and that such evidence must be correlated with evidence of twinning and further evidence of mixed cell populations in more than one blood group. (Annotations B.M.J. 1957. p 1467).

A/

A second method for detection of chimaerism has been the use of experimental skin grafts. In these experiments tolerance of skin grafts between chimaeras is taken as a manifestation of immune tolerance and hence blood chimaerism. This has been adequately shown in cattle (Anderson et al., 1951), and in sheep (Slee 1963).

The third and simplest method of detecting blood cell chimaerism is (in the case of the freemartin) by sex chromosome analysis of peripheral blood leucocyte preparations or bone marrow preparations.

The use of differential red cell grouping, skin graft tolerance and chromosome analysis, as well as careful investigation of case history and demonstration of placental anastomoses, are all complementary methods of studying chimaerism. The additional use of nuclear sexing studies and fibroblast cultures of other tissues, helps to establish if other cell lines are involved and whether mosaicism exists.

In this study on intersex sheep leucocyte cultures, fibroblast cultures and sex chromatin studies were used together with all the ancillary clinical data available to define the cause of intersexuality.

Intersexuality/

Intersexuality in The Sheep.

There is no evidence that true hermaphrodites occur in sheep and reports on the occurrence of freemartinism are rare. Twinning rates in most British breeds of sheep are high, figures of 130-200 lambs born per 100 ewes mated are quite common for well fed lowland sheep, such as the Dorset Horn breed (Fraser 1951). Therefore if the freemartin condition occurred in sheep in a high percentage of mixed twin pregnancies there would be a large number of infertile ewes in many flocks. Lillie (1917), in his extensive study on freemartinism was not able to present any evidence that the condition did in fact occur in sheep.

Reference is made to the freemartin condition in sheep in Lisle's Husbandry in 1722, but since no details are given, little significance can be attached to the quotation.

The first authoritative report of a possible freemartin-like condition in the sheep was presented by Roberts and Greenwood (1928). By anatomical comparison of the genitalia of a Southdown-Welsh Mountain crossbred lamb with a black Welsh calf these authors suggested that this sheep intersex was precisely similar to the bovine freemartin. They also/

also considered that the latter was a genetic male. In this case the gonads were described as testis-like bodies lying beneath the skin in the inguinal region. There was no vagina but an enlarged penile clitoris. The case was summarised as being a "female lamb with testis".

The second reported case of a sheep freemartin was that of Ewen and Hunmason (1947) in a purebred Rambouillet ewe born co-twin to a ram. This case apparently was initially a typical ewe but developed full sized horns (normally found only in the male in Merinos). The external genitalia of this sheep were those of a normal ewe, except that the clitoris was described as being much enlarged and mis-shapen. The mammary glands were similar to those of a yearling ewe. One testis-like gonad was situated beneath the skin immediately above the right mammary gland while the other was in the normal position for an ovary. Histological examination of the gonads showed that they consisted of collections of tubules lined with simple epithelium. The tubules were separated by a cellular connective tissue. No structure resembling epididymis, seminal vesicle, prostate gland or uterus was found.

An/

An interesting case of freemartinism in the sheep was diagnosed on the findings of erythrocyte mosaicism by Stormont, Weir and Lane (1953). This case, superficially, was a normal female but was described as having the vagina of a freemartin. The anatomical description was not recorded but it was stated that examination left no doubt that it was a true freemartin sheep.

Moore and Rawson (1958), while using skin homograft acceptance as a criterion for the development of acquired tolerance in sheep observed one pair of tolerant twins out of five pairs examined. In this pair of twins one was a male and the other appeared to be a freemartin judging by the abnormal appearance of the vulva and clitoris and failure to show oestrus.

The fifth reported case of freemartinism was that of Slee (1963), whose diagnosis was based on observed immunological tolerance to skin grafts in three pairs of lambs of large litters (three to five lambs). Of the pairs showing tolerance between grafts, one pair included opposite sexed lambs. One was male and the other showed external signs of freemartinism (see Case No. I).

Alexander and Williams (1965), were the first investigators to show a freemartin sheep associated with/

with a demonstrable placental anastomosis. In this case three lambs were involved, a male, a female and an apparent freemartin. The anastomosis occurred only between the placenta of the male lamb and the freemartin. Anatomically, the freemartin lamb resembled the description given by Fraser-Roberts and Greenwood (1928) with the external genitalia of a ewe, except for an enlarged clitoris. No posterior vagina nor uterine horns were found present but two testicle-like gonads were found lying subcutaneously in the inguinal region.

Chromosomal evidence of the freemartin condition in sheep added another means of confirming the existence of this condition (Gerneke 1965). In this report two sheep with extreme freemartin genitalia, again similar to those reported by Roberts and Greenwood (1928) and Alexander and Williams (1965) were described.

In one case by using bone marrow chromosome preparations, mixed XY and XX mosaicism was demonstrated. In the other, all the cells were XX female. The somatic sex was described as female from tissue cultures of kidney and gonad of the first case only. In view of the difficulty in interpreting nuclear sex from buccal cells of the sheep and related animals no

significance/

significance can be placed on this author's findings on sex chromatin from this tissue in these animals.

A summary of the above cases is included in Table 23.

From the above cases it can be seen that no cytogenetical study on a number of sexually abnormal sheep has been reported previously. Further, in the preceding cases of freemartinism in sheep there is very incomplete evidence that these were precisely analogous to the bovine freemartin or whether they were mixed forms of intersexes. In this study a unique opportunity arose to add to the knowledge of this subject in the sheep.

Numerous requests by the author for intersex sheep were largely unsuccessful, emphasising either the probable rarity of such animals, or the difficulty of identification. Seven of the ten animals used in this study were made available from the Animal Breeding Research Organisation of Edinburgh. The value of this contribution deserves special recognition because in each case a complete history was available, so that the two important aspects of history and phenotype could be compared with the cytological findings on the sex chromosomes from various tissues of these animals.

The/

The Examination of Intersex Sheep.

In view of the ultimate findings from the ten intersex sheep examined, they have been divided into two groups for consideration. The details of each stage of investigation of the individual animals in each group are given together to give continuity of discussion and assist in comparison.

The following headings are used:-

A. Female Intersexes.

B. Male Intersexes.

A. Female Intersexes.

It must be emphasised that although the findings on this group of sheep lead to a diagnosis of free-martinism, they were initially accepted for study as unknowns. Six animals were studied in this group. in five,* a detailed comparison of anatomy and histology of the genitalia was undertaken together with chromosome studies from four tissues and sex chromatin studies of nerve tissue. The last case was not studied in such detail, but is included because of its interesting case history.

* Fibroblast cultures from Case No. 5 did not grow satisfactorily although taken for examination.

CASE HISTORIES AND DESCRIPTION OF EXTERNAL GENITALIA.

Case No.1. (Figs. 50 and 51).

This animal was born to a Welsh ewe on 13/4/61, as one of quadruplets, the other lambs being two male and one female. It was recorded at birth as a female. It was used by Slee (1963) in skin graft experiments and was shown to have skin graft tolerance with one of its litter mates. It was concluded that it was a natural blood chimera and red cell mosaicism tests confirmed this. Two other litter mates also showed homograft skin tolerance. Slee concluded that this was a freemartin sheep.

Externally, it had the appearance of a male animal with large horns and heavy forequarters and it showed strong male libido towards two ewes on heat. In each case it proceeded with the body motions of copulation. It was very aggressive towards other male animals. In appearance the external genitalia were, however, similar to those of a ewe except for an enlarged glans clitoris and two small gonads which could be palpated subcutaneously in the region of the external ring. There was no scrotum. Teats and mammary tissue were present/

present but the vagina was very short and blind.

Case No. 2. (Figs, 52 and 53)

Case No.2 was a purebred Clun ewe born 18/4/61 co-twin to a ram. At birth it weighed 5.75 lbs., approximately half the weight of its latter mate of 10.50 lbs. At twelve weks of age it was still very small, being 36 lbs., compared with 78 lbs., for its twin. Eventually it grew to normal size for its breed and although run with the ram during 1962 and 1963 it did not breed. Externally it resembled a ewe and was quite passive towards other animals. Its external genitalia resembled those of a ewe except for an enlarged glans clitoris. Mammary tissue was present but no vagina. Gonads could not be palpated.

Case No.3. (Figs. 54 and 55).

An F.I. lamb born 11/4/59, out of a Scottish blackface ewe by a Welsh ram, it also was co-twin to a ram. Like the previous case it had the chance to mate in 1960 and 1961 but did not come to heat. It was a typically over-fat barren ewe in appearance, with female external genitalia but a slightly enlarged clitoris. The vagina in this case was present in part and a finger could be inserted for two and a half/

half inches. Mammary tissue was present and a milky secretion could be drawn from the teats.

Case No. 4. (Figs. 56 and 57).

This was a F.I. lamb from a Scottish blackface ewe by a Welsh ram, but not the same sire as in the previous case. It was born a triplet on 10/4/59, and its litter mates were male and female. Like Case No. 3, it failed to come on heat, though run with a ram in 1960 and 1961.

The appearance of Case No.4 was similar to the previous case except that the external genitalia could have passed on casual examination as those of a normal ewe. The glans clitoris was very small while the vagina was larger than Case No. 3 and the anterior end could not be palpated. Teats and mammary tissue were present but no secretion. There was no scrotum nor palpable gonads.

Case No. 5. (Figs. 58 and 59).

The record of this animal is incomplete. It was born in 1964, co-twin to a male, being a Cheviot-Scottish Blackface cross.

Externally this animal resembled a ewe. The external genitalia were normal and the glans clitoris was not evident until the vulval lips were parted.

Case No.6 /

Case No. 6. (Figs. 60 and 61)

This animal, a purebred Cheviot ewe born April, 1965, was admitted to the Department of Veterinary Reproduction of Glasgow University as it had failed to breed. It had won the ewe lamb championship for its breed at the Royal Northern Show, Aberdeen in 1965. There was no record of its having been a twin. There was evidence that its appearance and external genitalia had changed as this animal passed the scrutiny of stock judges as a ewe lamb of high quality. However, when it was presented for examination to the author approximately eight months later, it had developed a definite masculine appearance. This was depicted particularly by the heavy shoulder appearance and "weaker" hindquarters similar to those of Case No.1. While there was no obvious glans clitoris, the external genitalia did have an unusual appearance. This was shown by a greatly enlarged vulval tip which protruded backwards about 2 cms. and caused the animal to urinate in an upward direction.

