A STUDY OF CYTOGENETICS IN THE PIG

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by:

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INTRODUCTION TO THE STUDY

The broad aims of this study are to determine the normal in the pig in relation to chromosome content, both numerical and morphological, to the sex chromatin content of various tissues and the replication pattern of the chromosomes by means of autoradiographic studies as a morphological aid. Finally, the results obtained from these detailed studies will be used in the examination of animals with suspected chromosomal abnormalities and a more accurate determination of any abnormalities present in these animals thus obtained.

In more detail, the thesis consists of four main sections within which all subsections are connected by some common factor.

Section 1 Determination of the Normal Chromosome Content

A technique for the culturing of white blood cells will be described and using this, an analysis of the chromosome count in a large number of pigs made. From this, the normal number of chromosomes will be determined, together with the range of normality found in this species. Further to numerical analysis, the morphological characteristics of the pig will be studied in order to determine the number of homologous pairs which can be positively identified and also those sets of pairs in which this cannot be done. As a further aid to the identification of chromosomes, the frequency with which secondary constrictions are observed on each chromosome will be analysed and from these results, the possible use of such constrictions as markers will be determined. As a final analysis of the chromosome content, an idiogram will be constructed, this showing, diagramatically, the dimensions of an average pig cell.

Thus, in this section, the normal chromosome picture, both numerical and morphological is obtained by a number of methods.

Section 2 Sex Chromatin Studies

The sex chromatin content in nerve tissue of both adult males and females will be studied in order to determine the exact frequencies found on both sexes. Using the Feulgen staining method for D.N.A. the nature of the bodies in both sexes will be studied. In addition, foetal nerve tissue will be studied in order to determine the differences, if any, between sex chromatin content in adult and foetal tissue. Further to the studies on nerve tissue, the presence of sex chromatin in other tissues will be studied. By this study, the frequency of sex chromatin in normal animals will be determined, thus giving a basis for future studies in animals with X chromosome aberrations. Using certain non nervous tissues, the potentiality of screening techniques in the live animal will be determined.

Section 3

As a further aid to chromosomal identification which was initiated in Section 1, an autoradiographical study will be carried out. As a result of this study, it is hoped that the replicating pattern of the chromosomes will be useful in identifying individual chromosomes and secondly, in separating pairs of homologues which might be morphologically similar.

An extension of this study will be the determination of the damaging effects of tritium on the chromosomes. By carrying out multiple cultures with tritium added at different times, it is hoped to determine the effect on chromosomes of tritium itself, and also of the time of contact it has with the chromosomes. Finally, analysis of the results may indicate if any damage caused by the tritium will be a random process throughout the cell or whether certain areas are more sensitive to the radiation than are others.

Section 4

A study of clinical cases is envisaged in the hope that chromosome aberrations may be detected. Peripheral blood cultures will be carried out on these cases, and any which display chromosomal abnormalities will be further studied cytogenetically. In this section it is hoped to demonstrate the usefulness of chromosomal studies in the pig and its potential as a routine examination of animals with abnormalities, both congenital and apparently acquired.

SECTION I

Introduction to Cytogenetics.

GENERAL HISTORICAL INTRODUCTION

Cytogenetics, or the study of both the normal and the abnormal structure and behaviour of chromosomes, has, within the last 15 years undergone an explosion of knowledge. However, it was as early as 1880 that Fleming had observed the mechanisms of division in living cells and had shown that the chromosomes of the Salemander split longitudinally. This period towards the end of the 19th Century was an exceptionally fruitful one, in that many original observations were made which were to form the basis of man's knowledge of heredity. Among these discoveries, which are too numerous to catalogue, two stand out as being of extreme importance. The first was the reports of Hertvig, (1876 and 1885) on the basis of fertilization and heredity. Hertvig found in the eggs of the sea urchin that at fertilization two nuclei united, one from the egg and the other from the spermatozoa. From this he concluded that these nuclei must carry the materials which were inherited from parents to offspring. The second observation, although perhaps not so basic as those of Hertvig, has turned out to be the forerunner of possibly the most fruitful field in modern cytogenetics yet discovered. Henking (1891), while studying the cell division of spermatocytes in Pyrrhocorus Apterus, noticed a chromatid body which appeared to be more intensely stained than the others and which lagged behind the remainder of the chromosomes. He further noted that