Fig. 50

INTERSEX SHEEP. No.1

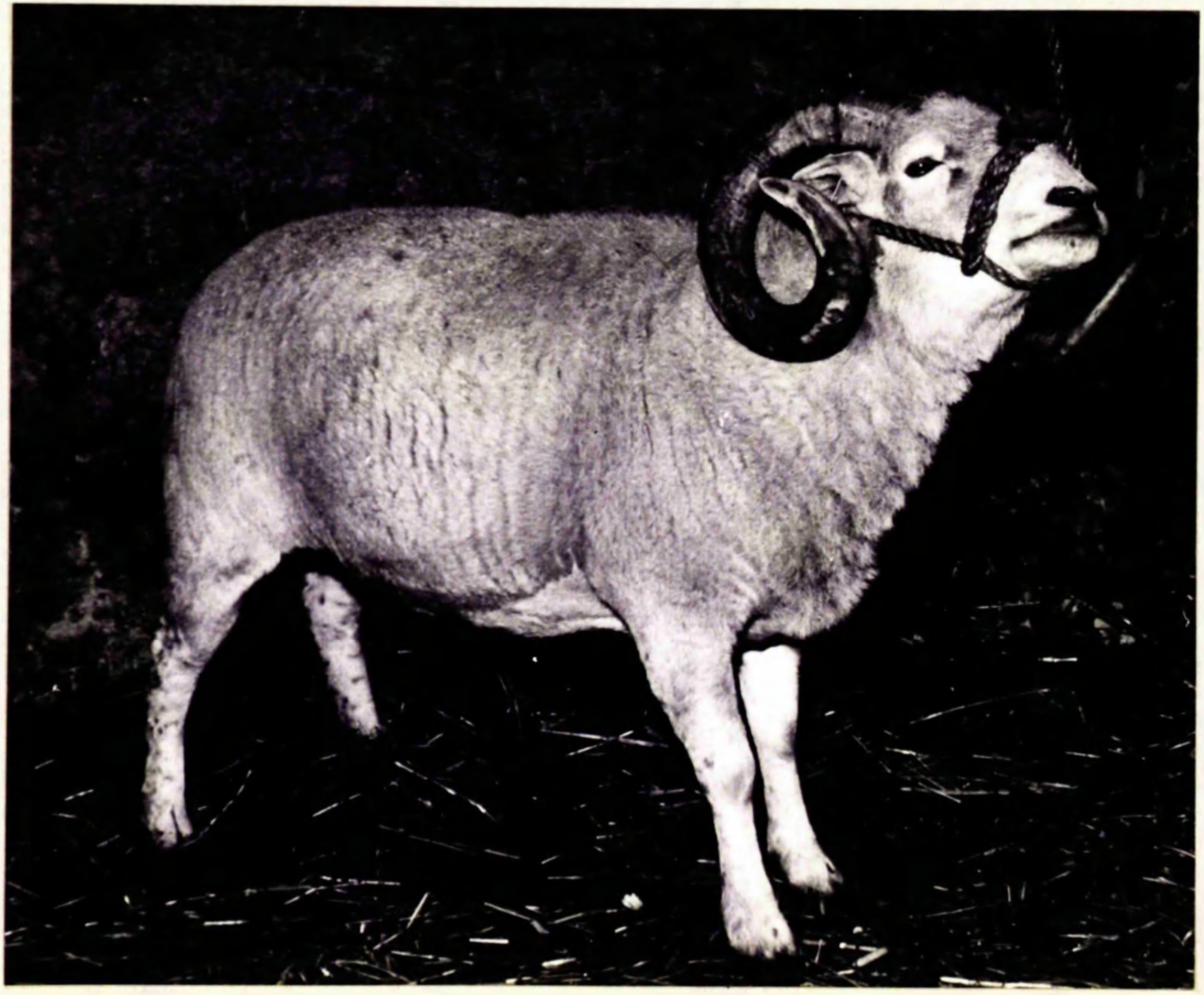


FIG. 57

THE EXTERNAL GENITALIA OF

INTERSEX SHEEP No. 1

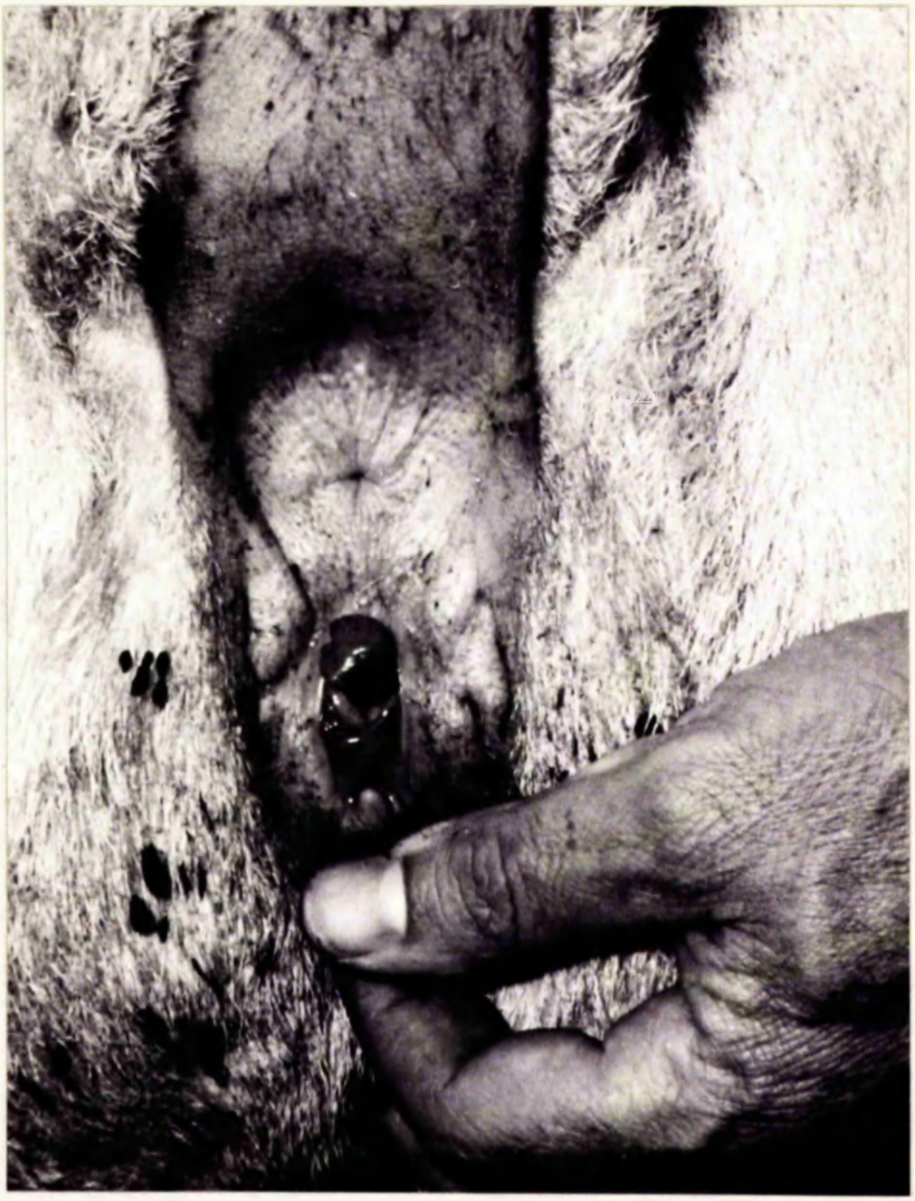


Fig. 52

INTERSEX SHEEP No. 2

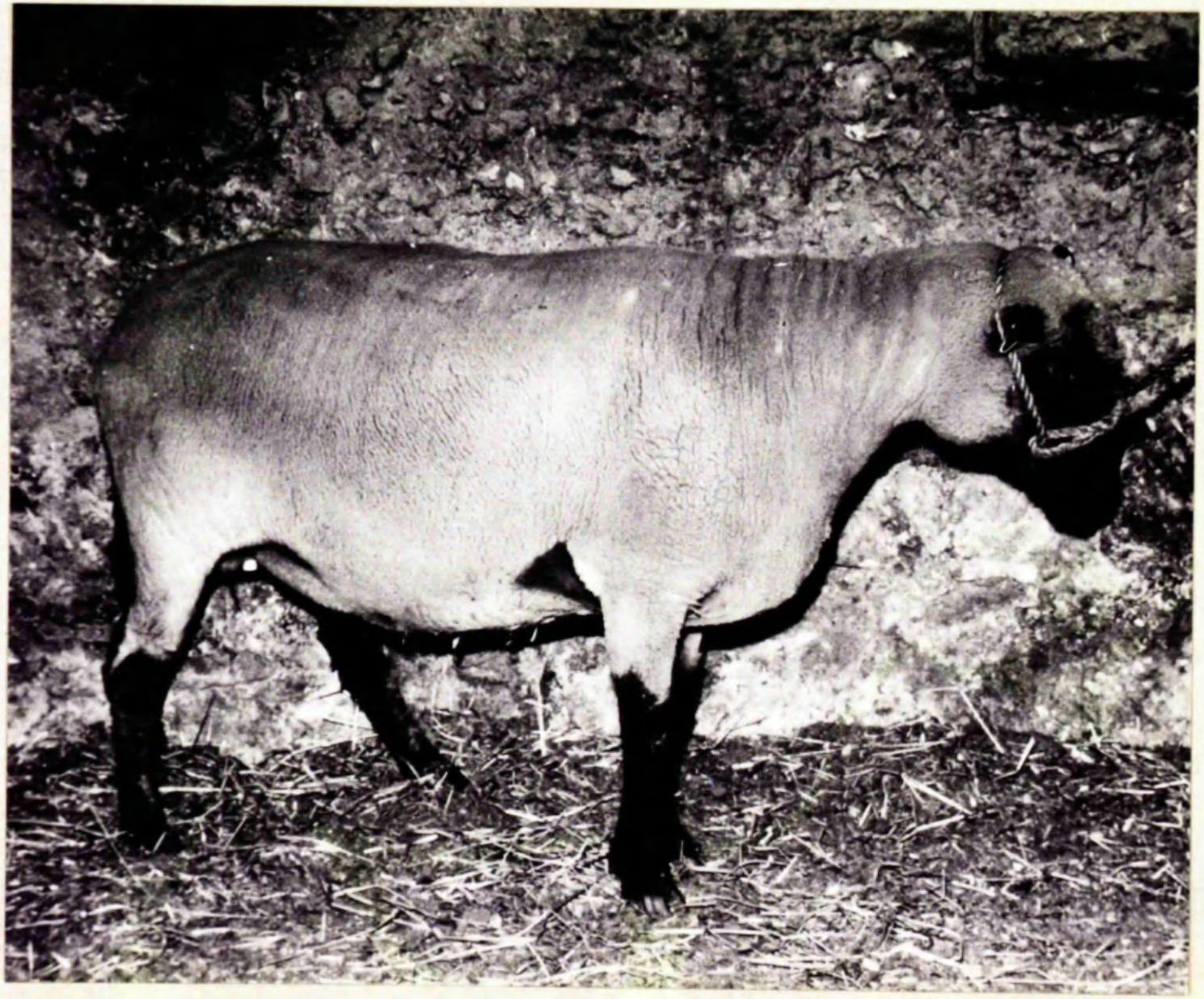


Fig. 53

THE EXTERNAL GENITALIA OF

INTERSEX SHEEP No. 2

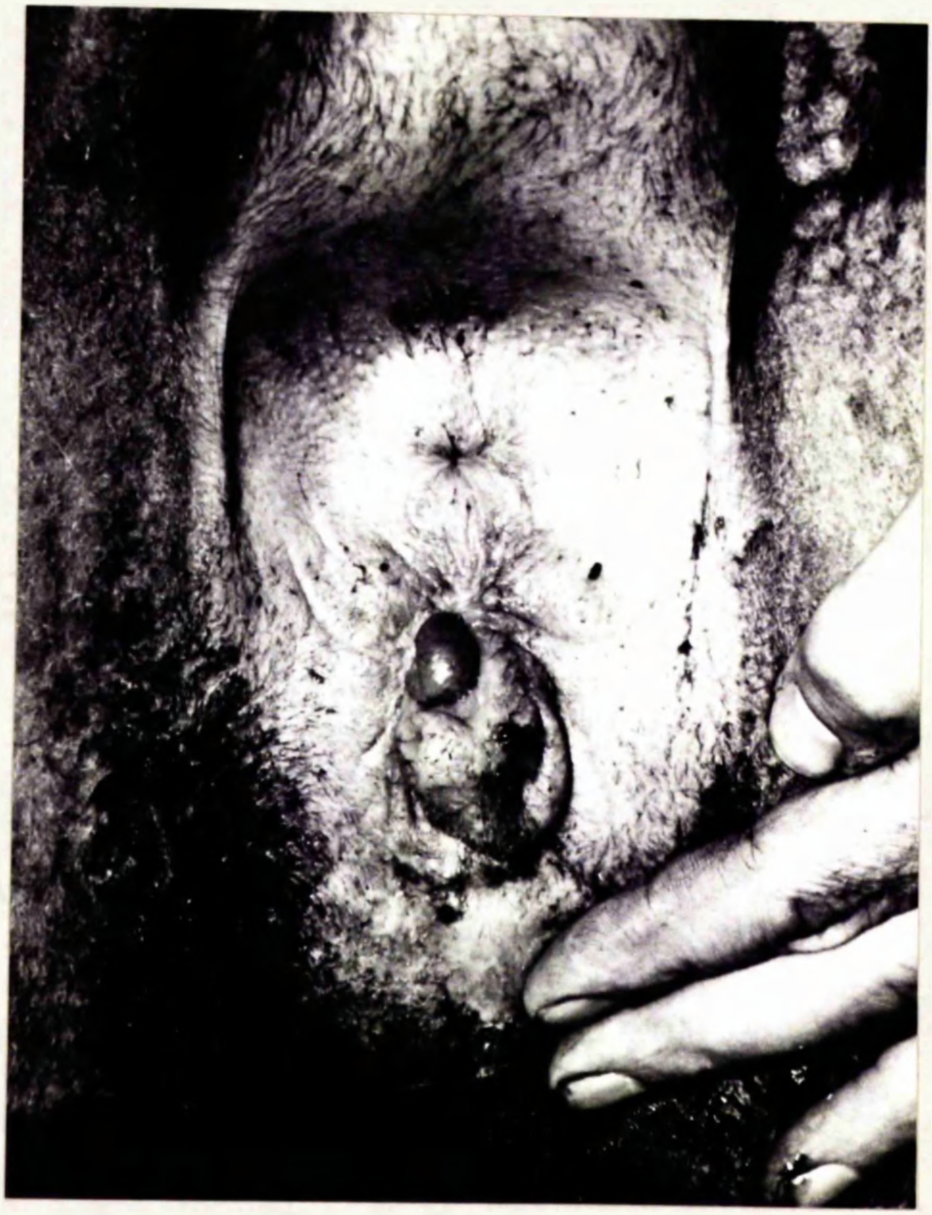


Fig. 54

INTERSEX SHEEP No. 3

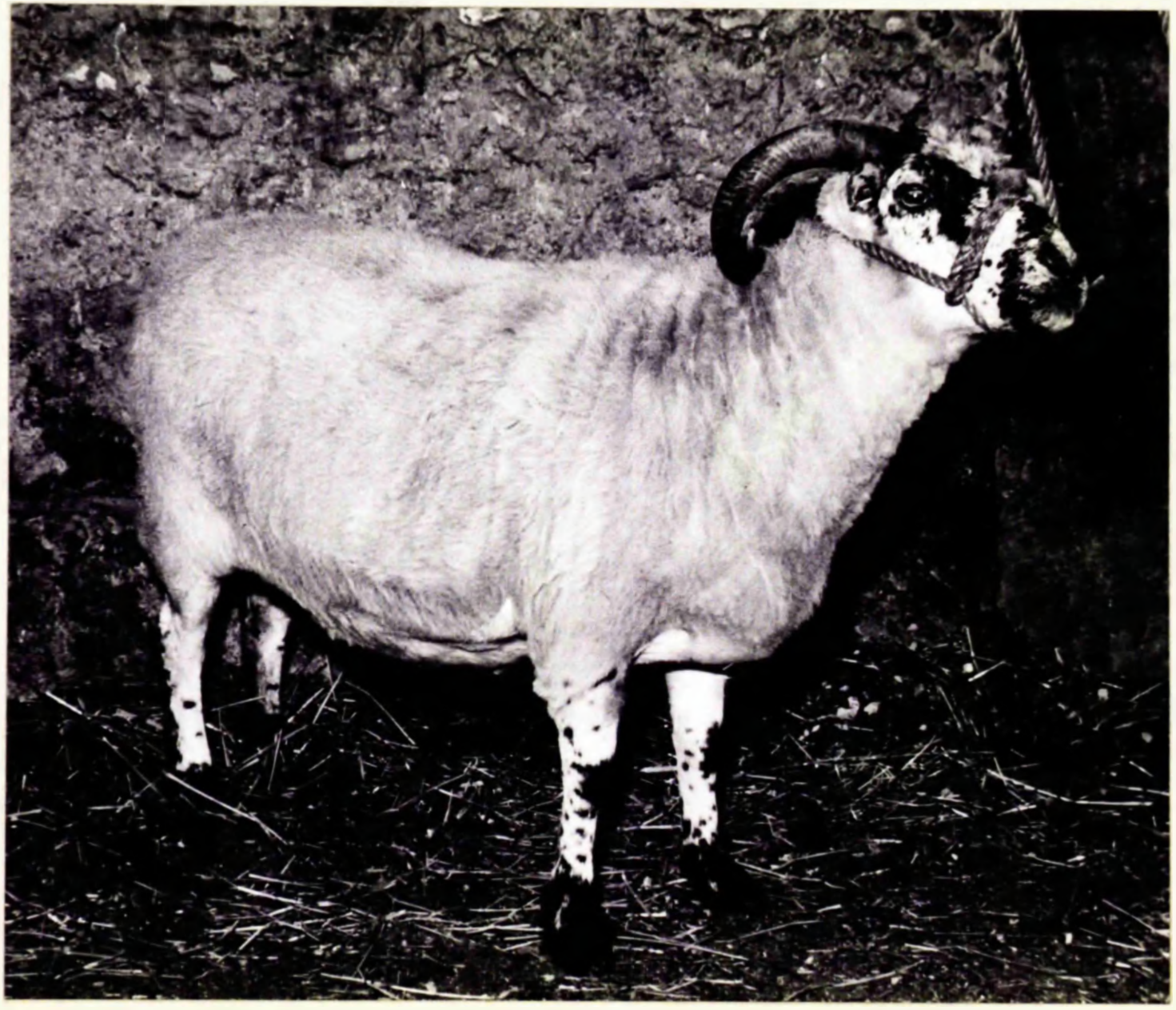


Fig. 55

THE EXTERNAL GENITALIA OF

INTERSEX SHEEP No. 3

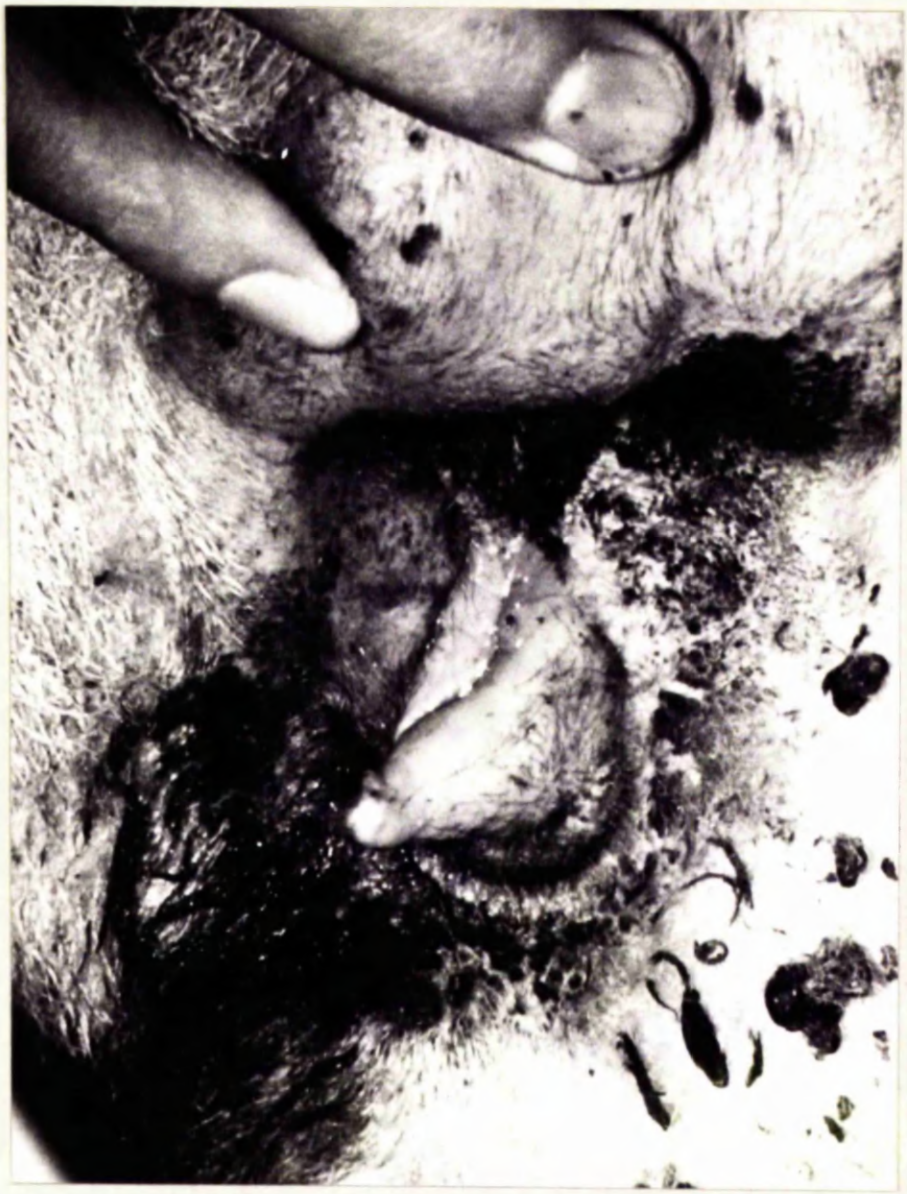


Fig. 56

INTERSEX SHEEP No. 4

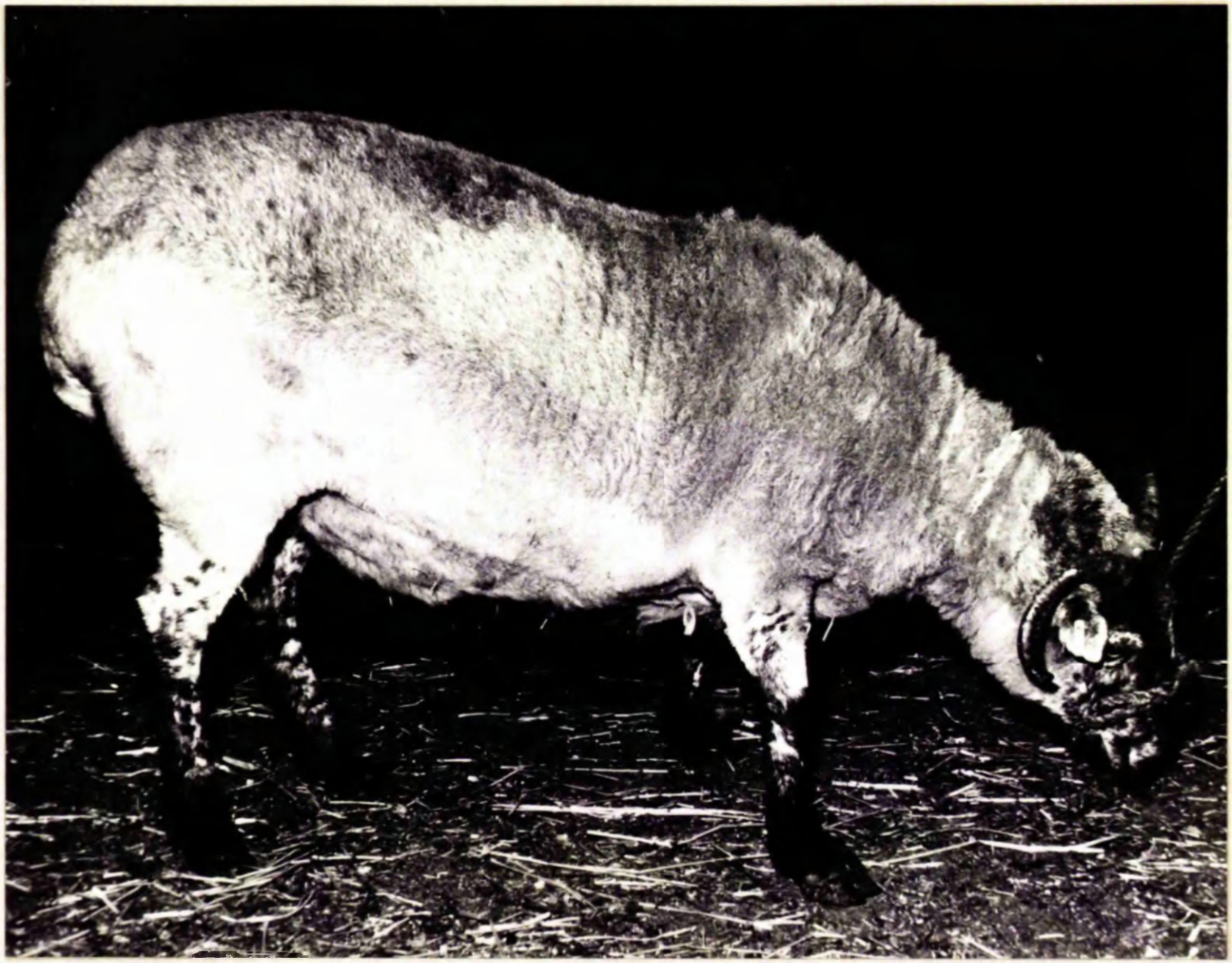


Fig. 57

THE EXTERNAL GENITALIA OF

INTERSEX SHEEP No. 4.

Fig. 58

INTERSEX SHEEP No. 5

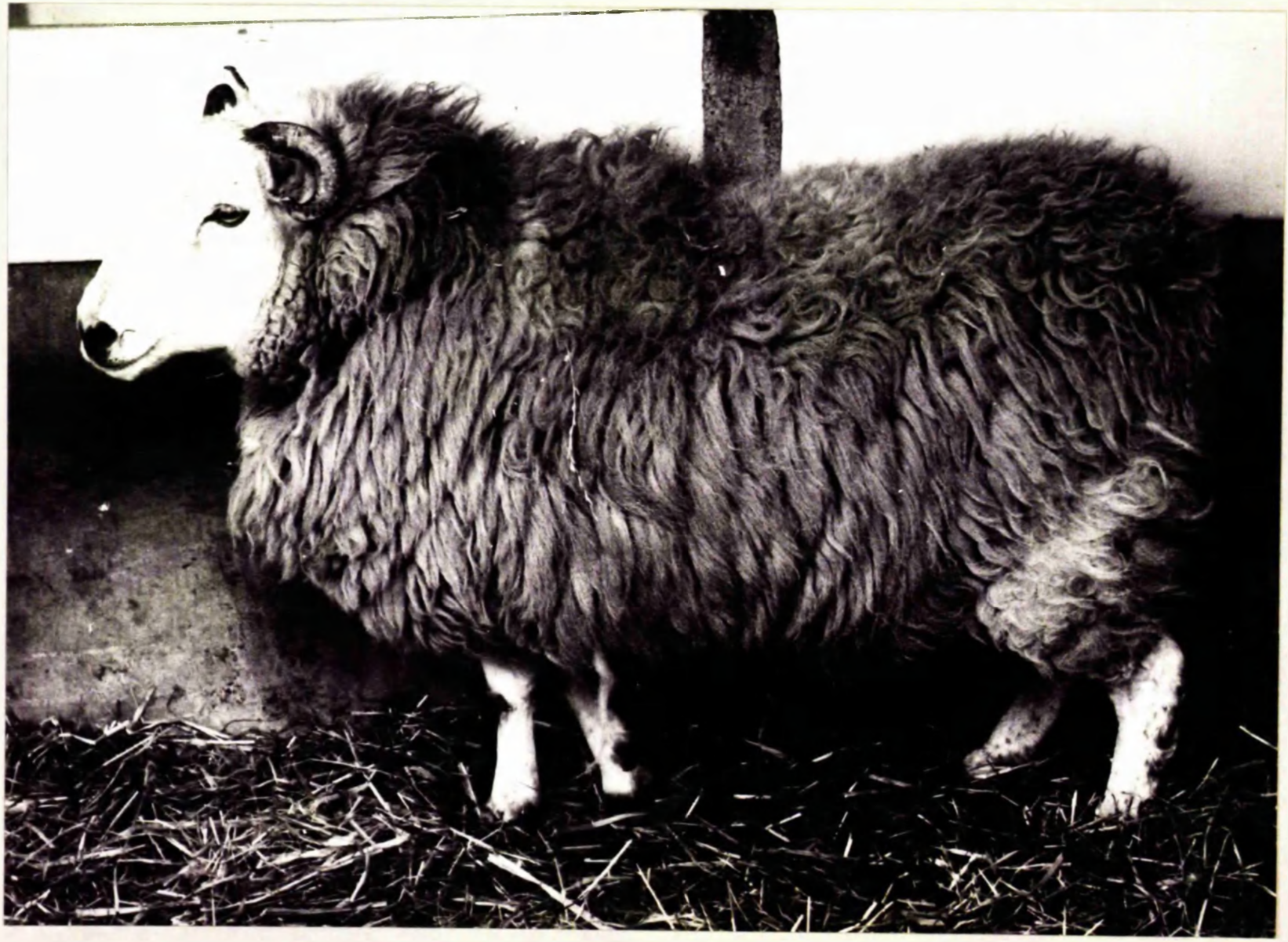


Fig. 59

THE EXTERNAL GENITALIA OF

INTERSEX SHEEP No. 5

Fig. 60

INTERSEX SHEEP No. 6

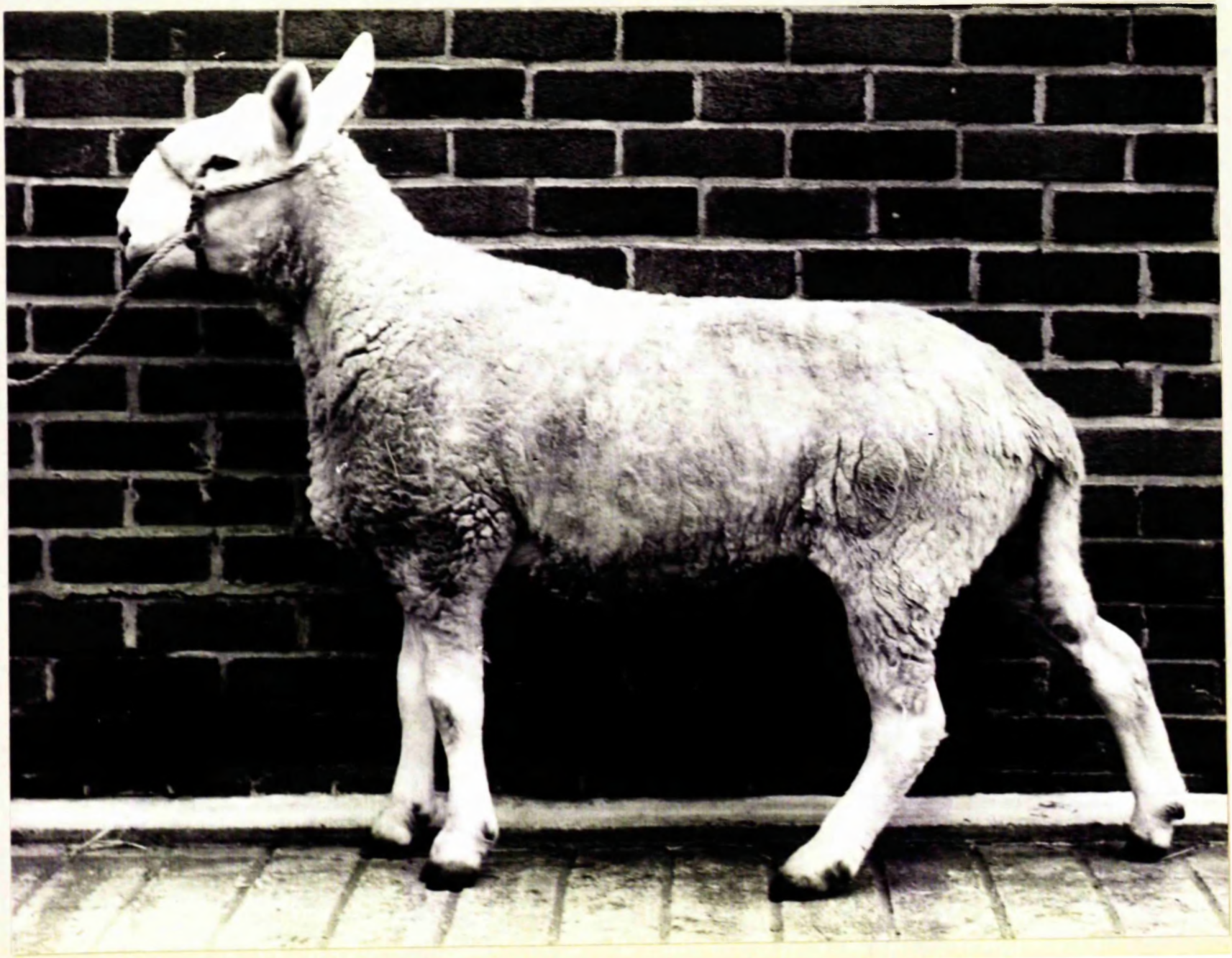


Fig. 61

THE EXTERNAL GENITALIA OF

INTERSEX SHEEP No.6

THE ANATOMY and HISTOLOGY of the GENITAL SYSTEMS INTERSEX SHEEP.

Since it is necessary to compare these anatomical and histological results with those of the bovine freemartin a brief review of the anatomy and histology and the terminology used to describe these is important.

Anatomy and Histology of the Bovine Freemartin Genitalia.

There is no exact counterpart in either testis or ovary for some of the cell formations found in the freemartin gonad. Most of the terms for describing reproductive glands and organs, however, are used in the usual sense. The sex cords, which in the male become the seminiferous tubules and in the female the medullary cords and seminiferous tubules in the freemartin. The choice of term depends largely on the degree of organisation in the gonad (Chapin, 1917). All the material between seminiferous tubules, including interstitial cells and connective tissue stroma is referred to as interstitial material. The term albuginea in the freemartin is used to designate the tissues lying just beneath the peritoreum and surrounding the sex cords (seminiferous tubules) and includes/

includes both the tunica albuginea and the tunica vasculosa.

The transformation in the freemartin gonad presents a series of gradations between "near female" and "near male" and the degree of transformations of other genital organs (Vas deferens, seminal vesicles, uterus) is correlated approximately with this (Willier 1921).

Sex Cords (Seminiferous Tubules).

In some freemartins, the sex cord region is simply an unorganised homogeneous mass of cells but in others it comes to resemble seminiferous tubules (Chapin 1917). Chapin stated that no second set of sex cords (cords of Pfluger) develops in the freemartin as in the normal female calf, but Bissonette (1924) suggested that they may appear and degenerate. Whatever happens they are never present in the adult freemartin, hence the absence of ovarian follicles.

The Rete.

The rete is present in the indifferent stage of gonad development and in the early stages of differentiation. In the male it grows with the testis, in the female it diminishes. In the freemartin the rete continues to grow to the point of becoming/

becoming even larger than in a male of corresponding age. (Chapin 1917 and Willier 1921).

Interstitial Cells.

The interstitial cells increase in number as the gonad is transformed in the male direction. (Willier 1921).

Epididymis.

According to Lillie (1917) and Willier (1921), the epididymis is absent from gonads showing low degrees of transformation but a typical epididymis often larger than the male equivalent is present in highly transformed gonads.

Other Structures.

The blood vessels connected with the genitalia in the freemartin range from typically ovarian to testicular. Mesonephric ducts, Mullerian ducts and seminal vesicles all show a similar gradation in development or recession depending on the degree of male transformation. The saccus vaginalis in the freemartin is variable between the deep structure of the male and the shallow structure of the female.

The Anatomy of Intersex Sheep.

Although chromosome studies of all these animals were started before the post-mortem examinations were/

were made, for the sake of continuity the anatomical descriptions of the genitalia are given first.

Case No. 1. (Fig. 62).

No female external genitalia were present in this animal except for the rudimentary posterior vagina (urogenital passage) which narrowed abruptly and became continuous with the urethral opening from the bladder. The glans clitoris was a contorted bundle of erectile tissue lying between the ventral part of the vulval lips. It had a galea glandis but no urethral process as seen in a normal ram.

Both gonads were testicular in appearance and situated subcutaneously at the external inguinal ring. The right gonad was approximately 2.5 cms. x 1.5 cms. and was smaller than the left gonad which was 3.5 cms. x 2.5 cms. The left epididymis was very large and greatly out of proportion. All the contents of the spermatic cord were present including a well developed external cremaster muscle, spermatic artery and vein and ductus deferens. The ductus deferens developed into two ampullae, which however did not appear to open into the urogenital sinus.

Case No. 2. (Fig. 63).

This case resembled Case No. 1. The gonads

were/

were lying extra-abdominally beneath the mammary gland. They measured approximately 2 cm. x 1 cm. and were almost completely enveloped by a massive overgrowth of epididymis and plexus of blood vessels. There was strong adhesion between the tunica vaginalis, gonads and epididymis. The epididymis was cystic and some of the cysts contained 1-2 ml. of fluid. The spermatic cord was identical with the previous case. Seminal vesicles and ampullae were present. The glans clitoris was smaller and had no galea glandis nor erectile tissue.

Case No. 3. (Figs. 64 & 65).

The gonads in this case were very small, measuring about 1 cm. x =.5 cms. and were situated at the brim of the pelvis in a relatively ovarian position. In appearance they resembled small testes, one (Fig. 65), having a very extensive plexus of blood vessels covering it and the associated epididymis. Running ventrally from each glad was a well defined gubernaculum which was inserted into the shallow saccus vaginalis. Mesonephric ducts ran from both gonads for 10-12 cms. and then gradually faded out into cellular connective tissue. There was no connection to the urogenital passage. No seminal vesicles nor prostate gland were present.

The/

The clitoris was an enlarged conical mass of fibrous tissue but did not contain a body resembling the deformed glans clitoris as seen in the previous two cases. The urogenital passage was vagina-like and about 8 cms. in length.

Case No. 4. (Figs. 66 - 68).

The gonads in this case were very small and poorly defined being about the size of a wheat grain. They were situated in a position more sub-lumbar than normal ovaries. Each was suspended by ovarian ligament-like structures and a gubernaculum ran from each gonad to the saccus vaginalis.

Running from each gonad was a duct which in several places was dilated to form cystic bodies containing straw-coloured fluid. Posteriorly the ducts joined to form a common body which was not continuous with the urogenital sinus. No seminal vesicles were present. The glans clitoris was very small and no erectile tissue was seen.

Case No. 5. (Fig. 69).

As in the previous case the gonads in this animal were small but were more easily defined. They were ovarian in position and suspension and a very prominent gubernaculum was present on both sides.

The/

The ducts running from each gonad had some resemblance to uterine horns particularly near their junction., As in the previous case the uterine body did not unite with the urogenital sinus which in this case was about 10 cms. long.

Case No. 6. (Fig. 70).

The gonads were in an ovarian position the left lying further forward than the right. They were very similar in appearance to those of Case No. 3, being small, testicle like and measuring about 1.5 cms. in diameter. Ducts ran from each gonad and each dilated in one place into a small cystic body. The ducts did not unite nor was there any connection with the urogenital sinus. Again, prominent gonadal ligaments and blood vessels were present as well as gubernacula on both sides.

The urogenital sinus ended abruptly at the opening of the urethra. Embedded in a minute clitorine prepuce with a small preputial opening, was a well formed glans clitoris. This was not visible until the vulval lips were parted. It had a well developed galea glandis, a processus urethrae and a tortuous body of erectile tissue about 3 cms. long in the connective tissue on the floor of the vestibule. No seminal vesicles nor bulbo-urethral glands/

Key to Figs. 62 - 70.

a = ampulla

b = bladder

dd = ductus deferens

e = epididymis

g = gubernaculum

go = glans clitoris

gl = gonadal ligament

sv = seminal vesicle

ug = urogenital sinus

uh = abortive uterine horn

G = Gonad

Fig. 62

THE INTERNAL GENITALIA OF

INTERSEX SHEEP No. 1

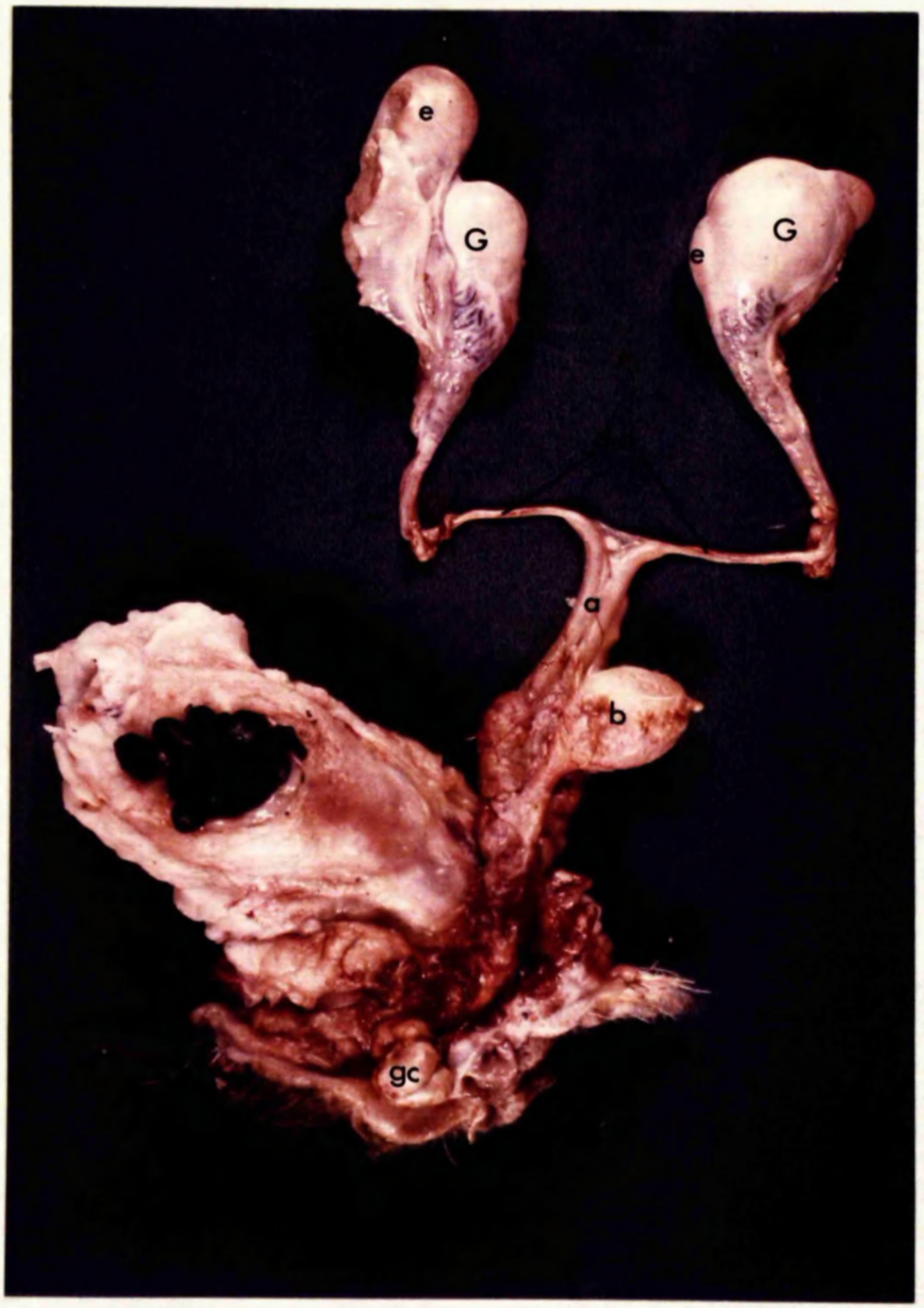

e
G
e
G
a
b
gc

Fig. 63

THE INTERNAL GENITALIA OF

INTERSEX SHEEP No. 2

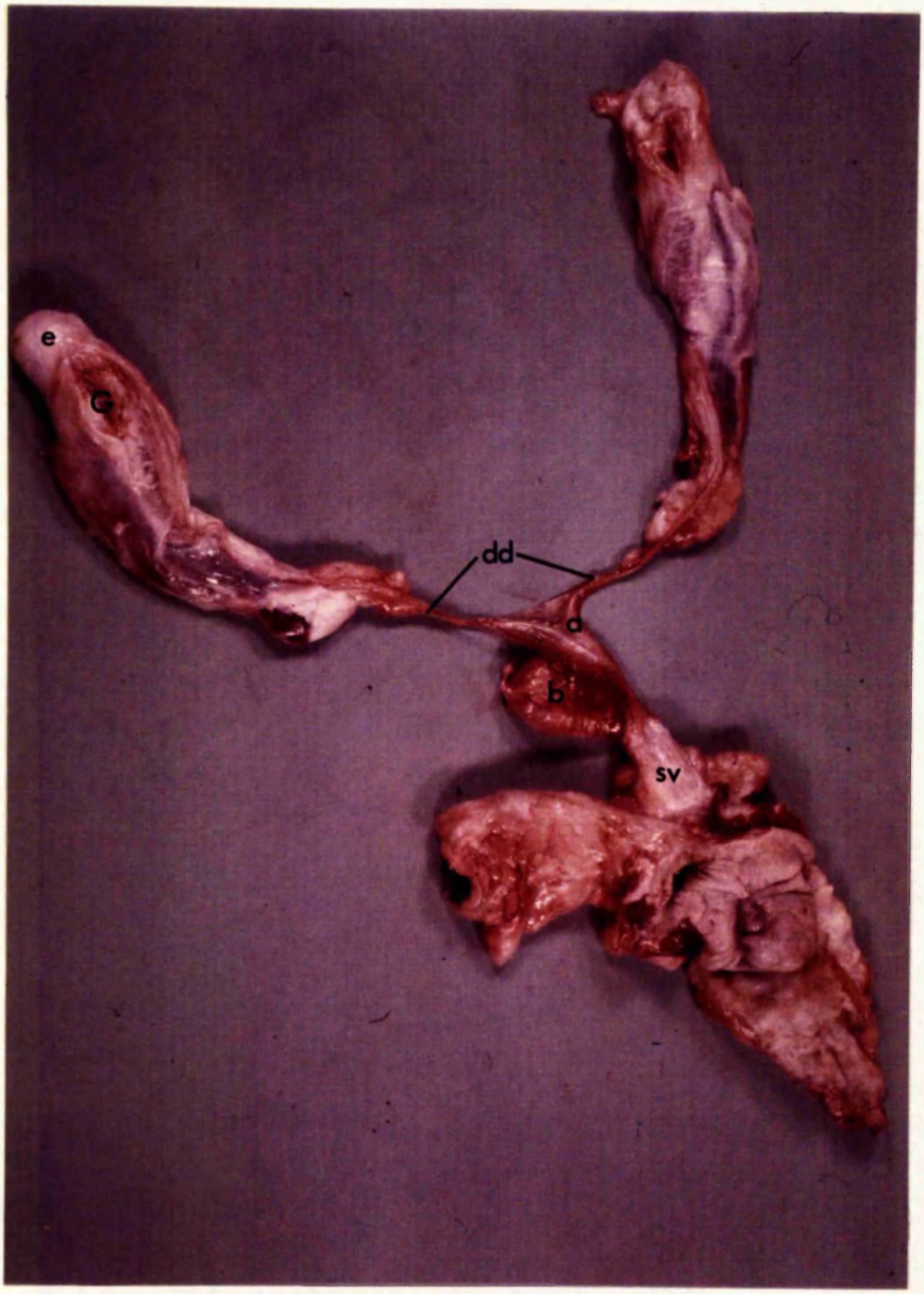

e
G
dd
a
b
sv

Fig. 64.

THE INTERNAL GENITALIA OF

INTERSEX SHEEP No.3

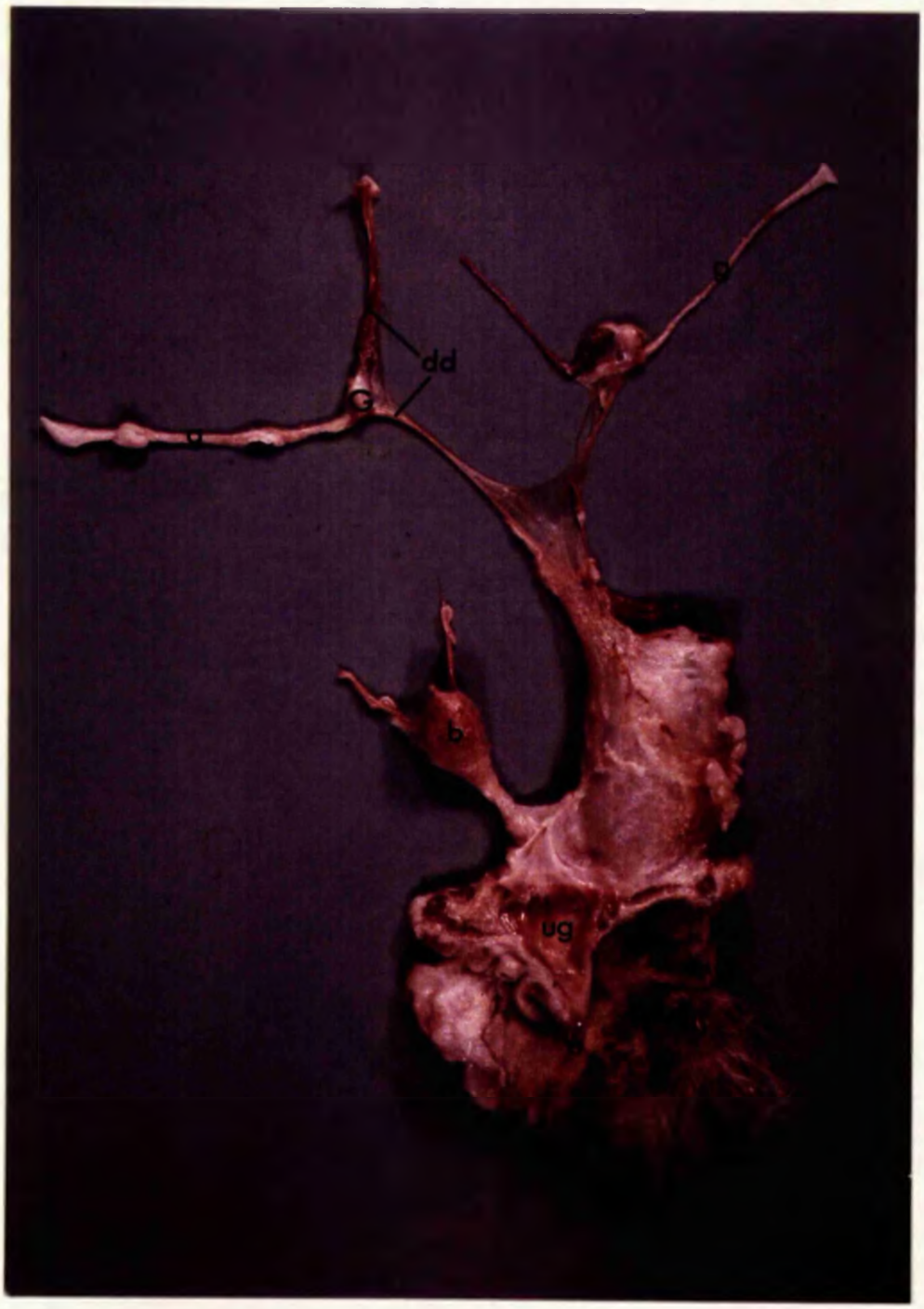

dd
b
ug

Fig. 65

LEFT GONAD OF INTERSEX

SHEEP No. 3

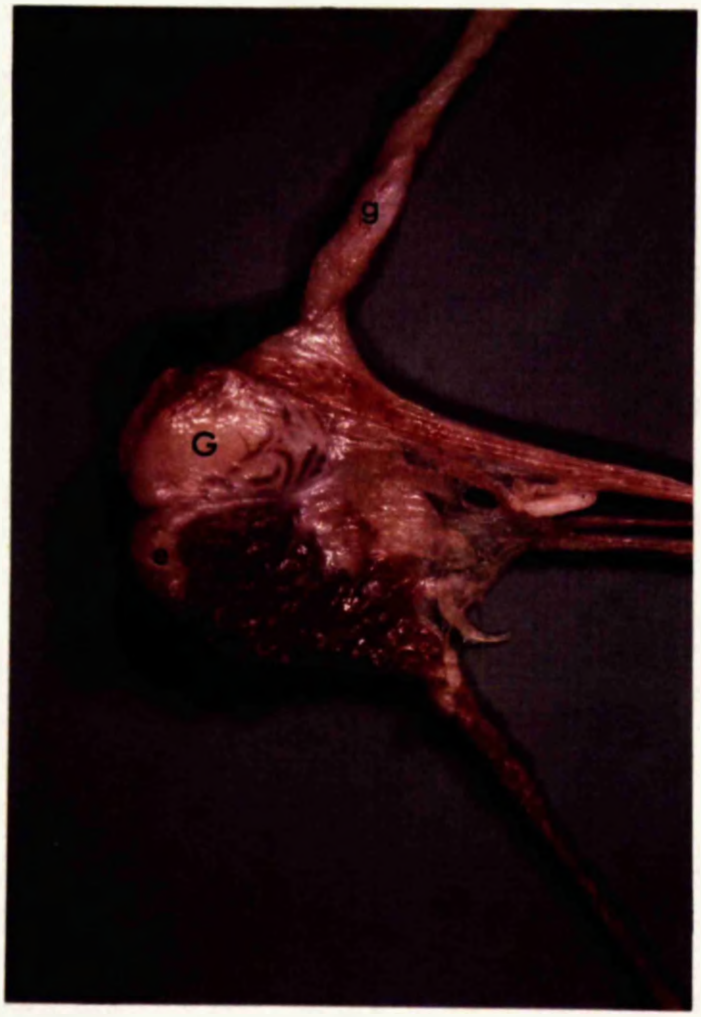

g
G

Fig. 66

THE INTERNAL GENITALIA OF

INTERSEX SHEEP No.4

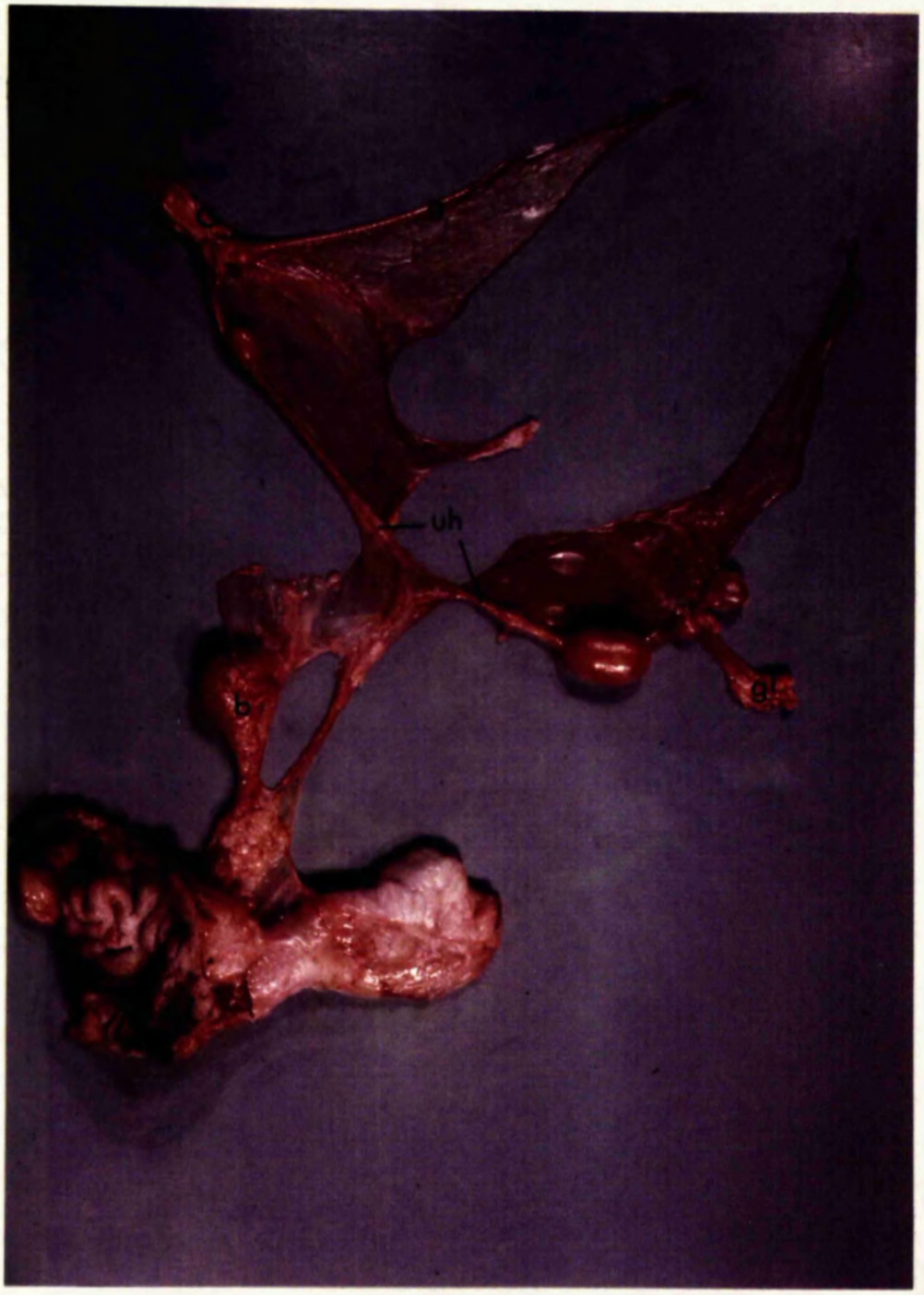

uh
b
gf

Fig. 67

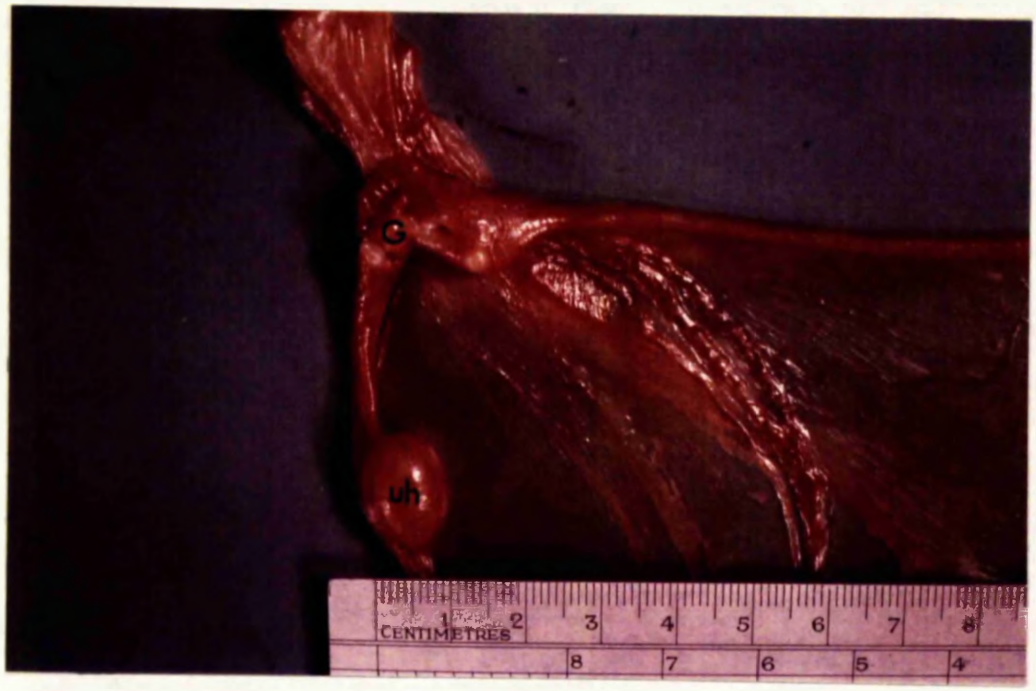
THE LEFT GONAD OF INTERSEX

SHEEP No. 4

Fig. 68

THE RIGHT GONAD OF INTERSEX

SHEEP No. 4


G
uh
CENTIMETRES

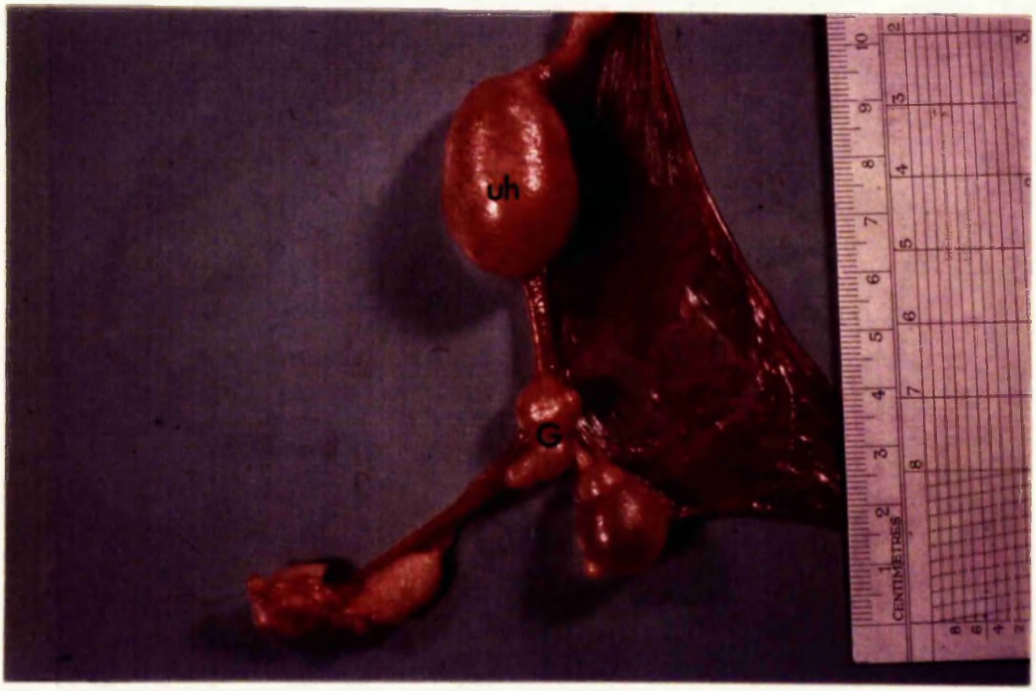

uh
G
CENTIMETRES

Fig. 69

THE INTERNAL GENITALIA OF

INTERSEX SHEEP No. 5

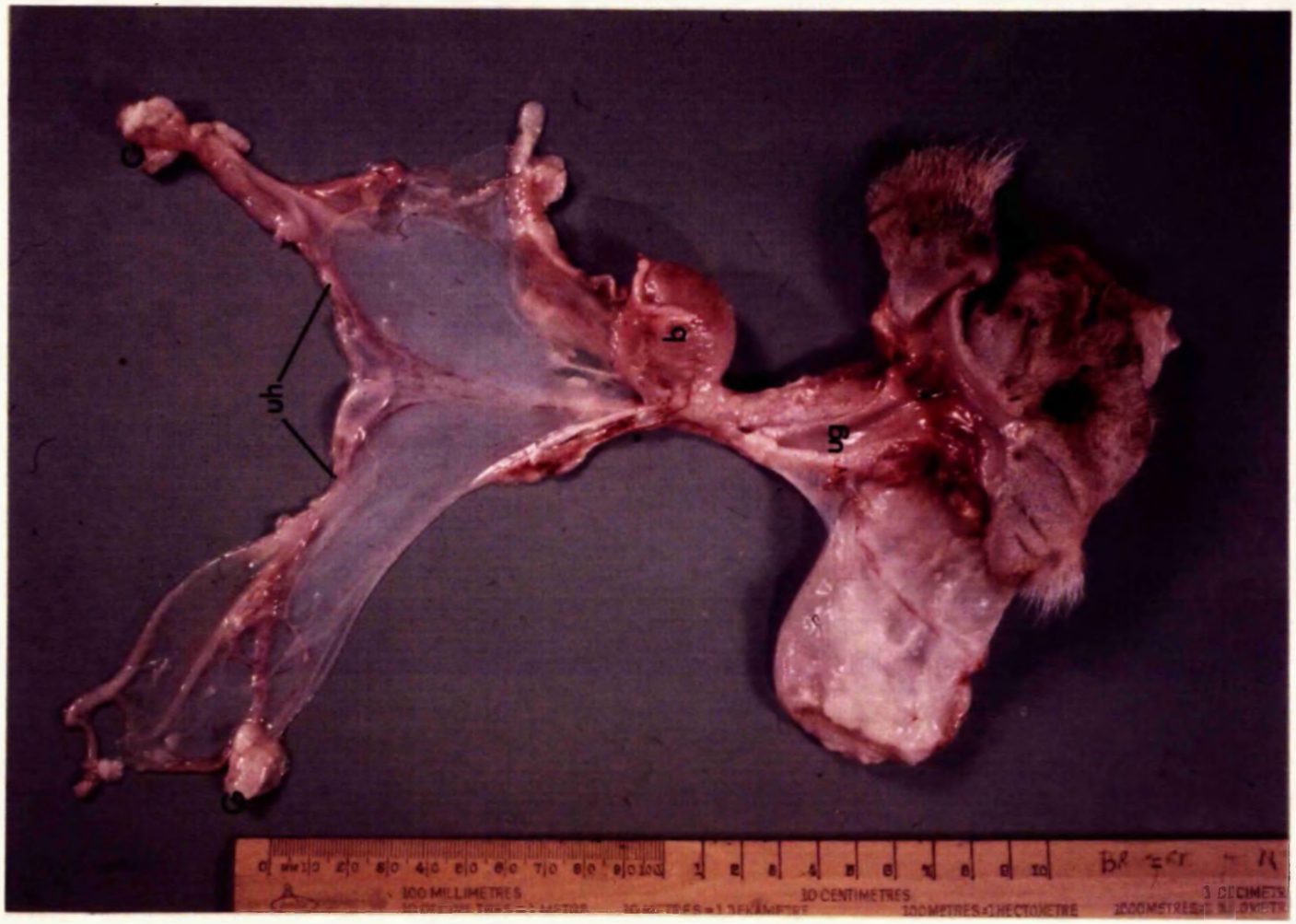
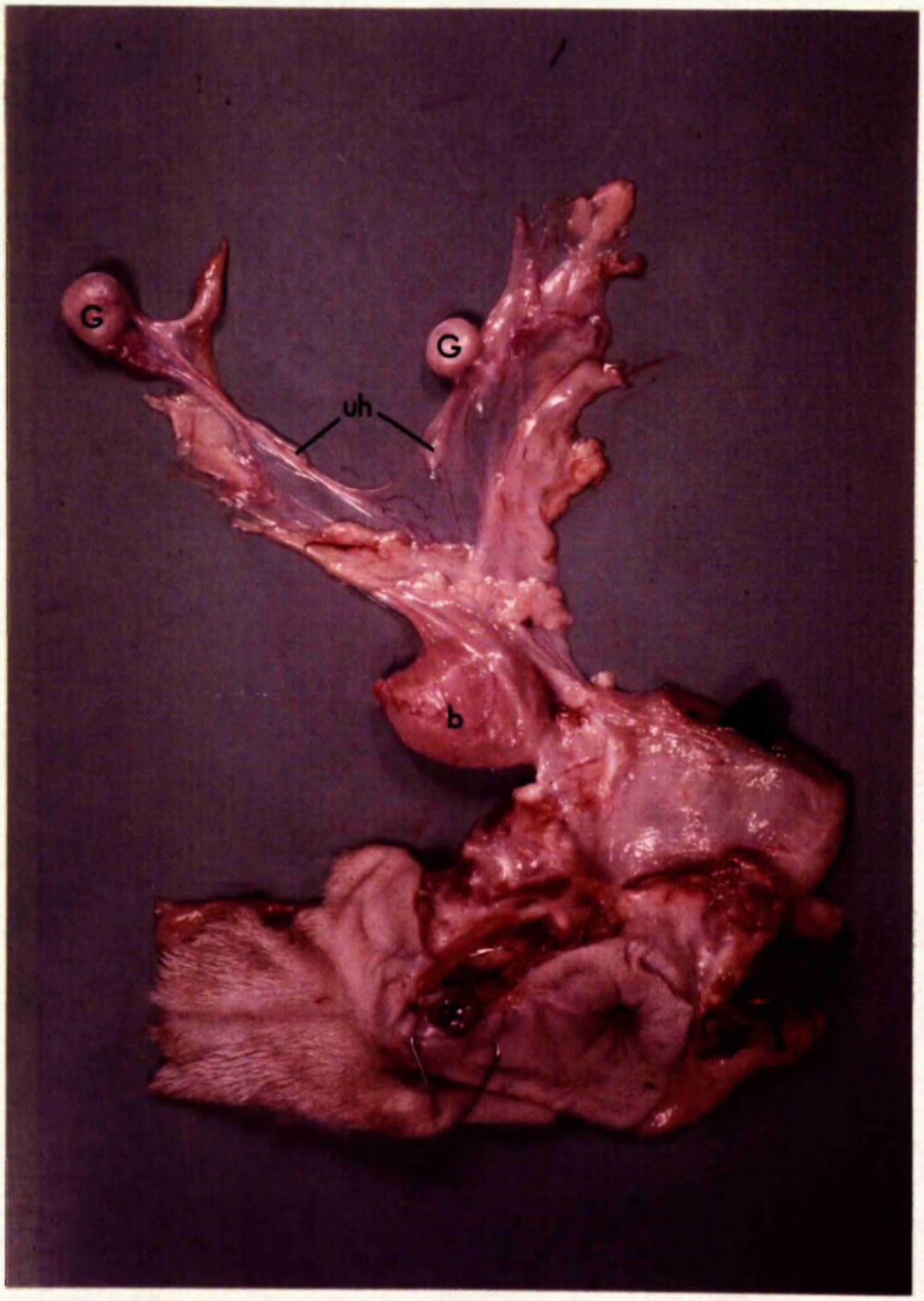

uh
b
gh
100 MILLIMETRES
10 CENTIMETRES

Fig. 70

THE INTERNAL GENITALIA OF

INTERSEX SHEEP No. 6


G
G
uh
b

glands were seen.

Histology of Intersex Gonads and Genitalia.

Similar cells were found in all gonads but their organisation and distribution varied considerably. There was a close correlation between the histological and anatomical findings.

The gonads of Case 1 presented uniformly distributed sex cords* (seminiferous tubules) consisting of a single layer of cells (Fig. 71). The cells composing this layer generally had rounded nuclei situated near to the basement membrane. Cytoplasmic strands streamed from the basement membrane towards the lumen of the tubules producing a pale syncytial mass. The cells resembled Sertoli cells, but the nuclei had a different shape due presumably to the uncrowded arrangement. No cells resembling gonocytes or spermatogonia could be seen. Surrounding the sex cords was an interstitial mass composed of interstitial cells and connective tissue cells. The gonads in Case No. 1 resembled closely those of a cryptorchid sheep.

In Case No. 2, the amount of intestitial tissue was increased and the sex cords tended to be grouped into/

*The term sex cord is preferred since no sperm producing cells were present.

into clusters (Fig. 72). Similar arrangements of ducts could be seen in the gonads of Cases 3 4 and 5. In the latter cases however, the amount of interstitial tissue increased and the sex cords in some regions had no apparent lumen (Fig. 75).

An area of cells resembling a rete testis could be seen in the gonads of Nos. 1, 2, 3, 4 and 5 (Fig. 76). This area was pushed to one side or end of the general mixture of connective tissue cells. The cells lining the irregular ducts of the rete were low columnar of cuboidal. A thin tunica albuginea covered gonads 1, 2, 3 and 5.

The epididymis in Cases 1, 2, 3 and 5 was lined by typically ciliated columnar cells (Fig. 77).

Sections of various ducts which resembled spermatic cords in Cases 1, 2 and 3, and abortive uteri in Cases 4 and 5, were examined. In Cases 1 and 2, the histological findings were as expected, namely ductus deferens, large blood vessels, muscle and connective tissue. In cases 4 and 5, sections taken of the cystic dilatations showed in some areas a resemblance to uterine formation. The innermost part of these sections resembled endometrium in which the uterine glands were well developed (Figs. 80 & 81).

In/

In Case 5 they contained melanotic pigment. In both cases a thin layer was present analogous to the myometrium of a normal uterus. The outer layer was composed of connective tissue blood vessels and peritoneal tissues typical of the serous layer of a normal uterus.

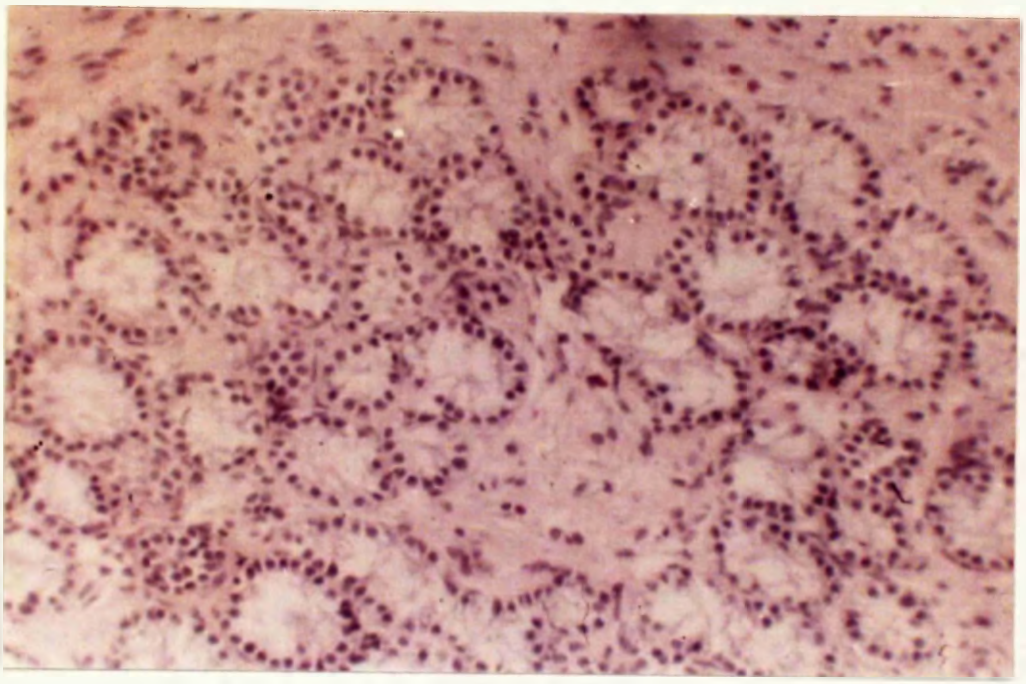
Histologically, the seminal vesicles resembled those of the castrated male sheep (Aitken 1955). Tall columnar epithelial cells with prominent nuclei lined the convoluted tubules of these glands. Treatment of sections of this gland with Periodic Acid Schiff (P.A.S.), showed strong positive staining of the cell cytoplasm while prior treatment of sections with diastase followed by P.A.S. resulted in a poor staining reaction. This would indicate the presence of intracellular glycogen (Figs. 78 & 79).

Fig. 71. Case No.1

GONAD. H and E. X 100

Fig. 72. Case No. 2

GONAD. H and E. X 100



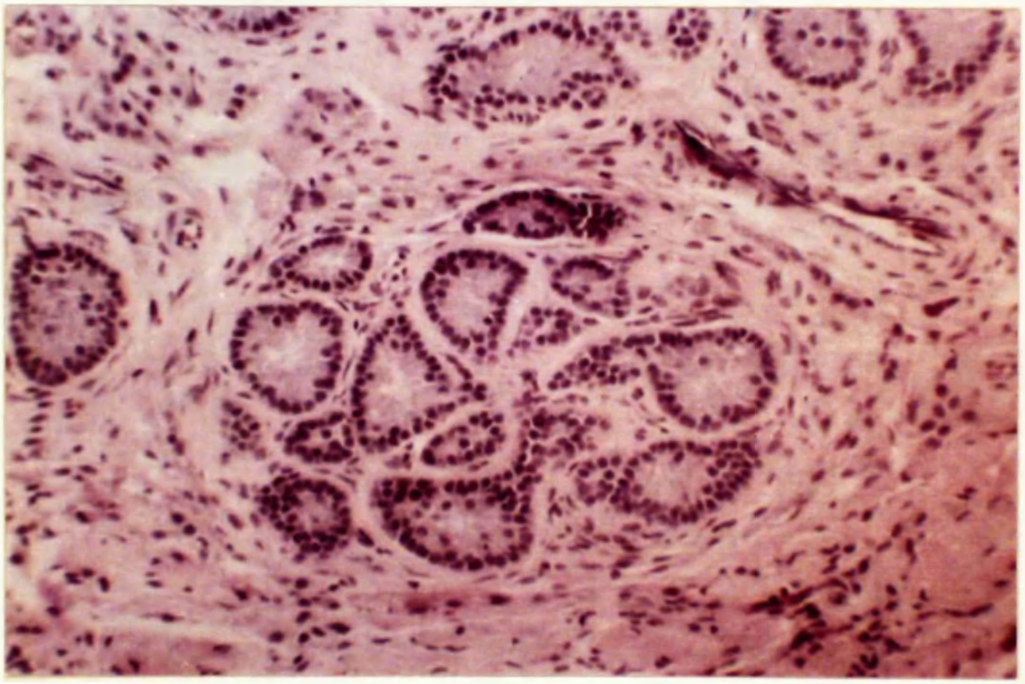
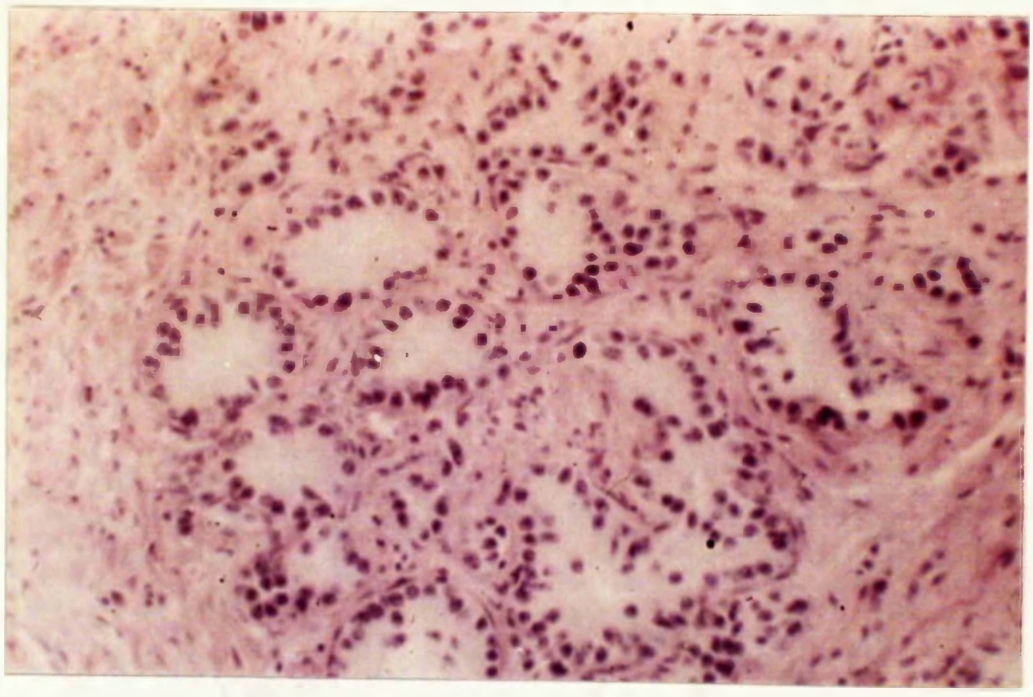


Fig. 73. Case No. 3

GONAD. H. and E. X 100

Fig. 74 Case No. 1.

GONAD H. and E. X 400



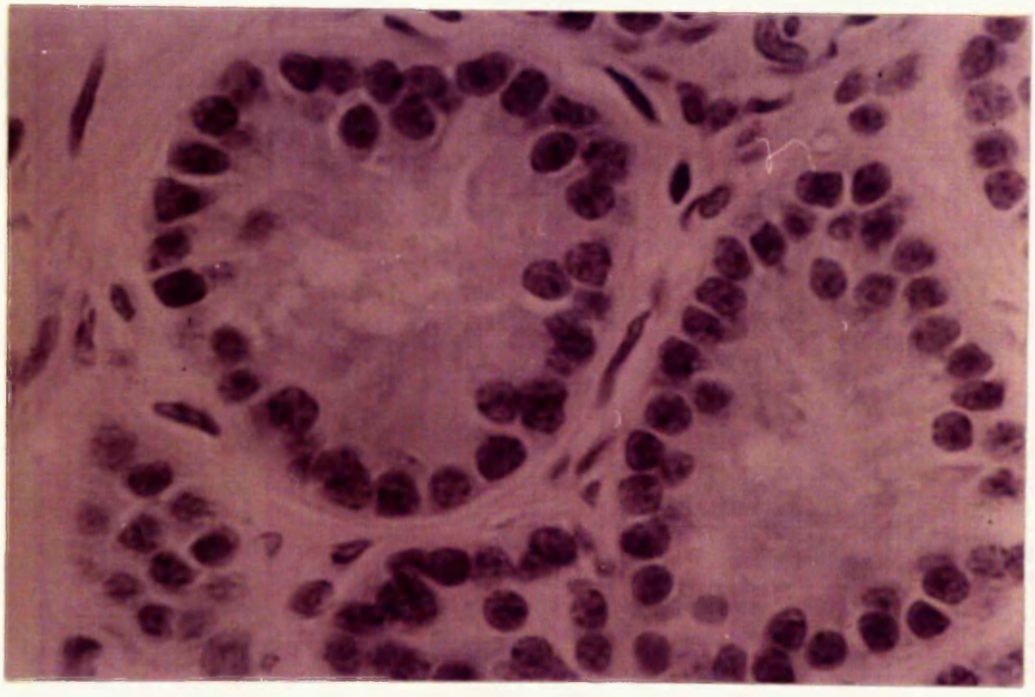


Fig. 75.

GONAD H and E. X 60

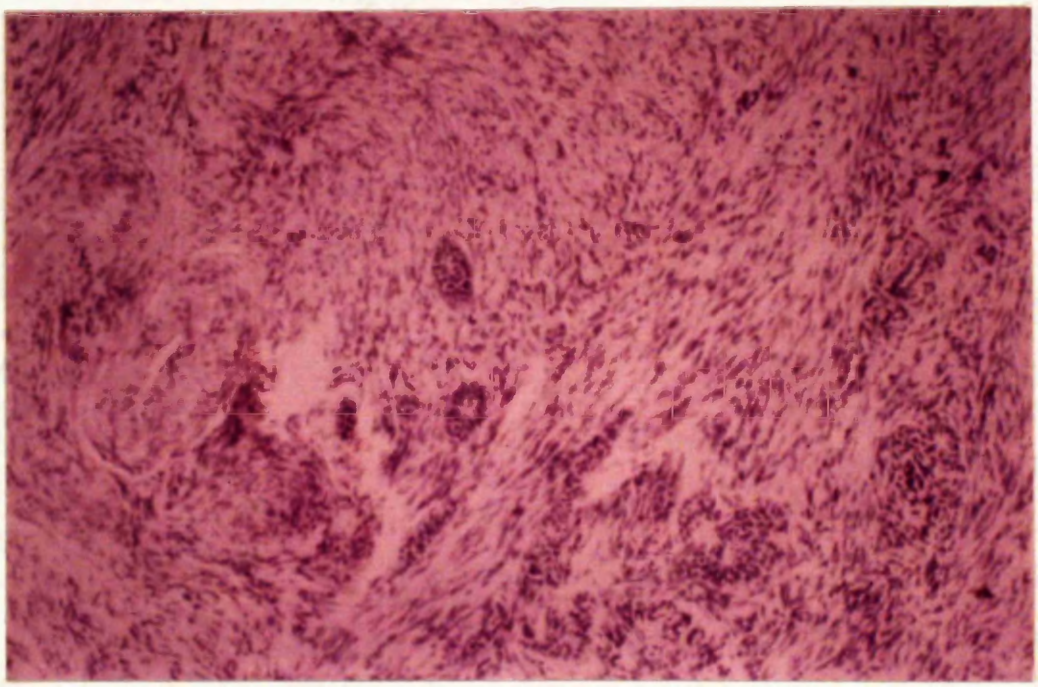
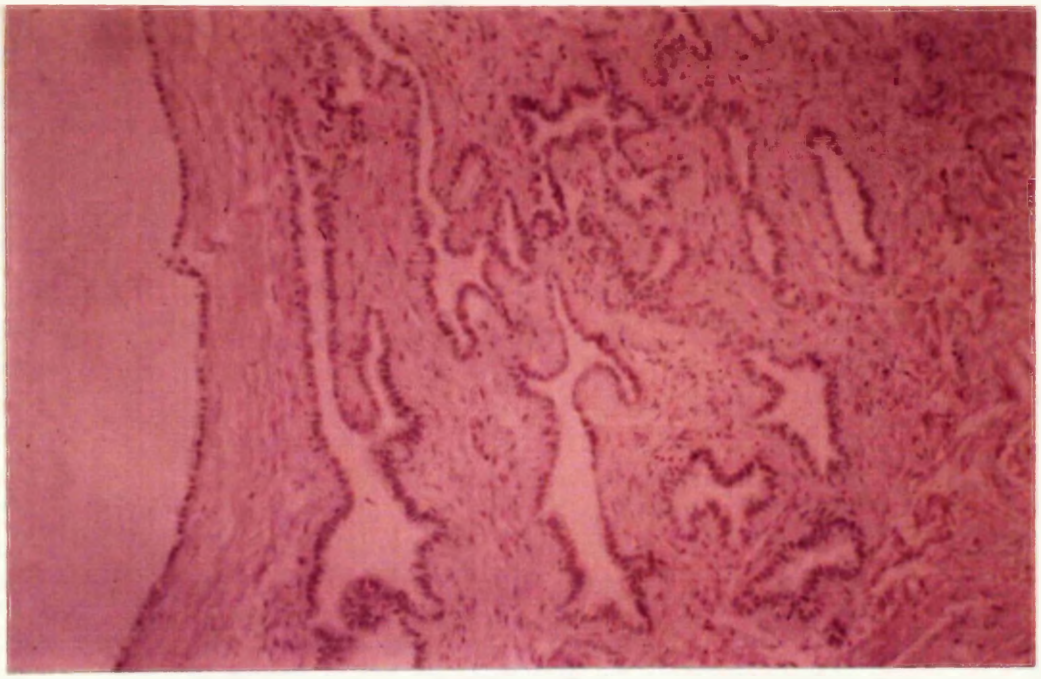


Fig. 76. Case No. 3.

RETE TESTIS H and E. X 60

Fig. 77. Case No. 1.

EPIDIDYMIS. H and E. X 100



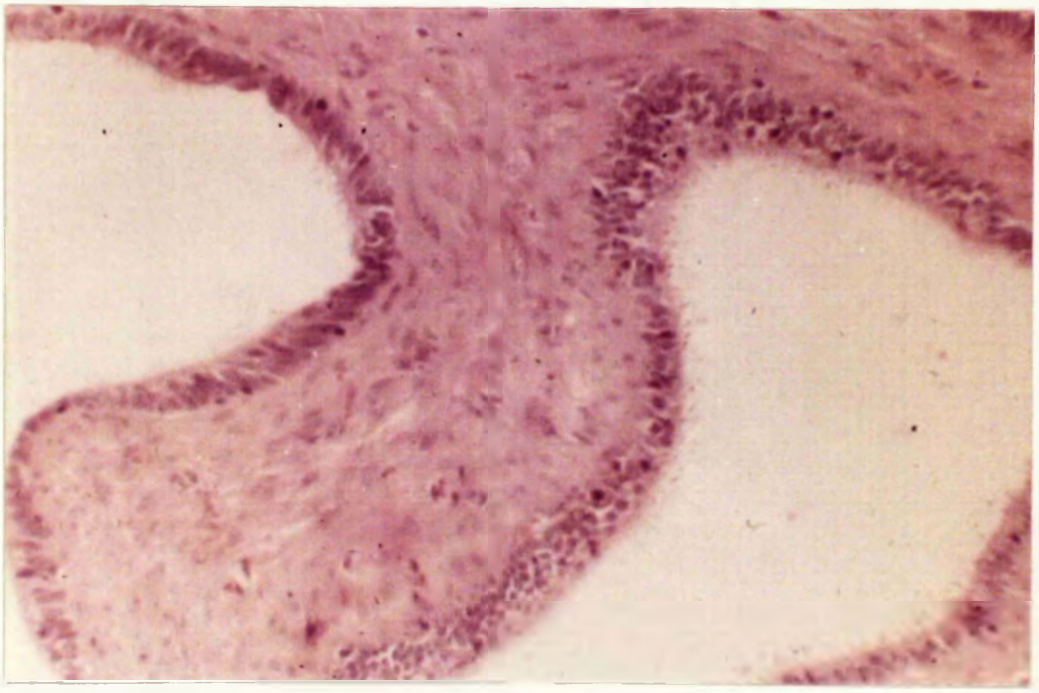
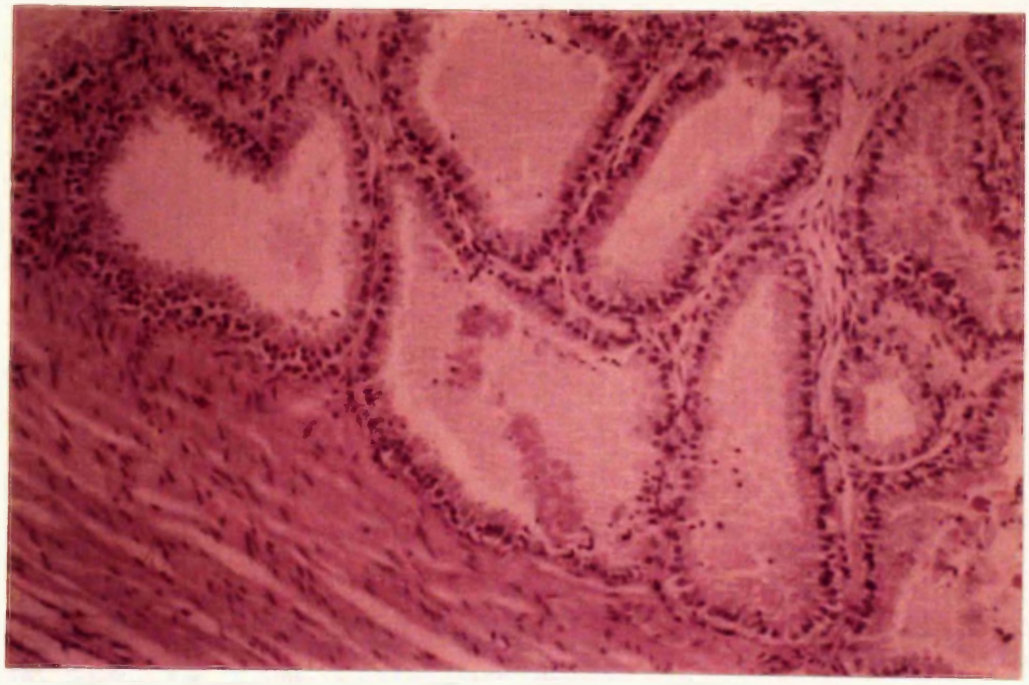


Fig. 78. Case No. 4

SEMINAL VESICLE. H and E. X 60

Fig. 79. Case No. 4

SEMINAL VESICLE. P.A.S. X 400



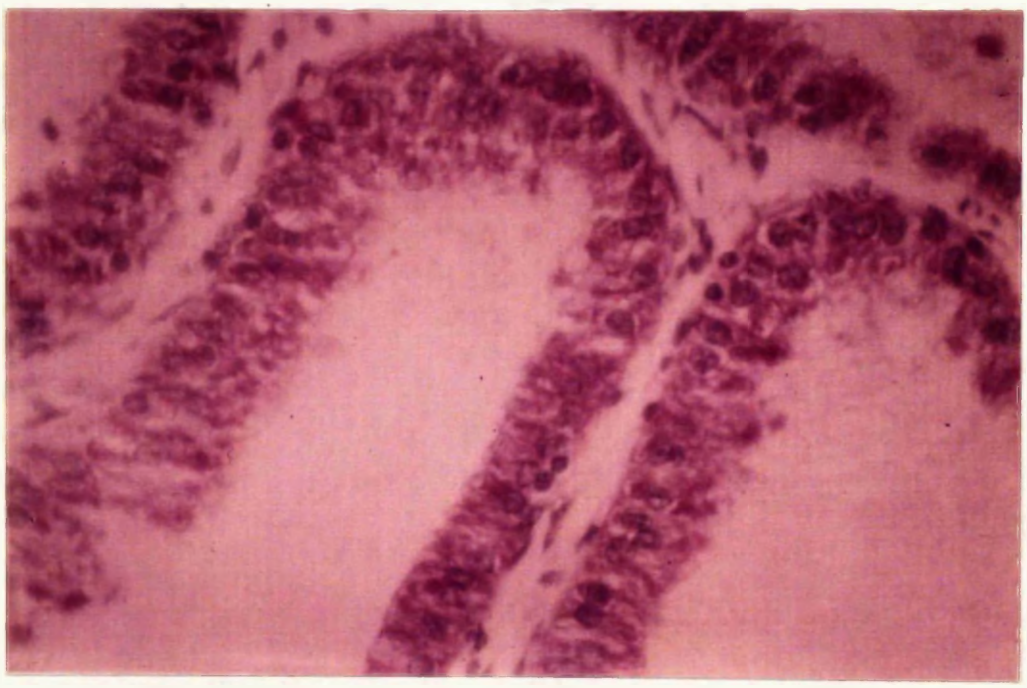
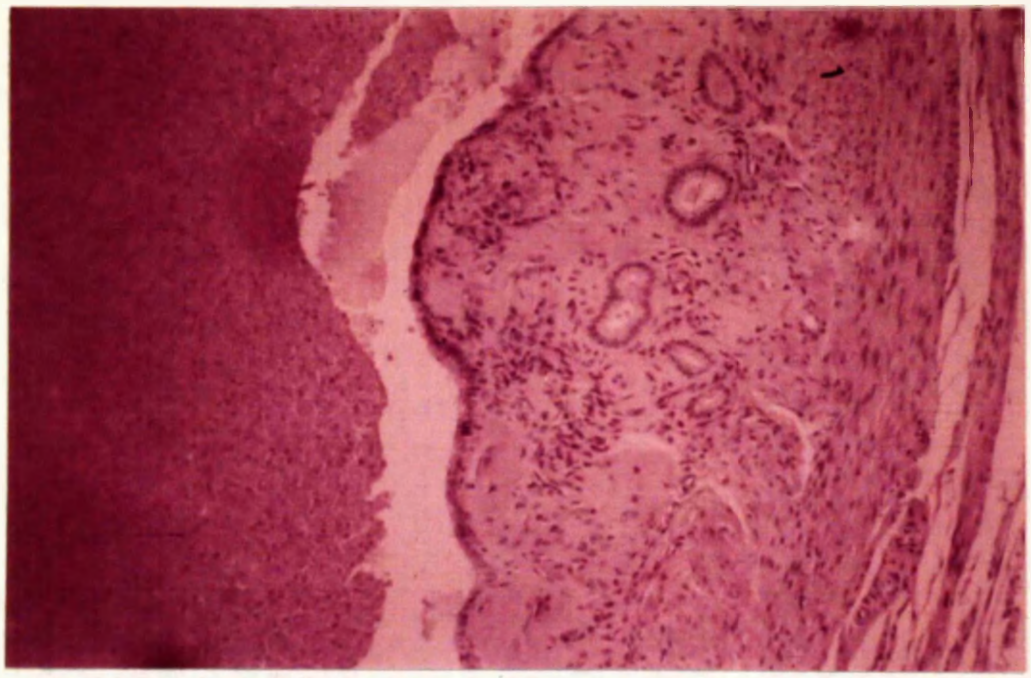


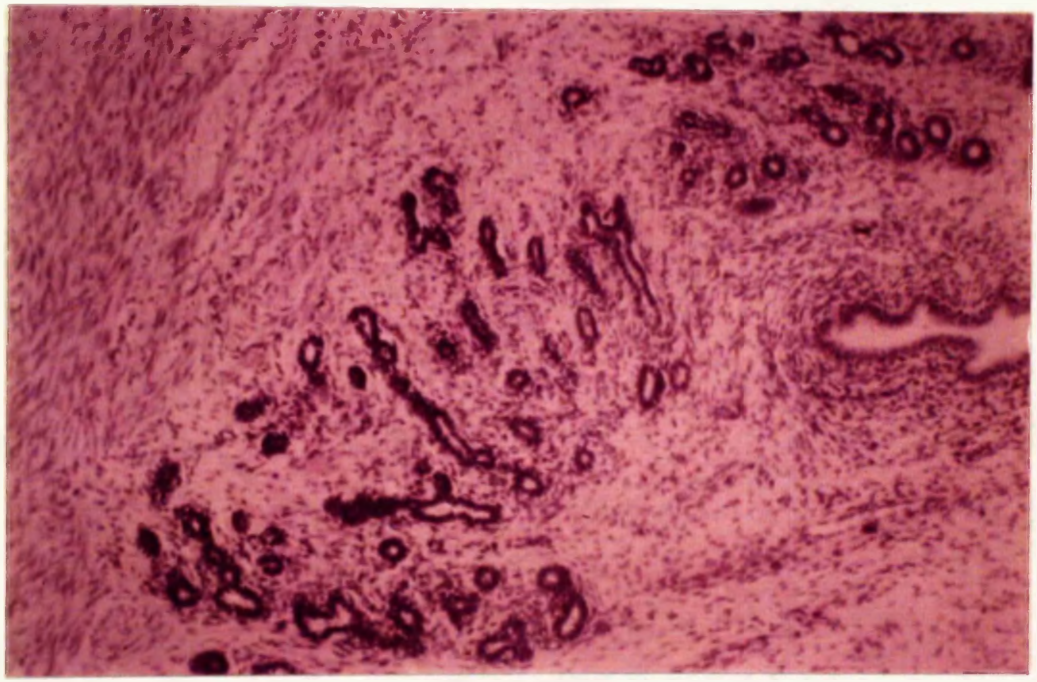
Fig. 80. Case No. 4

SECTION OF uh , Fig. 66 H and E. X 60

Fig. 81 Case No. 5

Section of uh , Fig. 69 H and E. X 60





CHROMOSOME STUDIES FROM LEUCOCYTE CULTURES.

Chromosome preparations were made from leucocyte cultures of all the intersex cases described. In each case the chromosomes from at least two satisfactory cultures were examined and more than was actually necessary to establish chimaerism were counted. The reason for this was to compare the larger number of non-modal cells found in chromosome counts of these animals with those of the normal sheep. It was thought that a third cell line may have been present in some of these animals.

Results.

The results of chromosome counts from the six intersex animals is given in Table 15.

It is clear from the above tables that each sheep was a blood chimaera, metaphases with both XX and XY sex chromosome complements being present. With the exception of Case 5 the predominant cell line was male but in some individual cultures there was a reversal of this in successive cultures to female. This is evident in Case 1 between the first and fourth cultures and in Case 3 between the first and second cultures.

On only two occasions was the diploid male set

of/

TABLE 15.

LEUCOCYTE CULTURES OF INTERSEX SHEEP Nos. 1-6

INTERSEX No. 1.

Chromosome No.	50	51	52	53	54	55	Poly	XX*	XY*
Culture A265	1	1	0	8	58	0	1	1	58
" R265	0	0	0	1	6	0	0	6	0
" T265	1	0	0	0	15	1	1	10	6
" K365	2	1	2	4	54	2	1	45	11
Total Cells 160	4	2	2	13	133	3	3	62	75

INTERSEX No. 2.

INTERSEX No. 2.

Chromosome No.	50	51	52	53	54	55	Poly	XX*	XY*
Culture C265	1	1	2	4	12	0	0	1	11
" F265	3	1	1	10	60	0	1	7	55
" Q265	2	0	1	2	14	0	2	2	14
" S265	1	0	1	2	45	0	1	7	40
Total Cells 167	7	2	5	18	131	0	4	17	120

XX and XY Chromosomes counted only on cells with 54 Chromosomes and Tetraploid cells.

TABLE 15 (contd.)

INTERSEX No. 3.

Chromosome No.	50	51	52	53	54	55	Poly	XX*	XY*
Culture 2165	0	0	3	0	43	1	1	35	10
" L265	4	3	3	12	80	2	2	6	78
" M265	0	0	0	1	3	0	1	0	3
" N265	3	0	2	11	25	1	3	2	27
Total Cells 204	7	3	8	24	151	4	7	43	118

INTERSEX No. 4.

Chromosome No.	50	51	52	53	54	55	Poly	XX*	XY*
Culture E265	0	0	1	1	19	1	0	1	19
" K265	10	3	11	15	130	5	1	10	125
" W365	2	1	9	20	171	2	7	11	170
Total Cells 409	12	4	21	36	320	8	8	22	314

* XX and XY Chromosomes counted only on cells with 54 Chromosomes and Tetraploid Cells.

TABLE 15. (contd.)

INTERSEX No. 5.

Chromosome No.	50	51	52	53	54	55	Poly	XX*	XY*
Culture 066/1	2	3	5	7	90	2	1	86	7
" P66/1	0	2	3	13	169	3	3	167	11(2)
Total cells (303)	2	5	8	20	259	5	4	253	18(2)

INTERSEX No. 6.

Chromosome No.	50	51	52	53	54	55	Poly	XX*	XY*
Total Cells(206)	1	1	6	11	186	1	0	80	107

XX abd XY Chromosomes counted only on Cells with 54 Chromosomes and Tetraploid Cells.

of chromosomes found beside the diploid female set of chromosomes (Fig. 82). Usually cells of the same sex were together.

Except in No. 2 the incidence of both hypermodal and hypomodal cells were greater than in normal sheep (Tables 16 & 17).

Photographic karyotypes of all the hypermodal cells were prepared to see if a common chromosome was triplicated (Figs. 83 & 84) show metaphases with 55 and 56 chromosomes respectively. In none of these cases was it possible to establish the identity of the additional chromosome. It was however always a member of the acrocentric group. Hypermodal cells were both of male and female karyotypes, therefore it seemed unlikely that this was an additional X chromosome as seen in certain types of mosaicism in man. The possibility of the additional chromosome having been acquired from neighbouring cells due to disruption and chromosome separation is low. "Foreign" chromosomes usually can be detected as they tend to differ from their host chromosomes by showing a different degree of contraction. This is caused by the varying stages at which colchicine has affected the cells. The

only/

TABLE 16.

INDIVIDUAL CHROMOSOME COUNTS OF SIX INTERSEX SHEEP
LEUCOCYTE CULTURE.

SHEEP	AGE	NO. CELLS EXAMINED	HYPOMODAL %	MODAL %	HYPERMODAL (55) %	POLYPLOID %
No. 6	18 months	206	9.22	90.29	0.47	0
No. 5	4 years	303	11.55	85.48	1.65	1.32
No. 4	5 years	160	13.12	83.13	1.88	1.88
No. 1	5 years	167	19.16	78.44	0.00	2.39
No. 3	7 years	409	17.84	78.24	1.96	1.96
No. 2	7 years	204	20.58	74.02	1.97	3.43

TABLE 17.

COMPARISON OF CHROMOSOME COUNTS FROM 22 NORMAL SHEEP WITH 5 INTERSEX SHEEP.

SHEEP	AVERAGE AGE	CELLS EXAMINED	HYPOMODAL %	MODAL %	HYPERMODAL (55) %	POLYPLOID %
22 Normal	2 years	1832	10.81	87.44	0.32	1.36
5 Freemartin		1243	16.45	79.86	1.49	2.20
% Difference			+ 5.64	- 7.58	÷ 1.17	÷ .84

only analysis that could be applied to the hypomodal cells was a comparison of the ratios of male and female cells between hypomodal cells and modal and hypermodal cells. In Table 18 it can be seen that in the case of each animal the percentage of cells with or without Y chromosomes did not vary significantly between the two groups. This would suggest that there was little likelihood of a regularly occurring hypodiploid cell line.

Part of the increase in hypomodal cells would correspond to the increase in hypermodal cells provided these were the result of non-disjunction and that the hypomodal cells remained viable. A large proportion of the hypomodal cells however, must be accepted as artefacts caused by preparation procedures and perhaps over-scanning of the slides. Whether or not the increase is hypomodal cells is artificial does not reduce the significance of the increase in hypermodal cells.

The increase in aneuploid cells could be associated with freemartinism, or it could have been an effect of the age of these animals. An increase in aneuploid cells has been shown to be associated with aging in man (Jacobs, Court-Brown and Doll, 1961)./

TABLE 18.

ANALYSIS OF HYPOMODAL CELLS OF INTERSEX SHEEP

ANIMAL	HYPOMODAL CELLS		MODAL & HYPERMODAL CELLS	
	Y Present	Y Absent	Y Present	Y Absent
No. 1	13 61.9%	8 38.1%	75 54.75%	62 45.25%
No. 2	24 75%	8 25%	120 87.59%	17 12.41%
No. 3	35 83.33%	7 16.66%	118 73.29%	43 26.71%
No. 4	65 92.86%	5 7.14%	314 93.40%	22 6.6%
No. 5	2 5.71%	33 94.29%	18 6.64%	253 93.36%

(1961). The intersex sheep Nos. 1-5 were aged between four and seven years, while the normal sheep with which they were compared were all under two years of age. It is not possible to check the possibility of the latter alternative, since no chromosome counts were made on normal sheep of comparable age. Conversely however, the chromosome counts from Intersex sheep No. 6 which was eighteen months old were similar to those of the young sheep (Table 16). In this case there were 90.29% of modal cells out of a total of two hundred and six counted. Only one genuine aneuploid cell was seen.

Figs. 85-87 show fusion of chromosomes, presumably due to breakage and reconstitution of non-homologous fragments. This phenomenon was recorded only in preparations from the intersex sheep. Similar phenomena are seen in human preparations (Ferguson-Smith, personal communication). A case reported in man by Trujillo, Stenius, Ohno and Nowack (1961) showed a translocation between the second largest autosome and a member of the 6-12 group in a number of metaphases from a first leucocyte culture. In a second culture from the same/

Fig. 82.

MALE and FEMALE CELL IN APPOSITION.

Y CHROMOSOME ARROWED.

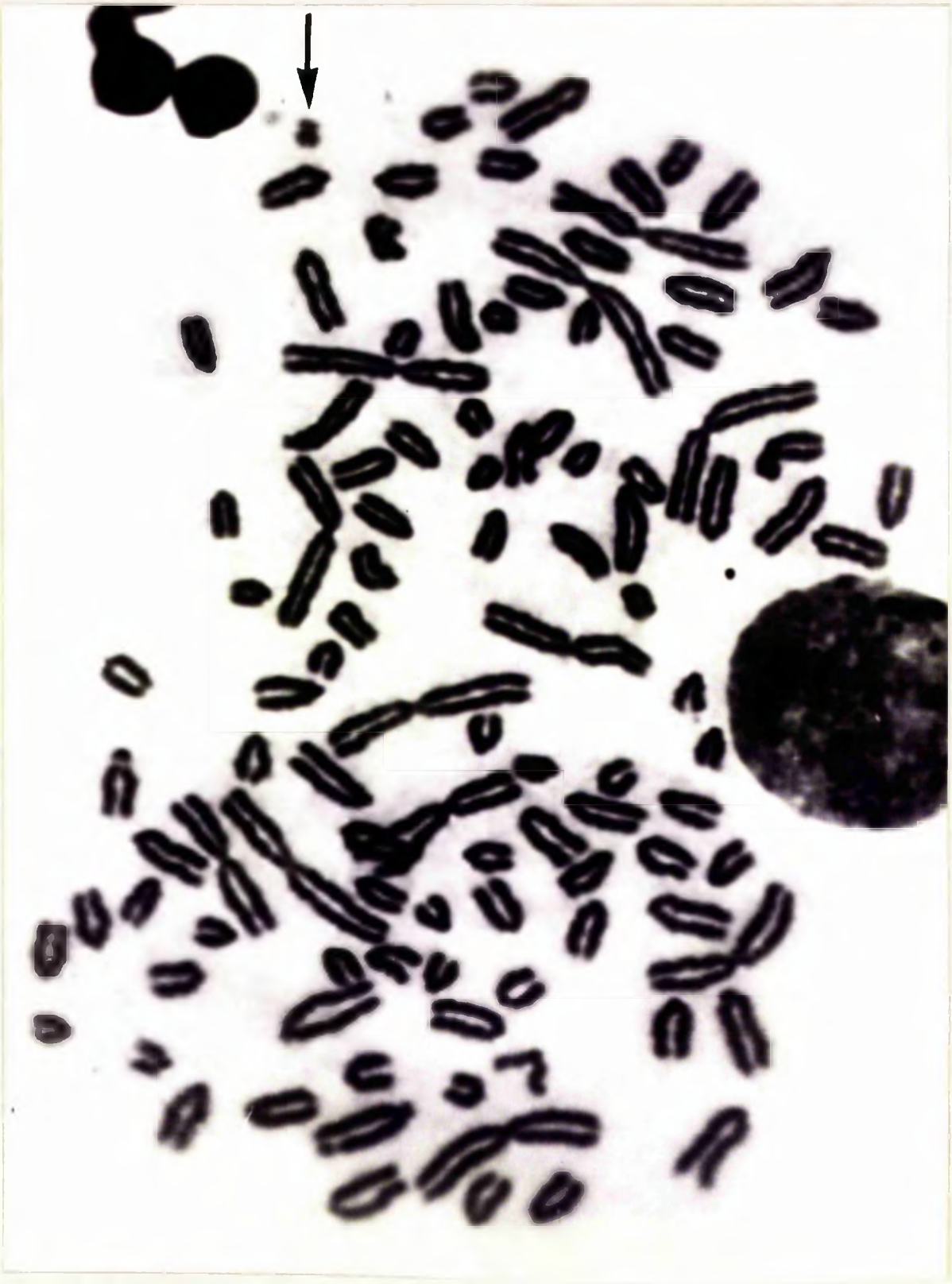


Fig. 83.

HYPERDIPLOID CELL (55 Chromosomes.)

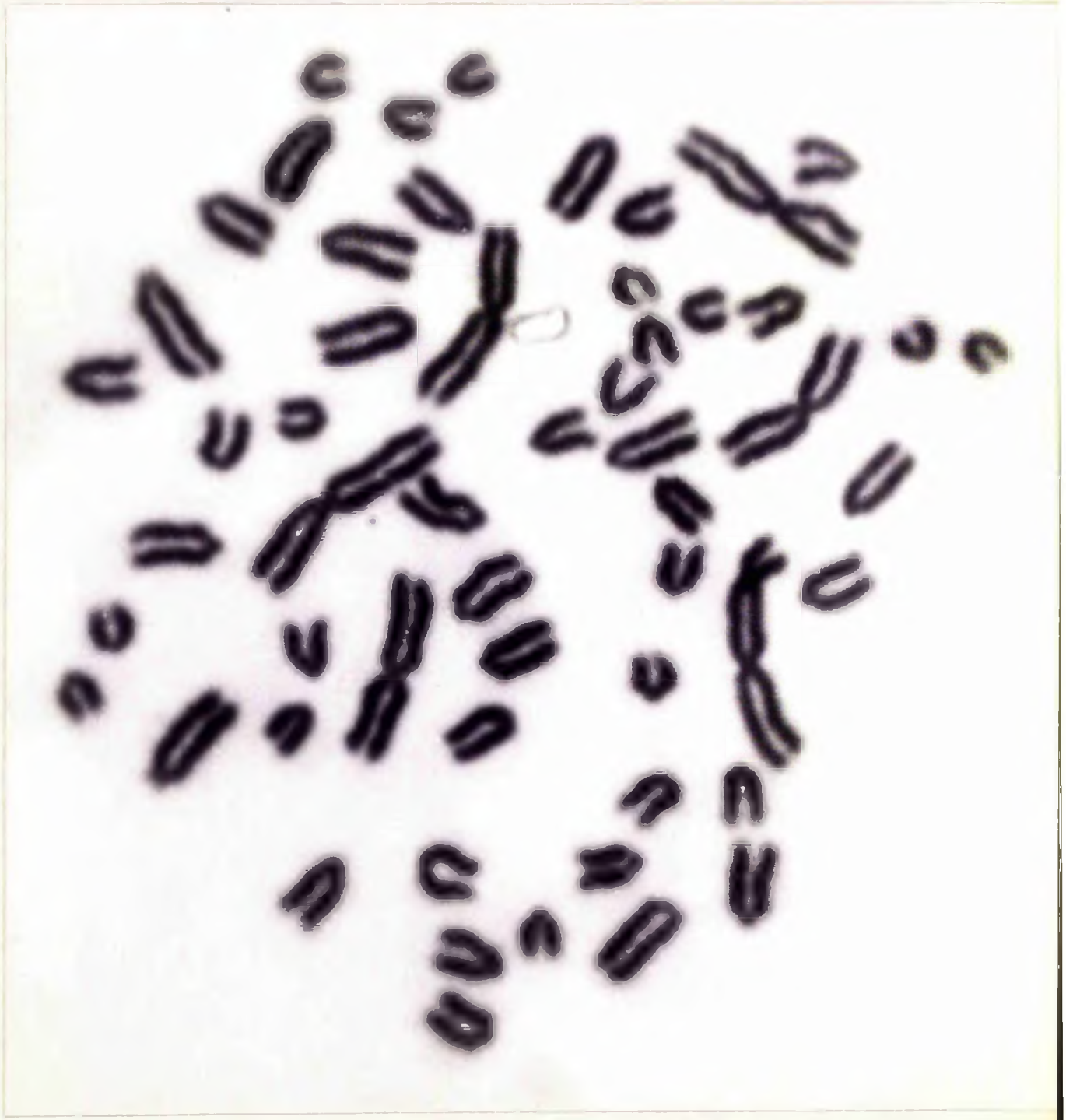


Fig. 84.

HYPERDIPLOID CELL. (56 Chromosomes).

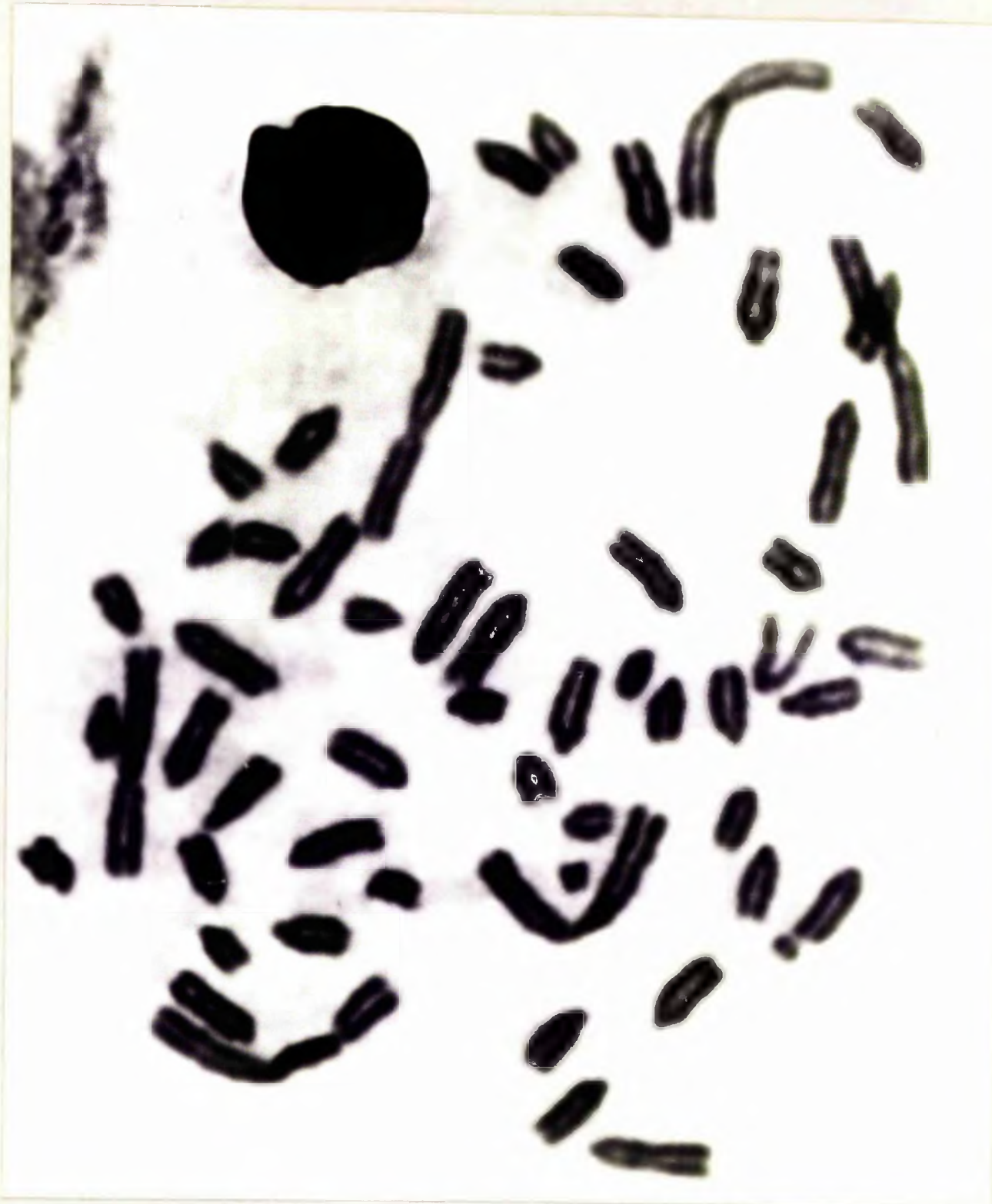


Fig. 85

BREAKAGE OF A METACENTRIC CHROMOSOME AND FUSION WITH AN ACROMETRIC CHROMOSOME.

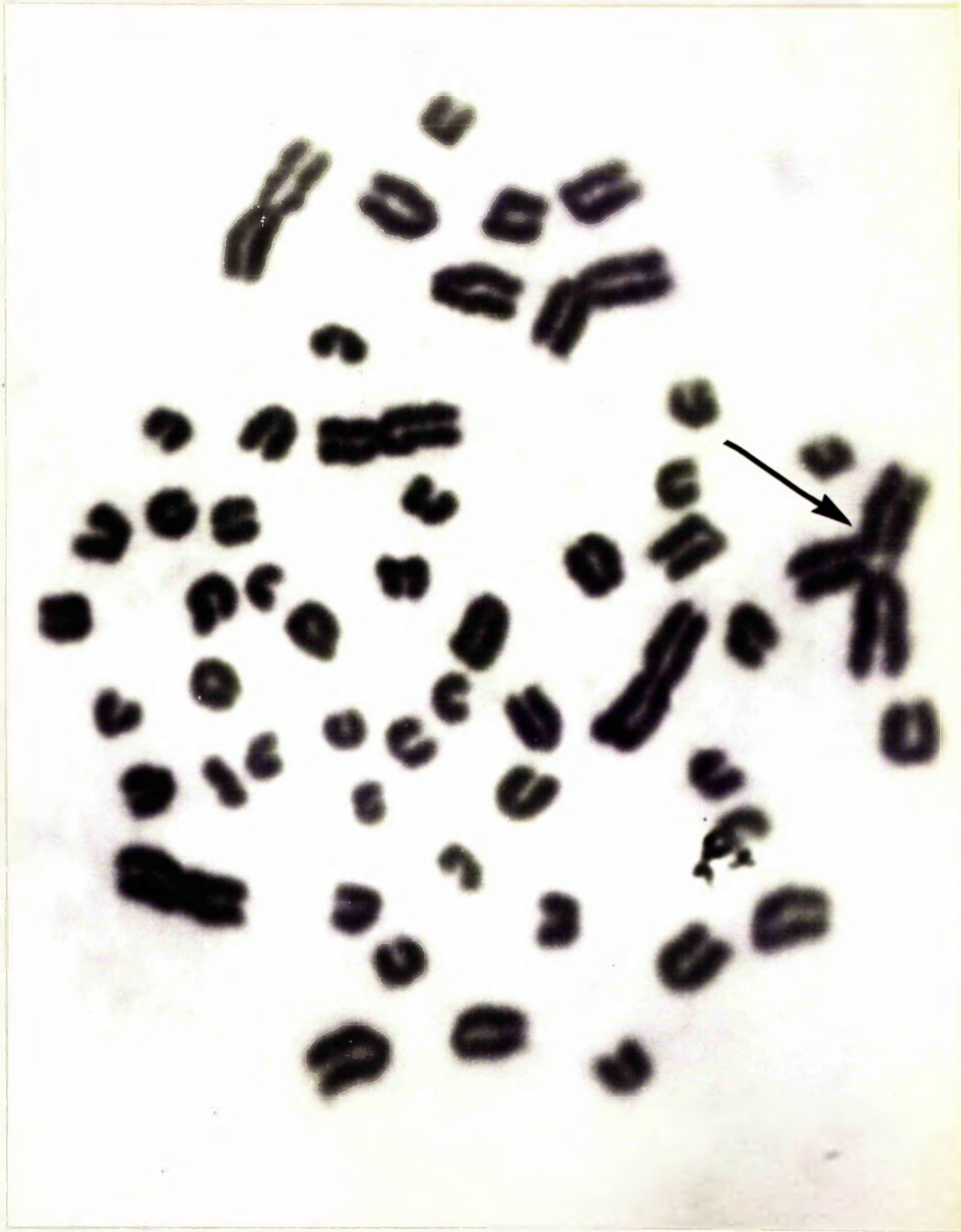


Fig. 86.

TRIAD FUSION OF 3 ACROCENTRIC CHROMOSOMES.
Presumably due to breakage and reconstitution of fragments.

Possible acrocentric association arrowed bottom left.

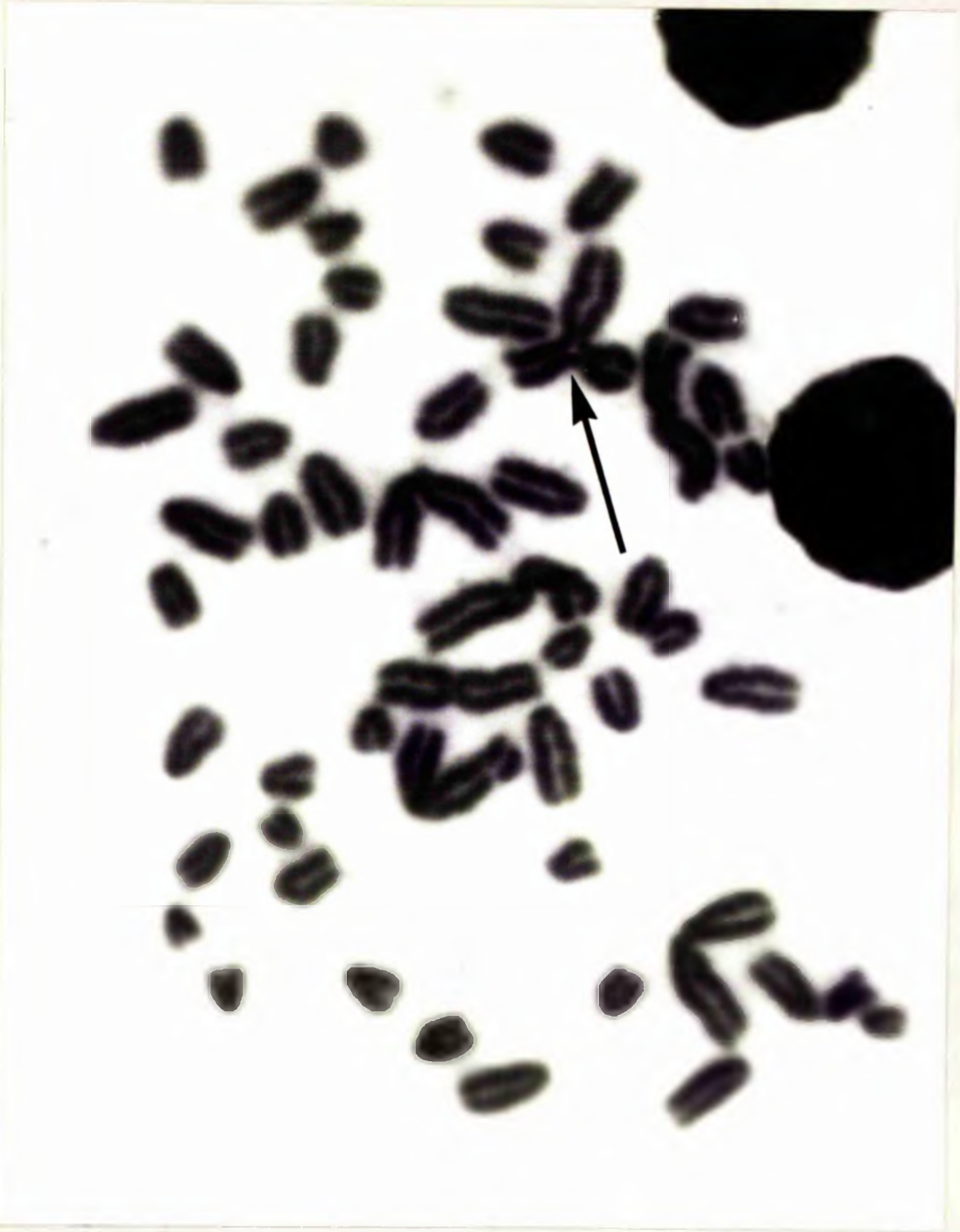
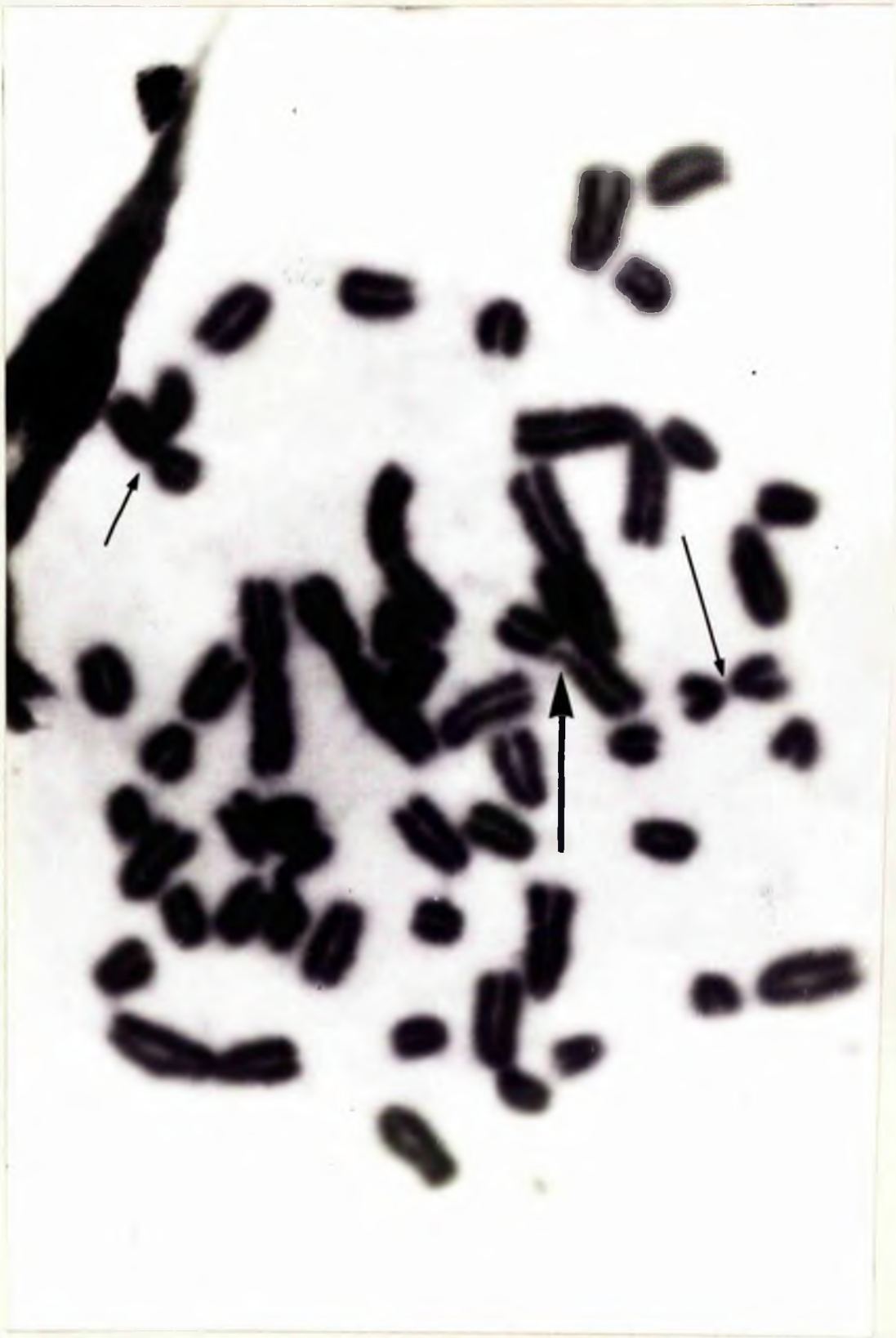


Fig. 87

TRIAD FUSION OF 3 ACROCENTRIC CHROMOSOMES AS IN Fig. 86.

Two possible acrocentric associations arrowed, top left and bottom.



same patient, none of these cells were seen. These authors concluded that the translocation observed in the first culture must have arisen in vitro. In the absence of any regular findings or great numbers of these cells in the sheep it is assumed that they arose in a similar manner.

CHROMOSOME STUDIES FROM FIBROBLAST CULTURE OF OTHER TISSUES.

The examination of the chromosomes in other tissues in the intersex sheep was of great importance, in order to establish whether mixed cell lines were present in any tissues other than blood. For the culturing of the various tissues the author is indebted to Mrs. J. McNab of the Department of Genetics of Glasgow University who supplied the following description of the method.

Culture Method.

Explants of muscle kidney and gonads were collected aseptically in Waymouth's MB 752/1 medium supplemented by 10% millipore filtered calf serum (see appendix 4). Antibiotics consisting of 20,000 units penicillin and 0.002 gm. streptomycin (Crystamycin, see appendix 4) per 100 ml. medium were/

were added to inhibit bacterial growth. Explants were stored at 4°C. until culture. Explants stored for up to 4 days at this temperature grew well.

Before transferring the explant to a sterile 2 oz. medical flat containing growth culture medium, the explant was well cleaned. Five to six changed of Hanks Balanced Salt Solution containing 200 units penicillin and 0.002 gm. streptomycin per 1 ml. were pipetted over the explant and blood and fat were removed as far as possible. The tissue was then chopped into pieces of about 2-4 mm. diameter and allowed to stick on to the glass surface of a 2 oz. medical flat bottle. Waymouth's growth medium supplemented by the before mentioned antibiotics and by 10% filtered calf serum was introduced into the bottle and the bottle kept, by means of plasticine, at a slope of about 30° to the horizontal so that the explant just touched the medium and still remained stuck on to the glass surface. This is essentially an adaption of the original Carrel Flask Technique, without the use of a plasma clot.

The medium was buffered by the incorporation of Tris HCl 2.5 mM final concentration (Appendix 4) buffered to pH 7.1 in the original Waymouth's medium.

After/

After several days cells of fibroblast morphology began to grow out from the explant and when they grew to a diameter of about 5-10 mms. they were considered ready to transfer. Before transfer the explant was carefully removed and set up in a new 2 oz. medical flat where it would repropagate in fresh culture medium.

Cell outgrowth from the original explant was washed carefully in 3 ml. versene buffer (0.2 gm %) (Appendix 4) and trypsinized in 1 ml. trypsin versene buffer (Appendix 4) in 0.002 gm % versene) at 37°C for about 5 mins. Suspended cells were then propagated in 10 ml. of Waymouth's medium + 10% calf serum in 6 oz. medical flats. Stocks were maintained in medical flats in the culture medium by serial passage using 2:1 splits. Cells could be stored in 5% glycerol at -70°C and these cells regenerated well on regrowing in Waymouth's medium + 10% calf serum.

CYTOLOGY

Bottles for cytology were allowed to reach confluence and then split 2:1 as routinely. After 24 hours' growth, colcemide at a concentration of 4 ugms per 1 ml., was added to arrest mitoses and allowed to act overnight or for approximately 8-10 hours.

Medium/

Medium from the cultures, together with versene buffer wash and trypsinized cell suspensions, was then spun (600 revs/min.) for 10 mins. in the centrifuge. Slides were made of the chromosomes according to the technique of Moorhead et al., (1960) and stained by 2% aceto-orcein.

Results and Discussion.

In viewing the metaphase preparations of the various fibroblast cultures, there was unfortunately a very high wastage of material due to the longer time required for colchicine treatment. The effect of this was to produce a number of metaphases in which chromatid separation had occurred, so that the number of suitable metaphases for scoring was reduced considerably.

Sufficient metaphases were examined, however, from four animals and from various tissues of these animals to show that no male cell line was present in any of them (Table 19). This result showed that these animals were genetic females but the full implication of this is discussed in the general discussion together with the sex chromatin studies.

The number of cells counted from fibroblast cultures was too small to apply an analysis of the chromosome/

TABLE 19.

FIBROBLAST CULTURES FROM INTERSEX SHEEP Nos. 1-4

INTERSEX No. 1.

Chromosome No.	50	51	52	53	54	55	Poly*	XX	XY	Total
Muscle	1	3	1	1	24	0	0	24	0	30
Right Gonad	1	0	2	2	6	0	1	8	0	13
Left Gonad	0	0	0	0	2	0	0	2	0	2
Right Kidney	0	1	1	0	19	0	1	21	0	23
Left Kidney	0	0	0	1	13	0	0	13	0	14
Total Cells	2	4	4	4	64	0	2(4)	68	0	82

INTERSEX No. 2.

Chromosome No.	50	51	52	53	54	55	Poly	XX	XY	Total
Muscle	3	0	1	6	16	2	0	18	0	28
Left Gonad	0	0	1	2	21	0	0	21	0	24
Left Kidney	0	0	0	0	5	0	0	5	0	5
Right Kidney	0	1	3	2	52	1	0	53	0	59
Total Cells	3	1	5	10	94	3	0	97	0	116

* Counted as two cells when Tetraploid.

TABLE 19 (contd).

FIBROBLAST CULTURES FROM INTERSEX SHEEP Nos. 1-4.

INTERSEX No. 3.

Chromosome No.	50	51	52	53	54	55	Poly.	XX	XY	Total
Muscle	6	0	3	6	51	0	0	51	0	66
Right Kidney	3	1	2	7	37	0	10	57	0	70
Total Cells	9	1	5	13	88	0	10(20)	118	0	136

INTERSEX No. 4.

Chromosome No.	50	51	52	53	54	55	Poly.	XX	XY	Total
Muscle	2	0	4	2	31	2	0	33	0	41
Right Kidney	1	0	1	3	18	1	0	19	0	24
Right Gonad	0	0	0	0	2	0	0	2	0	2
Total Cells	3	0	5	5	51	3	0	54	0	67
	TOTAL ALL CULTURES									
Total Cells	17	6	19	32	297	6	12(24)	337	0	401

chromosome counts and further no such preparations had been made from normal sheep with which to compare them. It is perhaps pertinent to note however, that six genuine cells with 55 chromosomes were seen. This strengthens the suggestion that the increase in aneuploidy seen in leucocyte cultures of these sheep may be associated with one of the factors described in Part III.

Sex Chromatin Studies.

From six* of the intersex sheep nerve tissue sections were prepared by the method described in Section I. In four of these, nerve tissue was examined for the presence of sex chromatin bodies from the Purkinje cells and the ventral horn cells of the cervical, thoracic, lumbar and sacral spinal cord. In two animals, cases Nos. 1 and 6, material from the lumbar spinal cord only was examined.

The detailed findings from the examination of 2,695 cells from the above regions are given in Table 20. Table 25 shows the comparison between the percentage of cells showing chromatin bodies in four ewes and the five freemartin sheep. The details of the ewe data are given in Table 1, Section I.

Discussion.

The sex chromatin studies in the six intersex sheep supported the findings of the fibroblast cultures, that all these animals were genetic females./

*Case No. 6 was presented when these studies were completed but the examination of one hundred and eight nerve cells showed that 68.5% were chromatin positive and that the animal was a genetic female.

TABLE 20.

SEX CHROMATIN STUDIES FROM 5 INTERSEX SHEEP

	Nucleolus	Nucleoplasm	Peripheral	None	Total	% Nucleolus	% Nucleoplasm	% Peripheral	% Total
CEREBELLUM									
Intersex 1				Not Examined					
" 2	173	4	6	4	187	92.50	2.14	3.21	97.85
" 3	93	9	60	48	210	44.29	4.29	28.57	77.15
" 4	189	2	10	18	219	86.30	0.92	4.56	91.78
" 5	45	18	57	24	144	31.25	12.50	39.58	83.33
CERVICAL SPINAL CORD.									
Intersex 1				Not Examined					
" 2	24	6	9	14	53	45.28	11.32	16.98	73.58
" 3	44	15	23	26	108	40.74	13.89	21.30	75.93
" 4	38	9	19	44	110	34.55	8.18	17.27	60.00
" 5	41	16	13	38	108	37.95	14.81	12.04	64.80

THORACIC SPINAL CORD

Intersex	1				Not Examined					
"	2	50	19	14	25	108	46.30	17.59	12.96	76.85
"	3	48	12	18	34	112	42.86	10.71	16.07	69.24
"	4	53	15	11	29	108	49.07	13.89	10.19	73.15
"	5	41	12	18	37	108	37.96	11.11	16.67	65.74

LUMBAR SPINAL CORD

Intersex	1	72	53	60	67	252	28.57	21.03	23.84	73.44
"	2	38	24	13	33	108	35.19	22.22	12.04	69.45
"	3	37	20	23	28	108	34.26	18.52	21.30	74.08
"	4	47	13	19	29	108	43.52	12.04	17.59	73.15
"	5	42	14	28	28	112	37.50	12.50	25.00	75.00

SACRAL SPINAL CORD

Intersex	1				Not Examined					
"	2	80	7	10	11	108	74.07	6.48	9.26	89.81
"	3	42	20	14	32	108	38.89	18.52	12.96	70.37
"	4	77	4	10	17	108	71.30	3.70	9.26	84.26
"	5	29	37	15	27	108	26.85	34.26	13.89	75.00

TABLE 21.

COMPARISON OF SEX CHROMATIN IN GROUP OF 4 EWES AND 5 INTERSEX SHEEP AT FIVE LEVELS OF NERVE TISSUE

(4751 CELLS)

	TOTAL CELLS	TOTAL CELLS WITH SEX CHROMATIN	% CELLS WITH SEX CHROMATIN	% DIFFERENCE
CEREBELLUM				
Ewes	640	587	91.72	4.09 F = 3.1
Intersexes	760	666	87.63	
CERVICAL SPINAL CORD				
Ewes	347	305	87.90	20.9 F = 45 **
Intersexes	379	257	67.81	
THORACIC SPINAL CORD				
Ewes	355	295	83.10	11.77 F = 16.2 **
Intersexes	436	311	71.33	
LUMBAR SPINAL CORD				
Ewes	401	355	88.53	15.42 F = 46.9 **
Intersexes	688	503	73.11	
SACRAL SPINAL CORD				
Ewes	313	259	82.75	2.89 F < 1
Intersexes	432	345	79.86	

* Significant $P < 0.05$

xx Highly significant $P < 0.01$

female.

It can be seen however from Table 21 that when the percentage of cells with sex chromatin bodies are compared with the percentage of cells from normal ewes, there is a difference in favour of the ewes at every level examined. When these percentage differences were analysed statistically, using an angle transformation of the percentages in conjunction with analysis of variance, a highly significant difference was found in three out of the five levels examined. Individual levels of sex chromatin however did vary within each region (Table 20). It should be noted also that in the results of the normal ewes, there were two age groups, namely ewes and lambs. It was considered however that the number of animals was too small to make a statistical analysis within this normal group. Further, the ewes were of a comparable age with the intersex animals and the percentage of cells showing sex chromatin in these was higher in most cases than the lambs, so that in using the lamb data with the ewe data to compare with the intersex data, if any bias were present it was not in favour of the ewes. Since the original data on normal/

normal ewes had been collected over a year prior to examining the freemartin animals, there was a possibility that the difference could have been caused by technical factors. These included firstly, changed criteria for scoring sex chromatin bodies. Sex chromatin bodies in close association with or overlying the nucleolus, while staining darker than other nuclear material, do present possible ambiguities for observation. The decision as to whether or not such ambiguities had been scored in the ewe data was not recorded and while these bodies were not scored in the intersex sheep there was the possibility that some may have been scored in the ewes. Secondly, between the preparation of the two sets of material, both technician and microtome had been changed and finally, no consideration was given to observer bias.

For these reasons a blind trial was prepared of material from the four ewes and the five intersex sheep. This was carried out in the lumbar region of the spinal cord and a total of 960 cells was scored. The results of this trial are given in Table 22. In all cases except Intersex No.1, the/

TABLE 22.

BLIND TEST ON LUMBAR CORD OF 4 EWES and 5 INTERSEX SHEEP (960 Cells).

	NUCLEOLUS	NUCLEOPLASM	PERIPHERAL	NONE	TOTAL	% NUCLEOLUS	% NUCLEOPLASM	% PERIPHERAL	% NONE	% TOTAL
Ewe I	73	9	3	23	108	67.59	8.33	2.78	21.30	78.70
Ewe II	75	6	5	22	108	69.44	5.56	4.63	20.37	79.63
Ewe III	64	3	9	32	108	59.26	2.78	8.33	29.63	70.37
Ewe IV	27	35	21	25	108	25.00	32.41	19.44	23.15	76.85
Intersex 1.	39	9	26	22	96	40.63	9.37	27.08	22.92	77.08
Intersex 2	32	26	11	39	108	29.63	24.07	10.19	36.11	63.89
Intersex 3	26	23	22	37	108	24.07	21.30	20.37	34.26	65.74
Intersex 4	58	4	9	37	108	53.70	3.70	8 .33	34.26	65.74
Intersex 5	46	8	19	35	108	42.59	7.41	17.59	32.41	67.59

the total percentage of cells showing sex chromatin bodies was lower in the intersex sheep than the ewes. When this difference was tested statistically, by the same method as the previous data, it was found that $F = 8.7$, $P < 0.01$, which was again a highly significant difference.

DISCUSSION OF EXAMINATION OF FEMALE INTERSEX SHEEP.

Discussion of Chromosome and Sex Chromatin Investigation.

The presence of male and female cell lines in the blood of all these intersex animals but female cells only in other tissue confirms that each case was an established blood chimaera. When considered with the history of uterine association with a male twin together with the anatomical and histological findings, these findings confirm that the intersexuality shown by these sheep was analogous to the freemartin condition seen in cattle.

Chromosome studies, therefore, offer a simple and accurate method of classifying conditions such as freemartinism, where a wide variation in the development of genitalia is recorded. Firstly, the difficulty of verifying placental anastomosis and twinning has been overcome in these studies in sheep as in cattle (Herschler Fechheimer and Gilmore, 1966) by the demonstration of male and female chromosome complements /

complements in blood cultures. The fact that five freemartin sheep recorded as co-twins to rams showed blood cell chimaerism is conclusive evidence of the reliability of this method. Although no record was available of the sixth case it can be assumed, in the light of other findings, to have been born a co-twin to a ram. Besides the verification of hetero-sexual twinning being simplified by this method the time required to carry out these investigations is only a few days instead of months when using other techniques such as skin graft tolerance (Anderson et. al., 1951, and Slee, 1963). Further, when red cell mosaicism tests are used to verify chimaerism a negative result need not mean necessarily that chimaerism has not occurred. Evidence produced by Slee (1963) would suggest that red cell mosaicism may regress even in the presence of persistant skin graft tolerance. The permanence of blood cell chimaerism is adequately demonstrated in the sheep studied since it was still present in the two oldest sheep both aged seven years.

The absence of cells with the Y chromosome in any of the other tissues examined would suggest two facts. Firstly, the establishment of cell lines from/

from tissues other than blood does not take place in freemartin sheep and secondly, it verifies that the five freemartin sheep were genetic females. The presence of female cell lines only in tissues other than blood in freemartin sheep was also shown from kidney and gonad cultures in two cases by Gerneke (1965), Table 23. In the bovine freemartin there is controversial evidence on this point. Goodfellow et al., (1965) failed to show chimaerism in any tissue examined other than blood and bone marrow. However, Ohno et al., (1962) claimed the demonstration of chimaerism in gonadal cells of bull calves co-twin to freemartins but not in freemartin calves. In this latter case the cells were presumably XX spermatogonial cells. Similar findings have been reported in the marmoset monkey by Benirschke and Brownhill (1963), this case being conifrmed by the absence of XY sex bivalents in some of the meiotic preparations. Kanagawa et al., (1965) reported the finding of chimaerism* in several tissues in the bovine freemartin suggesting that chimaerism may extend to tissues even beyond the blood and gonads. The conflicting findings/

* The term chimaerism still applies since it is assumed to be a post zygotic exchange between different individuals.

findings in these various reports should be interpreted with caution. In the case of the present studies in the sheep and those of Goodfellow et al., in the bovine, long term cell cultures were used with subculture of explants, while Ohno, Benirschke and Brownhill based their observations on short term cultures of several hours and squash preparations. Kanagawa et al., (1965) apparently used a culture method in which the incubation time was ninety-six hours. Since the last case lacked detail and in view of the short culture time, some white blood cells may have survived and produced the small number of male cells recorded (13/969 cells counted), in these extraordinary findings in lung, kidney and gonadal tissue. There is no conflict of results, however, between those on the sheep and those of Goodfellow et al., (1965) on cattle, with the findings of Ohno et al., (1962) and those of Benirschke and Brownhill (1963), in spite of the use of different techniques, since the latter authors are not claiming chimaerism of tissues other than germ cells. On the other hand, the presence of XX cells in gonad, kidney and muscle cultures from the sheep, and muscle and gonad cultures from cattle, would suggest that no chimaerism/

chimaerism of other tissues occurs. This result, however, does not eliminate the possibility that germ cell chimaerism had occurred in these cases, so that neither the hormonal theory of Lillie, nor the germ cell theory of Ohno et al., is substantiated nor contradicted by these findings.

The difference between the incidence of sex chromatin in four ewes and five freemartin sheep (intersex sheep) is contrary to the findings of Moore, Graham and Barr (1957) for the bovine freemartin. These authors found no difference between new born female calves and freemartin calves, and freemartins and female calves of four months of age.

Recent evidence has shown in man that under certain circumstances, the incidence of scorable sex chromatin bodies is depressed. This has been recorded following the use of oestrogens, hydrocortisone and A.C.T.H. (Taylor, 1963). A similar depression has been reported in new born babies (Smith, Marden, McDonald and Speckhard, 1962; and Taylor 1963). There is also evidence that the incidence of sex chromatin is lowered during the menstrual cycle and that this is due to hormonal influence (Caratzali, 1963).

It/

It is possible, therefore, that the lowered incidence of sex chromatin in the freemartin sheep may be associated with similar factors but in the absence of information on hormone levels in these animals, no positive association can be established.

It must be emphasised that although this difference was shown to occur between normal ewes and freemartins, and in the absence of any incidence of mixed cell lines in other than blood, the freemartin sheep were interpreted as being genetically female from the sex chromatin findings.

DISCUSSION OF ANATOMICAL EXAMINATIONS.

As suggested by Slee (1963), the anastomosis of blood vessels of opposing placentae in the sheep is probably a freak situation caused by uterine overcrowding rather than the regular anastomosis seen in cattle. In the latter case the contributing factor is probably the early precotyledonary vascularisation of the chorion (Idllie, 1917). In the sheep there is no pre-cotyledonary vascularisation of the placentae as in cattle and anastomoses tend to be between cotyledons (Rotermund, 1930). There is no evidence as to the time at which this occurs in the sheep.

The anatomical and histological findings in these/

these sheep show great similarity to the bovine freemartin as described by many workers (Lillie, 1917, Chapin, 1917, Willier, 1921 and Bissonnette, 1924). Apart from the enlarged clitoris in Case 3 and presence of a glans clitoris in Cases 1 and 2, the external genitalia in the freemartin sheep were in no way modified from those of a normal female. This point was emphasised by Bissonnette (1924) who stated that the only modification in the bovine freemartin is the tendency to smaller size of external genitalia in some cases. In no case was a scrotum formed even when descent of the gonads had taken place and mammary tissue was present in all cases examined. The same observations applied to the sheep freemartins.

The gonads of the sheep freemartins were specially modified testis-like organs, as in the bovine freemartin (Lillie, 1917; Chapin, 1917) and not ovotestis as suggested by Goodfellow et al., (1965). In all the cases examined, sex cords were characteristically present in the gonads but nothing comparable to ovarian follicles was found, as seen in the true human hermaphrodite. This would suggest as in the bovine freemartin that the differentiation of the potentially female gonad does not take place

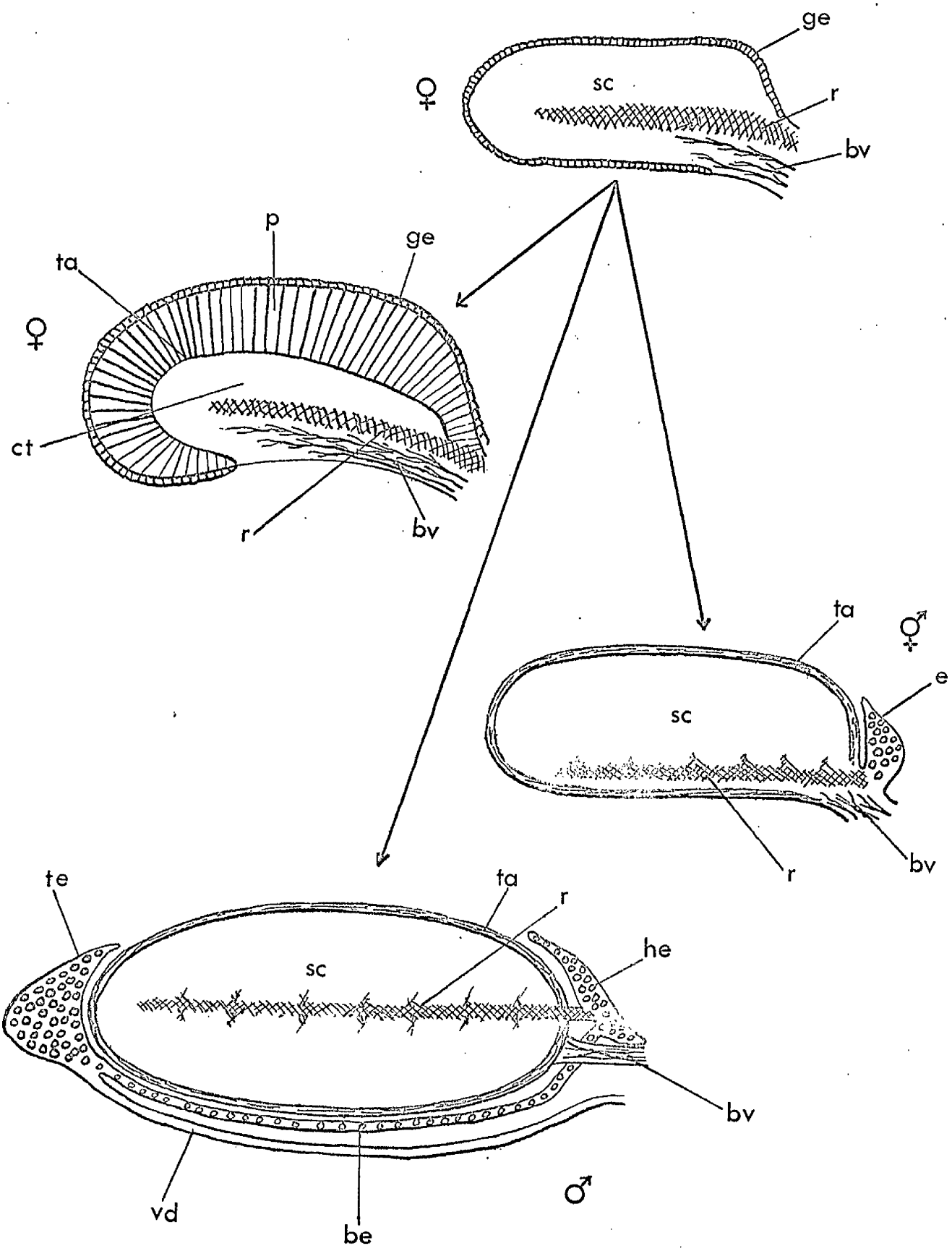
because/

because of the absence of the second in-growth of sex cords (Cords of Pflüger) (Chapin, 1917). The differentiation of male, female and freemartin gonads is demonstrated in Fig. 88.

The wide variation in genitalia between male and female types (Figs. 62 - 70), being similar to the bovine freemartins is no doubt directly related to the time at which the anastomosis between different sexed twin placentae took place. There do seem to be comparative differences, however. Roberts (1956) claimed that in the bovine freemartin the Mullerian duct system fails to develop and there is little evidence from reported cases reviewed that an organ with histological resemblance to a uterus has been recorded. (Goodfellow et al., (1965), mention a poorly developed endometrium in one of their cases). Cases 4 and 5 (Figs. 80 and 81), show that there is definite uterine development which would indicate that the Mullerian duct system had been able to differentiate to a more advanced stage probably due to the later introduction of the male modifying factor which thus acted for a shorter time. According to Bissonnette (1924) the degenar-ation of the Mullerian duct system in the bovine freemartin/

Fig. 88. The development of female, male and freemartin gonads (after Bissonnette, 1924). The top diagram represents the indifferent stage.

be = body of epididymis.
bv = blood vessels.
ct = connective tissue.
ge = germinal epithelium.
he = head of epididymis.
p = cords of Pfluger.
r = rete.
sc = sex cords (seminiferous tubules).
ta = tunica albuginea.
te = tail of epididymis.


ge
♀
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freemartin starts at a later date than in the male and is at a correspondingly more advanced stage of development. The degeneration goes on in both more or less uniformly but the anterior ends disappear first. This would account for the failure to trace any remnants of these ducts in the position of the uterine body in Cases 4 and 5 (Figs. 66 and 69).

The variation in the six cases studied is of considerable interest when examinedwith the other reported cases of freemartinism in the sheep (Table 23).

From the facts recorded in this table, it can be seen that the features of freemartinism can be divided into those which are apparently constant and those which show considerable variation. Among the former, the following features are listed.

1). All the freemartins are either co-twin, triplet or quadruplet to at least one male litter mate.

2). A mixing of blood between male and female litter mates has always occurred, as shown by either direct examination of placentae or the diagnosis of blood cell chimaerism.

3). Tissues other than blood are always genetically female as indicated by chromosome and sex chromatin studies.

4)./

TABLE No. 23.

SUMMARY OF REPORTED CASES OF FREEMARTINISM IN THE SHEEP.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.
AUTHOR & DATE	Fraser-Roberts & Greenwood(1928)	Ewan & Hunnason(1947)	Stormont Weir & Lane(1953)	Moore & Rowson(1958)	Slee(1963)& Author (1966)	Alexander & Williams(1964)	Gerneke(1965) Case I.	Gerneke(1965) Case II.	Author(1966) Case II.	Author(1966) Case III.	Author(1966) Case IV.	Author(1966) Case V.	Author(1966 Case VI.
BREED	Southdown X Welsh Mountain	Rambouillet	-	-	Welsh Mountain	Merino	Afrikaner X Persian	Karakul.	Clun	Scottish B.F. X Welsh	Scottish B.F. X Welsh	Scottish B.F X Cheviot	Cheviot
AGE	9 months	18 months	-	-	5 years	New Born	-	-	5 years	7 years	7 years	4 years	18 months
STATE OF PREGNANCY	Twin (Sex unknown)	Twin to Male	Twin to Male	Twin to Male	Quadruplet 2 male 1 female.	Triplet 1 Male 1 female	Twin to Male	No record.	Twin to Male	Twin to Male	Triplet Male Female(normal)	Twin to Male	No record
PUBERTAL CHANGE		Grew horns & clitoris.			Male Idbido								
SEX CHROMATIN STUDIES					Nerve Tissue Chromatin Positive		Buccal Cells Chromatin Positive ?	Buccal Cells Chromatin Positive ?	Nerve Tissue Chromatin Positive	Nerve Tissue Chromatin Positive	Nerve Tissue Chromatin Positive	Nerve Tissue Chromatin Positive	Nerve Tissue Chromatin Positive
SKIN GRAFT TOLERANCE				Tolerant	Tolerant to One male Twin only.								
ERYTHROCYTE MOSAICISM			Demonstrated to Twin		Demonstrated to same male								
BLOOD CHROMOSOME ANALYSIS					XX/XY		XX/XY (Bone marrow)	XX (Bone marrow)	XX/XY	XX/XY	XX/XY	XX/XY	XX/XY
CHROMOSOME	Muscle				XX(24)				(-)	as (24)	as (33)		-
of	Kidney				XX(33)		XX		XX (5)	XX (57)	XX (19)		-
OTHER TISSUES	Gonads				XX(10)		XX		XX (2)		XX (2)		-
EXTERNAL	Vulva Distorted	Distorted	Normal Ewe	N.R.	Distorted	Slight Distortion	Distorted	Distorted	Distorted	Distorted	Distorted	Normal Ewe	Normal at birth Ewe & pubertal change.
GENITALIA	Clitoris +++	++	-	-	+++	+	+++	+++	++	+	+	-	++ (not obvious externally)
	Scrotum -	-	-	-	-	-	-	-	-	-	-	-	-
	Position Subcutaneous Inguinal	One Subcutaneous One Abdominal	N.R.	N.R.	Subcutaneous Inguinal	Subcutaneous Inguinal	Subcutaneous Inguinal	Subcutaneous Inguinal	Subcutaneous Inguinal	Abdominal	Abdominal	Abdominal	Abdominal
GONADS	Appearance Testis like	Testis like	N.R.	N.R.	Testis like	Testis like	Testis like	Testis like	Testis like	Minute Testislike	Indifferent	Small Ovarianlike	Small testis
	Histology Sex Cords +++	Sex Cords +++	N.R.	N.R.	Sex Cords +++	Sex Cords +++ ?	Sex Cords +++	Sex Cords +++	Sex Cords +++	Sex Cords ++	Sex Cords +	Sex Cords +	
ACC. SEX GLANDS RECORDED	S. Vesicles	-	N.R.	N.R.	S.Vesicles	N.R.	S. Vesicles B.Urethral G.	S. Vesicles B. Urethral G.	Seminal Vesicles	-	-	-	
DUCT SYSTEM	Male	Vas.Deferens	N.R.	N.R.	Male	Male	Male	Male	Male	Male but incomplete	Abortive Uterine	Abortive Uterine	
UROGENITAL SINUS	No.	Vagina like	N.R.	N.R.	No	Recorded no Dimensions	No	No	No	Moderate 8 cms.	Large 10 cms.	Large 10-12 cms.	Small Ended abruptly 2-3 cms.

4). The external genitalia always include a vulva but acrotal pouches are never present.

The variable features on the other hand, include all the internal genitalia.

1). The Gonads.

a) Position either abdominal or subcutaneous.

b) Appearance either testis-like to small ovarian-like.

2). The duct system may vary from a well developed spermatic cord to an abortive uterine structure.

3). Accessory sex glands usually correspond to the degree of male or female development.

If then twelve of the cases* of freemartinism recorded in sheep (Table 28) are reviewed, three of these, Nos. 3 and 12 had the external appearance of/

* Case 4 is not considered because of lack of anatomical data.

of a ewe and four others, Nos. 6, 10, 11 and 13 ** would have passed on casual examination as ewes. These latter cases had only mildly deformed clitorae. The remaining cases, Nos. 1, 2, 5, 7, 8 and 9 all had noticeably enlarged glans clitorae, distorting the external genitalia and proclaiming them as intersexes.

The internal genitalia were of a corresponding nature, the latter six cases and Case 6 all having descended gonads, while the more ewe-like animals all had abdominal gonads and less tendency to masculinisation. When these cases are compared with cases of bovine freemartinism an interesting point arises. From many reported cases in adult cattle there are few cases in which the gonads had descended through the inguinal ring to a subcutaneous position together with the development of male-like genitalia. Three such recorded cases in the bovine have been described by Numan (1843), Lillie (1917)* and Fraser-Roberts and Greenwood (1928). By comparison,/

* This was a 26.5 cm embryo.

** History would indicate that a pubertal change in external genitalia had taken place in this case. Initially it passed as a ewe.

comparison, literature abounds with descriptions and photographs of bovine freemartins in which the gonads are abdominally placed and small and in which the external genitalia are similar to a normal female*.

Four such cases were described by Curson (1932), in two of these he refers to the gonads as ovaries. The gonads were abdominal in all cases as shown in his accompanying photographs. The external genitalia of the two cases shown, gave no evidence of abnormal glans clitoris development. Two similar cases were described by Williams (1934). In both of these the gonads were very small and abdominal and in one case only one gonad was recorded. The only abnormal feature of the external genitalia was the tuft of hairs common to some freemartins.

Clear descriptions are given of two of the freemartins used by Moore, Graham and Barr (1957) in their sex chromatin studies. Both of these are described/

* In the bovine freemartin, the majority of writers describe the external genitalia as being characteristic (c.f. Bissonnette) even in the presence of small internal gonads. The various characteristic features are described as a slightly smaller vulva and tuft of hairs, (Arthur 1964), a phallus-like clitoris (Roberts 1956) and a different urination from a normal heifer (Williams 1934).

described as having the external genitalia of a normal heifer with the gonads located in an ovarian position. An interesting report on multiple freemartins by Wallace (1960) with a clear photograph of the internal genitalia shows "ovarian" gonads and duct development of a female type. Two recent cases reported by Goodfellow et al., (1965) were also described as having normal female external genitalia and ovo-testes (presumably in an ovarian position). One of these cases was described as having a bicornuate uterus.

Of seventeen female animals born co-twin to bulls, studied by Swett, Matthews and Graves (1940), three had apparently normal internal genitalia and fourteen showed features of free-martinism. Table 24 gives details of these authors' studies. Only one case showed a single small gonad in a descended position. In eight cases the gonads were described as being small and occupying the position of ovaries. The remaining four cases did not mention gonads but the descriptions given suggested that no large descended gonads were present.

Judging from the descriptions of the external genitalia/

TABLE 24.

SUMMARY OF GONAD POSITION and DESCRIPTION OF EX. GENITALIA OF 15 FREEMARTINS (SWETT, MATTHEWS and GRAVES, 1940).

15 FREEMARTINS (SWETT, MATTHEWS and GRAVES, 1940).			
Case 691	Abdominal ?	Not Given	+ + +
834	Ovarian	Small Nodules	Normal
624	Ovarian ?	Not Given	No Record
1006	One Ovarian One Descended	Small Testicles	Normal
A70	Ovarian	Ovary Like Glands	Normal
296	Ovarian ?	Not Given	No Record
A82	Ovarian	Small Bodies	Normal
1209	Ovarian ?	Gonadal Lumps Resembling Testicular Tissue	+
1421	Absent Entirely	Typical Neuter	+
806	Internal	Vestigial Typical Neuter	No Record
1427	Only Rudiments of Femal Genitalia		+ + +
1229	Ovarian	Small Bulb like Objects	+
1212	Ovarian	Testicles	+ + +
1485	Ovarian	Small Follicle-like structures	+ + +
1200	Not Stated Undoubtedly an Intersex.		+ + +

* This apparently included Glans Clitoris and Ventral Commissure of Vulva.

genitalia of these cases, there would appear to be less correlation between the external and internal genitalia in the bovine freemartin than the sheep freemartin. This is difficult to interpret however from these authors' descriptions because of the confusing reference to a rudimentary penis (a prominent fold of skin running from vulva to umbilicis) which was not the glans clitoris referred to in this study and by other authors.

The apparent frequency of a larger proportion of male type freemartins, than female type freemartins reported in sheep as compared with cattle would suggest one of two possibilities. Either a number of freemartins have remained unidentified because like cattle there may possibly be more of the female type, or the anastomosis develops in sheep at a much earlier average age than in cattle, and hence the modification of the genitalia is more radical due to the earlier introduction of the modifying factor. It is noticeable that of the six cases discussed in this thesis, only two had very ambiguous external genitalia, Nos. 1 and 2 (Figs. 51 and 53) while Case No. 3 (Fig. 55) the distortion of the external genitalia was moderate.

There/

There is evidence too, that this distortion may become more apparent at puberty. In the case reported by Ewan and Hummason (1947), changes both in horn growth and external genitalia were noted at about the normal pubertal period for sheep. Also in the present series Case 1 and Case 6*, (Figs. 51 and 61) were classified as a ewe lambs at birth.

Cases 4, 5 and 6 (Figs. 57, 59, 61) were ewe-like in the appearance of their external genitalia. This fact is emphasised by the fact that Case 6 * had passed as a ewe under expert scrutiny when judged a show champion.

Beside the scarcity of individually reported cases of freemartinism in sheep, statistical evidence would suggest that its occurrence is extremely rare. Slee (1963) concluded this on the finding of no statistical difference in fertility between one hundred and eighty-seven ewes born twin to a ewe and one hundred and sixty-seven ewes born/

* The later ambiguous external genitalia was the result of pubertal change.

born twin to a ram. While statistical evidence does not suggest overt freemartinism, as pointed out by Slee (1963), the incidence of covert freemartinism may be higher than was previously recognised. The results of this investigation would tend to support this view. The estimate of 0.8% of freemartins among ewes by Stormont, Weir and Lane (1953) based on the estimation of 5% anastomosis in sheep twins may therefore not be as exaggerated as these authors originally suggested.

The future use of the blood cell culture method and the examination of chromosomes in cases of infertile ewes will undoubtedly help to clarify the situation.

D. MALE INTERSEXES.

Four cases were studied of what are commonly termed hermaphrodites by sheep breeders. These were cases of hypospadias, and since a similar pattern was common to all, full anatomical description of two cases is sufficient. Case 1 (Figs. 89 and 90).

This was a Suffolk ram aged eight months at the time of slaughter. On external examination, the/

the following anomalies were recorded.

The penis was smaller than normal and exposed by an incompletely formed prepuce. The external urethra was improperly closed at several places along its length, namely between the divided scrotal sacs and beneath the anus. The latter opening formed the external urethral opening through which urination took place. Urine dribbled constantly from this opening and produced a severe wool stain. Testes were descended into the scrotum.

At postmortem examination the internal genital organs resembled those of a normal ram (Fig. 91) except that the testes were smaller than usual, the left weighing 231 grams and the right 197 grams. Ampullae, seminal vesicles and bulbo-urethral glands were all present.

Sections of testis stained with H and E showed that spermatagenesis was taking place.

Case 2. Figs. 92 & 93.

This was the offspring of a Dorset horn crossed with an unspecified breed, which also was eight months old at the time of slaughter. The external genitalia showed similar anomolies to the previous case except that there was closure of the last three inches of the external urethra in this animal. The penis had no urethral process and the scrotum was bifurcated as in the previous case. The testes although descended, were quite small, weighing on the left side sixty-two grams and on the right, eighty grams.

The internal genitalia were similar to the previous case, being typically male in appearance. Sections of testis showed that spermatogenesis was taking place.

Case 3.

A Scottish blackface ram, one year old when destroyed, showing very similar external and internal genitalia to the previous case. The testes weighed, left 47.5 grams right 36.0 grams, respectively.

Case 4.

Another Scottish blackface ram destroyed at twelve/

twelve months of age and similar to the other cases except that the hypospadias was scrotal and not perineal as in the previous three cases. The testes weighed, left 63.0 grams and right 85.0 grams.

Chromosomes.

Chromosomes were studied from leucocyte culture preparations of all four animals, the counts being included with those for normal males in Table 5.

Sex Chromatin.

Examination of fifty spinal cord cells from each animal showed them all to be chromatin negative.

Discussion.

These four cases were all clearly cases of hypospadias and not hermaphrodites. The chromosome picture in each case was typically male and each animal was as expected, chromatin negative. Karyotypes prepared from these animals failed to show any chromosomal abnormality which could be associated with this anomoly. While such cases as these are commonly reported by shepherds there are few reports of such cases in the literature.

Van/

Fig. 89.

MALE INTERSEX No.I.

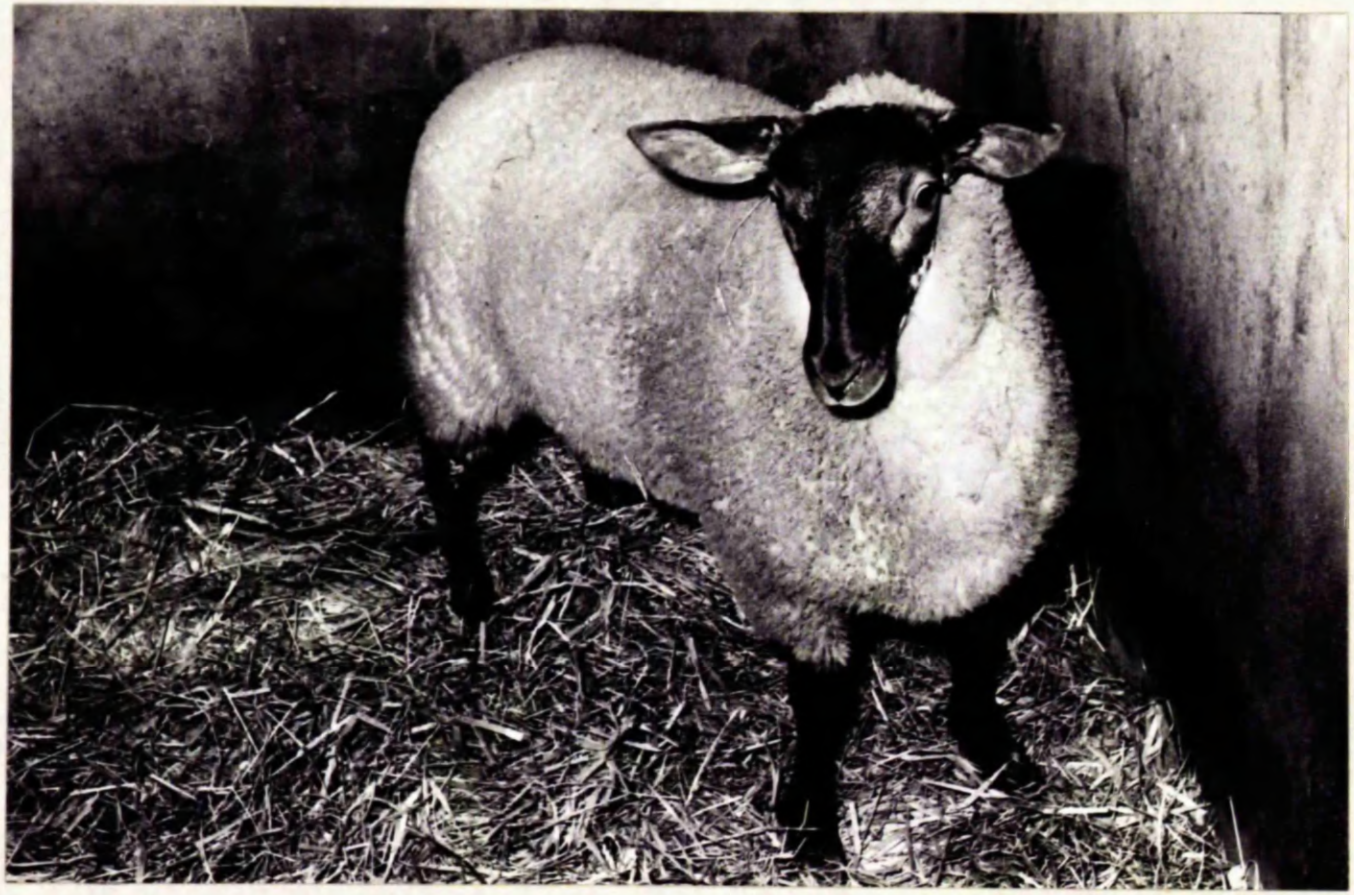


Fig. 90

THE EXTERNAL GENITALIA OF

MALE INTERSEX No.1

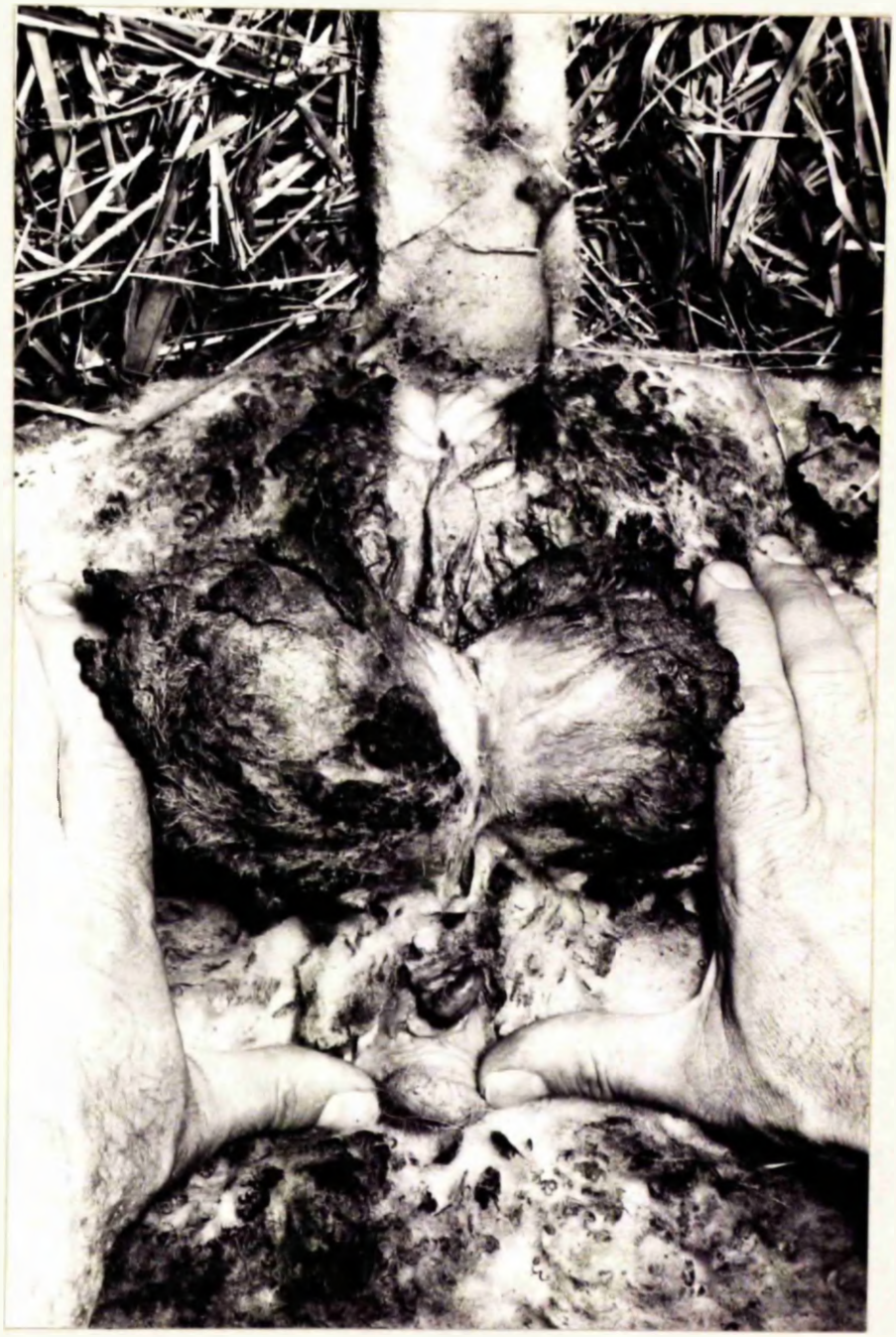


Fig. 91

THE INTERNAL GENITALIA OF

MALE INTERSEX No.1

Fig. 91.

KEY.

a	=	ampulla
bs	=	bifurcated scrotum
euo	=	external urethral opening
gg	=	glans clitoris
sv	=	seminal vesicle
t	=	testis
vd	=	vas deferens

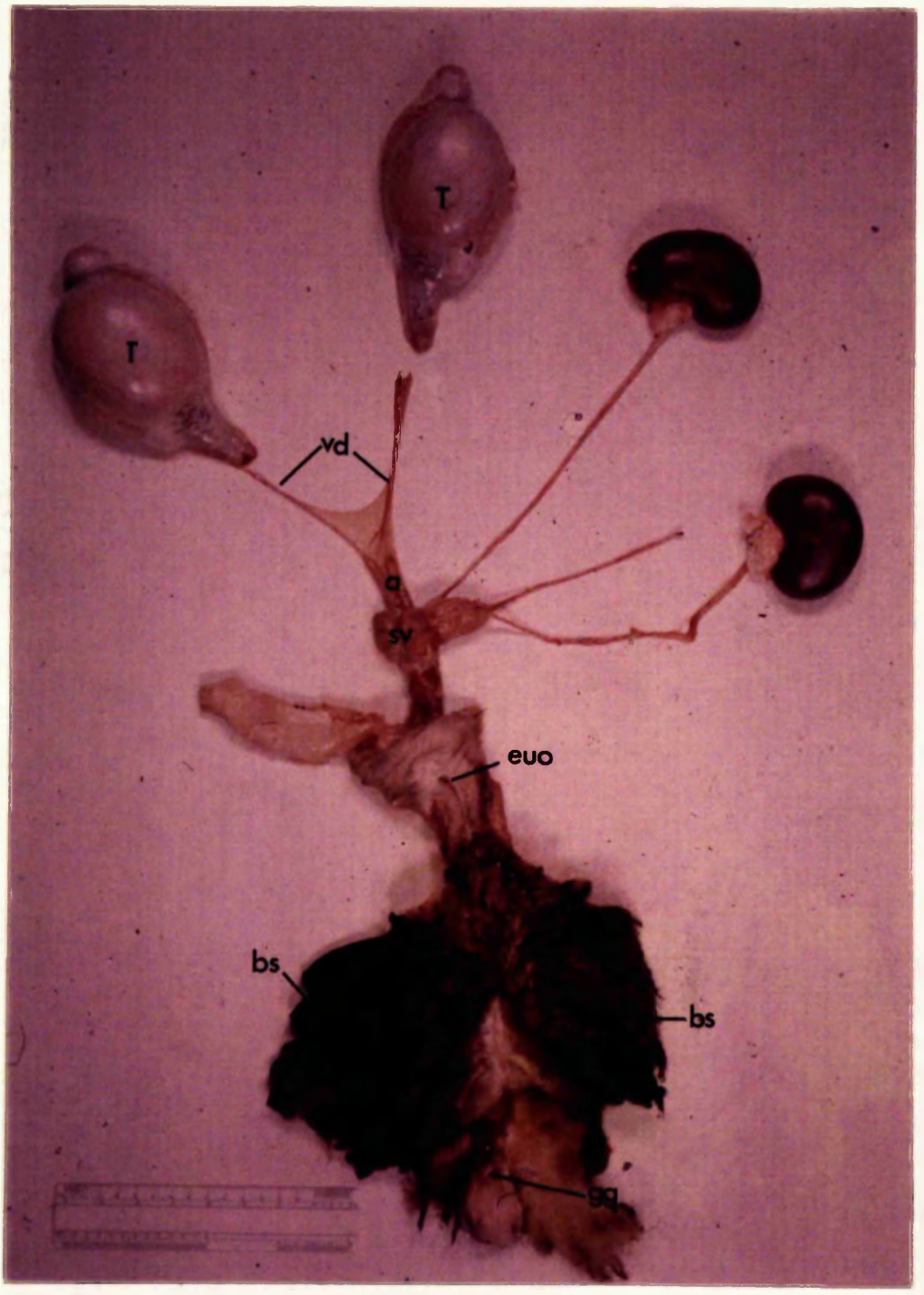

T
T
vd
o
sv
euo
bs
bs
gg

Fig. 92

MALE INTERSEX No. 2.

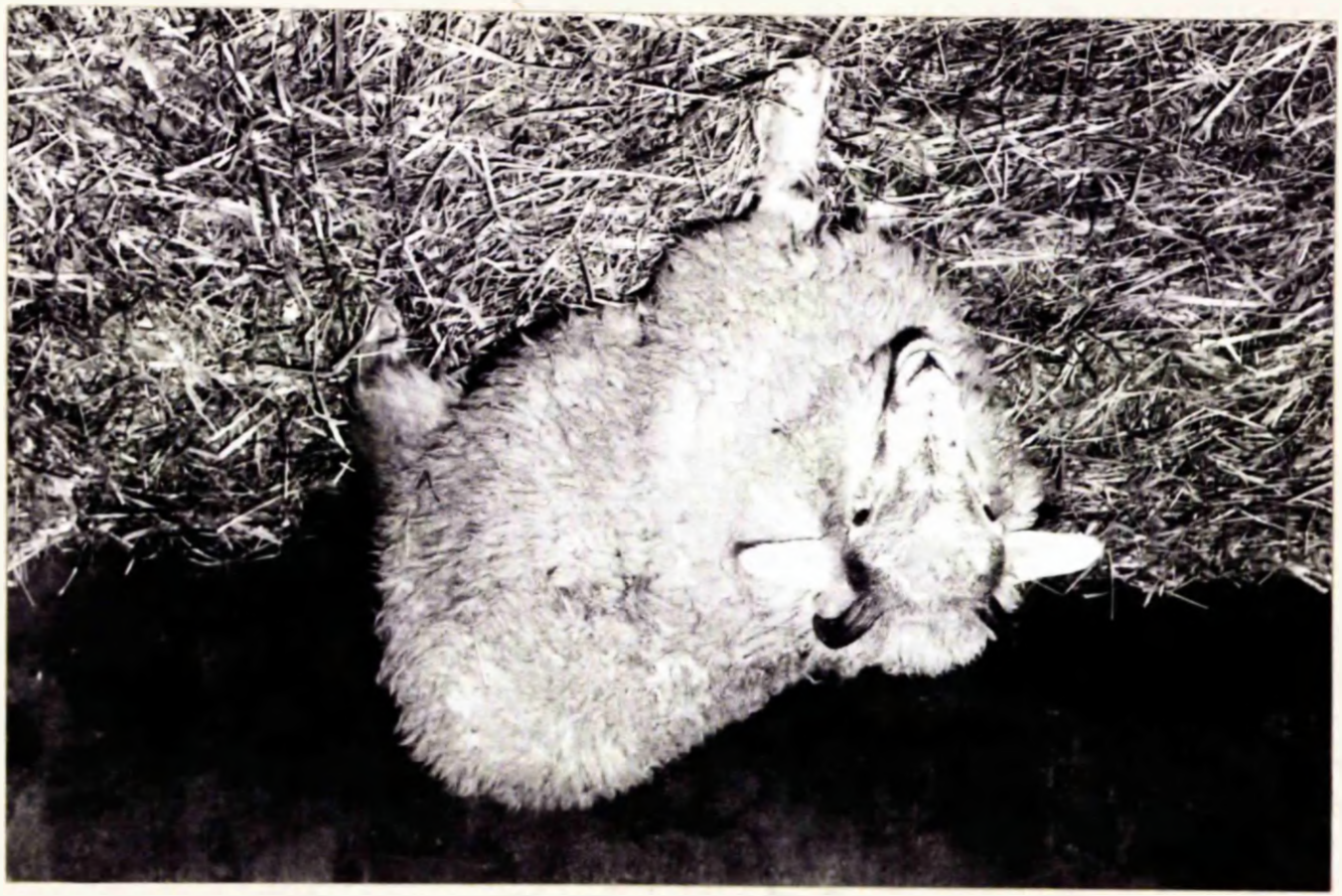
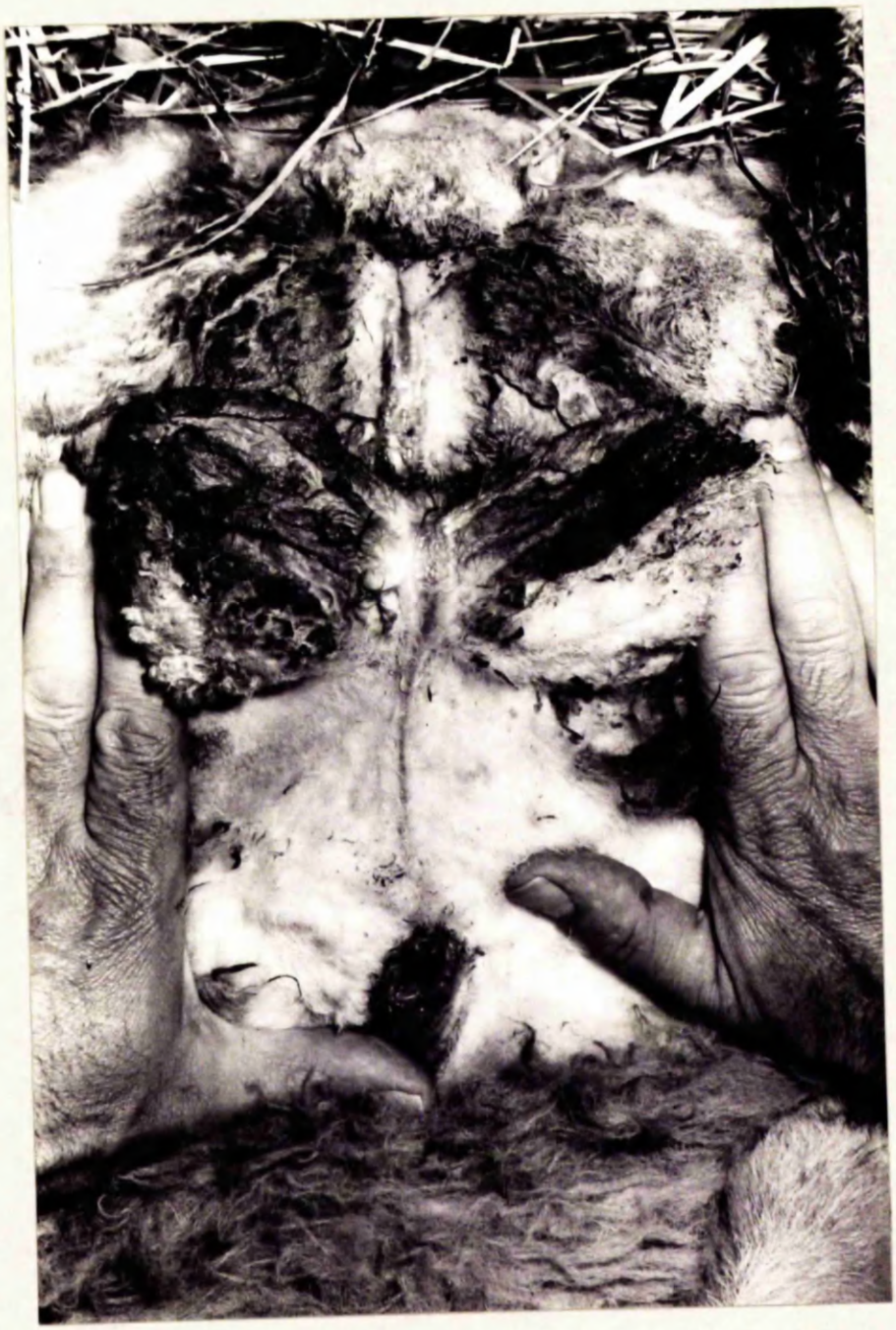


Fig. 93

THE EXTERNAL GENITALIA OF

MALE INTERSEX No.2.



Van Drimmelen and Thiel (1932) reported a case in a merino ram identical with these few cases and concluded that the abnormality was teratological in nature being due to incomplete fusion of the urethral folds. This and a similar case reported by Kitt (1921) would appear to be the only two reports available on this condition in the sheep.

General Discussion.

Work from this thesis has shown that although there is no simple diagnostic test for sex chromatin in the sheep comparable with the buccal cell test in man, the finding of sex chromatin in one other tissue than nerve cells would suggest that there is still scope for future investigation into other tissues. Further, the demonstrable presence of a sex chromatin body in female nerve cells alone is a most useful factor when used as an adjunct to chromosome studies.

While sex chromatin studies on the sheep are restricted at present, there is no reason why chromosome studies cannot be pursued along similar lines to those in man.

This study has shown that white blood cells of the sheep can be cultured and suitable chromosome preparations/

preparations made in a similar manner to those of man and other species. It would not be difficult therefore to establish chromosome studies as an integral part of diagnostic investigations in clinical departments of Veterinary Schools. Initially it would appear that chromosome studies in the sheep, as in man, may have their greatest value in investigations of sexual abnormality and embryonic death. The recent findings of McFeely (1966) and those of this thesis would tend to suggest this.

Although individual autosomes in the sheep karyotype cannot be identified easily at the moment the sex chromosomes can be identified and therefore present no obstacle to further investigations of cases of infertility in which they may be involved.

Future investigations should aim at using all the possible means of chromosome identification in order to establish the identity of individual chromosomes. The present studies have indicated that secondary constrictions do occur in sheep chromosomes. These are mainly demonstrable on the/

the large metacentric chromosomes which in the sheep are probably associated with nucleolus formation. Techniques which enhance the presence of secondary constrictions, such as those used by Hsu and Somers (1964) and Kabach, Saksela and Mellman (1964), could no doubt be applied to future studies in the sheep. These together with the use of autoradiography would seem to be the most hopeful means of furthering chromosome identification at present.

As well as following current human methodology on mitotic chromosomes investigations could be initiated into the meiotic chromosomes of the sheep. It is possible to identify the human sex chromosomes in pachytene preparations (Yerganian, 1957) and also the satellited chromosomes, Ferguson-Smith (1964). There would be a great advantage in having information on pachytene chromosomes in animals particularly in helping to recognise chromosome inversions and translocations.

The demonstration of the chromosome patterns in several tissues of the intersex sheep used in this study have shown the advantage that these can be, in assisting the classification of animals showing/

showing such a wide variation in sexual development. Further, the association of blood-cell chimaerism with freemartinism in sheep as in cattle offers a means of identifying cases of this anomoly which current work would suggest have been overlooked.

Finally, it can be suggested that similar anomolies as have been found in studies on human chromosomes are to be seen in animals. The suggestion of aneuploidy with age and the different sex chromatin pattern in the freemartin sheep serve to emphasise this point. It is therefore highly probable that future mammalian studies will have a complementary effect on human chromosome studies. Collaboration in this field would be of considerable benefit to both parties.

GENERAL SUMMARY.

SECTION 1.

1. A sex chromatin body was demonstrated in nerve cells of ewes, with no regularly significant difference in its incidence between new born lambs and adult animals.

2. The incidence of sex chromatin in nerve cells of the sheep (Purkinje cells and ventral horn cells) was similar to that of other members of the order Artiodactyla (goat, deer and cattle).

3. The sex chromatin body in the nerve cells of sheep behaved in a similar manner to that recorded in other species. The position in which it occurred varied between one of close association with the nucleolus and that of association with the nuclear membrane.

4. It was not possible to identify the sex chromatin body in either buccal cells or free amnion cells of the sheep. However, preparations made from amnio-chorion showed a sex chromatin body in 51.96% of female cells examined. This sex chromatin body was characteristically associated with the nuclear membrane and was 100% diagnostically reliable on a trial of fifty samples.

SECTION 2./

SECTION 2.

5. Metaphase chromosome preparations can be made from leucocyte cultures of whole blood of the sheep. Such cultures grew best when a minimum of 500 I.U. of heparin was added to each 10 ml. of blood collected.

6. Suitable growth of leucocytes was obtained from both Medium 199 and Waymouth's medium, but neither produced good growth without the addition of 10%-20% of homologous sheep serum.

7. The best yield of metaphases was obtained from an incubation time of 72-75 hours and of the last twenty-five cultures prepared, a satisfactory growth of leucocytes was obtained on twenty-two occasions.

SECTION 3.

8. This study showed that the modal diploid chromosome number of fifty-four occurred in 87.4% of 1,832 metaphases examined from 22 normal sheep. This chromosome number of fifty-four was confirmed for five additional breeds of sheep, including the Scottish Blackface, Scottish Blackface x Border Leicester (Greyface), Clun Forest, Welsh Mountain and Soay.

9./

9. The karyotype of the sheep can be divided into two groups of chromosomes for each sex. In the ewe there are six metacentric chromosomes in the first group and forty-eight acrocentric chromosomes in the second group, while there are six metacentric and forty-seven acrocentric chromosomes, and one small submetacentric chromosome in the ram.

10. The sex chromosomes can be identified in the sheep. The Y chromosome is quite distinct and the X chromosome is the largest of the acrocentric group of chromosomes and can be distinguished in a number of suitable metaphases.

11. Chromosome measurements were of little value in the identification of individual chromosomes, except for the six metacentric chromosomes and the first three pairs of acrocentric chromosomes (including the X-).

12. Secondary constrictions were recorded on several regularly occurring sites of the metacentric chromosomes in sufficient numbers to suggest that these are the main nucleolar organiser chromosomes. Secondary constrictions were also seen on many of the acrocentric chromosomes but in insufficient numbers/

numbers to assist in identification.

13. Certain figures interpreted as chromosome associations between acrocentric chromosomes, and acrocentric chromosomes and metacentric chromosomes were recorded. However, it was considered that in the absence of many of these and of demonstrable satellites, chromosome associations in the sheep appear to be of a more complex and delicate nature than in man, and that demonstration is difficult using current techniques.

SECTION 4.

14. The chromosome studies of the five Intersex sheep, which were known to be either co-twin or triplet or quadruplet to male lambs, have established that permanent blood cell chimaerism occurred in these sheep. This fact therefore verified that they were freemartins and that the freemartin condition could be diagnosed reliably in the sheep by the examination of chromosomes from cultured leucocytes.

15. Chromosome studies of 401 cells from three different tissues from each of four animals showed that only female cell lines were present and that the four sheep were therefore genetic females.

16./

16. Sex Chromatin studies on five of the freemartin sheep confirmed the above conclusion, but also showed that there was a significant difference in the incidence of sex chromatin in these animals when compared with normal ewes.

17. An increase in aneuploid cells (hypomodal and hypermodal) was demonstrated in five freemartin sheep, aged four to seven years, but not in a sixth, which was under two years of age. Comparison with the chromosome counts of twenty-two normal sheep all aged less than two years suggested that age aneuploidy occurred in these sheep in a manner similar to that shown in man.

18. Karyotype analysis of the hypermodal cells in the five freemartin sheep failed to identify the extra chromosome, which was however, always a member of the acrocentric group.

19. The anatomy of the genitalia of the freemartin sheep varied between a "male type" and a "female type", and there was a close correlation between this and the external genitalia as shown by the presence or absence of a glans clitoris and the descent of the gonads.

20. The histological findings of the gonads of/

of the freemartin sheep were similar to those of the bovine freemartin. Sex cords were present in each of the gonads in close accordance with the degree of masculinisation of the genitalia. No body comparable to an ovarian follicle was seen in any of them. These observations were in close agreement with Chapin (1917) on the bovine freemartin and would suggest that the ovine and bovine freemartins are the result of the same causative factor or factors.

21. Comparison with recorded cases of bovine freemartins and also in view of the four "female type" freemartins recorded in this study, it is suggested that covert freemartinism is probably more common in sheep than has been suspected previously.

22. The study of four cases of male pseudo-hermaphrodites confirmed that these were genetic males and cases of hypospadias.

Appendix to Part 1.

Sex Chromatin.

Cresyl Echt Violet. 2% aqueous solution.

Coleman and Bell, Ltd.

U.S.A.

Papanicolaou's Fixative.

50% Ether. (May and Baker).

50% Absolute ethyl alcohol.

Biebrich Scarlet. (Water soluble).

British Drug Houses.

Poole,

England.

Consisting of:

Biebrich Scarlet	1.0 Gm.
Phosphotungsic Acid (B.D.H.)	0.3 Gm.
Glacial acetic acid	5.0 ml.
50% ethyl alcohol	100.0 ml.

Fast Green F.C.F. (Harleco).

British Drug Houses,

Poole,

England.

Consisting of:

Fast Green F.C.F.	0.5 Gm.
Phosphomolybdic acid (B.D.H.)	0.3 Gm.
Phosphotungsic acid	0.3 Gm.
Glacial acetic acid	5.0 ml.
50% ethyl alcohol	100.0 ml.

APPENDIX to PART II.

Colcemid. Ciba Laboratories, Ltd.
Horsham, Sussex, England.
In 1 mg. tablets. Solution prepared by dissolving 2 tablets in 20 ml. of distilled water, and filtering. Solution was refrigerator stored.

Phytohaemagglutinin. Wellcome Research Laboratories, Beckenham, England.
Freeze dried material reconstituted in 5 ml. of sterile distilled water. (May and Baker).

T.C. 199. Tissue Culture Medium "199".
Glaxo Laboratories Ltd., Greenford, England.

Heparin. Heparin Inj. B.P. 1,000 Units per ml.
Boots Pure Drug Company, Ltd.
Nottingham, England.

Orcein Stain. Natural Orcein.
G.T. Gurr, Ltd., London, S.W.6, England.
Prepared by modification of the method of La Cour (1941), using 2% orcein in 65% acetic acid.

Waymouth's Medium.

The constituents were prepared by the Dept. of Genetics, Glasgow University, and mixed as required from the following stock solutions.

(1)	Stock solution of nutrients of Waymouth's medium 752/1 (Paul, 1961).	13.0 ml.
(2)	Hank's B.S.S.	73.0 ml.
(3)	Glutamine (0.7308 gm. in 50 ml.H.B.S.S.)	2.5 ml.
(4)	Sodium bicarbonate solution (6.6%).	2.4 ml.
(5)	Penicillin streptomycin (200U/ml.penicillin and 0.002 gm. streptomycin per ml. of medium.	0.4 ml.
	(Phenol red indicator in H.B.S.S.).	

The stock solution of nutrients, sodium bicarbonate, glutinine and antibiotics were stored in a deep freeze at - 68 C.

H.B.S.S. was kept in a refrigerator at 4 C.

Procedure for Cleaning and Sterilisation of Glasswear used for Leucocyte Culture.

Materials. Haemo-Sol.

Meineck and Co., Inc.

Baltimore, Maryland,

U.S.A.

Half an ounce to gallon of warm tap water.

Chloros., Industrial Sodium Hypochlorite.

I.C.I., Ltd.,

Millbank,

London, S.W.1.

Method. All traces of blood, etc. were well washed from culture and collecting bottles by repeated running in cold water. Washed glassware was then steeped in "Chloros" solution for 24 hours, (one tablespoon to a gallon of water). The bottles were again washed many times in tap water using a jet to ensure thorough removal of "Chloros" residue. They were then steeped in an "Haemosol" solution for several hours after which warm water was repeatedly run through, followed again by many rinsings of tap water. Following tap water rinsing, several rinsings were given in distilled water. All bottles were then placed upside down in a drying oven and thoroughly dried. Tops and rubber caps were washed many times and boiled in water for 10 minutes, the caps being dried in an oven and the rubber caps, with a clean cloth. All rubber caps and tops were then screwed on to culture bottles. Silver foil was placed over the tops and the bottles were autoclaved for 30 minutes at 15 lbs. pressure.

APPENDIX TO PART IV.

1. Waymouth's Medium.

See appendix to Part II.

2. Calf Serum. The calf serum used was prepared for routine culture work in the Department of Genetics, Glasgow.

3. Crystamycin.

Glaxo Laboratiles,
Greenford,
England.

4. Tris HCl. (Trizma HCl.).

Sigma Laboratiles,
London S.W. 6.
England.

5. Versene Buffer Solution.

Preparation.

To make 2 litres 10x Stock Solution.

NaCl	160.0 gm.
KCl	8.0 gm.
Versene	4.0 gm.
Phenol Red	0.4 gm.

For use dilute 200 ml. of 10x stock solution with 1900 ml. distilled water. Distribute in 95 ml. amounts in 6 Oz. M.F. bottles. Sterilise by autoclaving.

Store/

Store in fridg - 4 C.

Label - Versene Buffer Solution.

For use adjust pH to 7.4 with sodium bicarbonate.

6. Trypsin - Versene Solution.

Trypsin (Difco I : 250).

Difco Laboraties,
Michigan,
U.S.A.

Preparation.

Dilute 100 ml. versene buffer 10 x stock solution with 900 ml. distilled water.

Dissolve 5 gm. trypsin in this solution. Sterilise by millipere filtration, using prefilter, then GS pad.

Store in 50 ml. amounts in bottles in freezer at - 20 C.

Label 10 x Trypsin Versene Solution.

For use, add 10 ml. trypsin versene solution to one bottle versene buffer solution. Adjust pH to 7.6 with sodium bicarbonate.

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A STUDY in CYTOGENETICS in the SHEEP.

by A.N.Bruere.

Summary.

The purpose of this study was to apply some of the current methodology of medical cytogenetics, to a study of the sheep. The study was divided into four parts, the first being the investigation of the sex chromatin body in several tissues in the sheep while the second was an examination of the method of preparing mitotic chromosomes from leucocyte culture. The third stage was devoted to chromosome morphology and identification while the final part of the study involved the application of these methods to an investigation of intersexuality in the sheep. In this section particular emphasis was placed on the phenotype of each animal and its corresponding chromosome pattern in several tissues.

A sex chromatin body was demonstrated in nerve cells from the spinal cord and cerebellum of ewes, there being no apparent difference between new born lambs and adult sheep. In these areas of nerve tissue the incidence of sex chromatin was similar to those of other members of the same order of animals,

(Artiodactyla), and behaved in the same way.

It was not possible from the studies on buccal cells nor free amnion cells in the sheep to demonstrate a sex difference between rams and ewes. However preparations made from amnio-chorion showed a sex chromatin body in 51.96% of female cells examined. This sex chromatin body was characteristically associated with the nuclear membrane and was 100% diagnostically reliable when tested on a trial of fifty samples.

It was found that good metaphase preparations could be made from leucocyte cultures of whole blood of the sheep, but that certain factors were important for reliable growth of cells. These included the use of 500 I.U. of heparin to each 10 ml. of blood collected for culturing and the addition of 10%-20% of homologous sheep serum to either Medium 199 or Waymouth's medium, whichever was used. From the last 25 culture series of 122 carried out on the sheep, 22 satisfactory cultures were obtained and the best yield of metaphases was obtained following an incubation period of 72-75 hours.

This study showed that the modal diploid chromosome number of 54 occurred in 87.4% of 1,832 metaphases examined from 22 normal sheep. This chromosome

number of 54 was confirmed for five additional breeds of sheep including the Scottish Blackface, Scottish Blackface x Border Leicester (Greyface), Clun Forest, Welsh Mountain and Soay. Karyotype analysis of photographed metaphases showed that the chromosomes of the sheep can be divided into two groups for each sex. In the ewe there six metacentric chromosomes in the first group and forty eight acrocentric chromosomes in the second group, while there are six metacentric and forty seven acrocentric chromosomes and one small submetacentric chromosome in the ram. Karyotype studies further showed that the sex chromosomes can be identified in the sheep. The Y chromosome is distinct and the X is the largest of the acrocentric group and can be distinguished in a number of suitable metaphases.

The measurements made on individual chromosomes for idiogram construction were of little value in the identification of individual members, except for the six metacentric chromosomes and the first 3 pairs of acrocentric chromosomes including the X. The examination of 320 prepared karyotypes for secondary constrictions showed that these occurred on several sites on the metacentric chromosomes in sufficient

numbers to suggest that these are the main nucleolar organiser chromosomes. In the case of the acrocentric chromosomes secondary constrictions were recorded but in insufficient numbers to assist in identification.

From a number of metaphases certain figures interpreted as chromosome associations between acrocentric chromosomes, and acrocentric chromosomes and metacentric chromosomes were recorded. However, in the absence of many of these and of demonstrable satellites, chromosome associations in the sheep were considered to be of a more complex and delicate nature than in man and that demonstration is difficult using current techniques.

In a chromosome study of five freemartin sheep which were known to be either co-twin or triplet or quadruplet to male lambs, permanent blood cell chimaerism was shown to be established. This verified that the freemartin condition could be diagnosed reliably in the sheep by the examination of chromosomes from cultured leucocytes.

Chromosome studies of cells from three different tissues of each of four of these sheep showed that only female cell lines were present and that

they were genetic females. This conclusion was confirmed by the sex chromatin studies on nerve tissue from five of these sheep but it was also shown that there was a significant difference in the incidence of sex chromatin in the freemartins when compared with normal ewes.

When the chromosome counts from metaphases of the six freemartin sheep were analysed, an increase in aneuploid cells was demonstrated in five of these aged 4-7 years, but not in the sixth which was 18 months old. Comparison of these counts with those of 22 normal sheep aged less than 2 years suggested that age aneuploidy occurred in these sheep in a manner similar to that shown in man. Karyotype analyses of the hyperdiploid cells seen in these cases failed to show the identity of the extra chromosome which was however, always a member of the acrocentric group.

Anatomical studies of the internal genitalia of the above freemartin sheep showed that they varied between a "male" type and a "female type" and there was a close correlation between this and the external genitalia as shown by the presence of absence of glans clitoris and the position of the gonads.

Histologically the gonads of the freemartin sheep studied proved to be similar to those of the bovine freemartin. Sex cords were present in each of the gonads in close accordance with the degree of masculinisation of the genitalia. No body comparable to the ovarian follicle seen in true human hermaphrodites was seen in them. These observations would suggest that the ovine and bovine freemartins are the result of the same causative factor or factors.

The investigation of these cases has shown that the freemartin condition occurs in the sheep and that incidence of covert freemartinism in this species may be higher than previously suspected.

An examination of four cases of male pseudohermaphroditds confirmed that these were genetic males and cases of hypospadias.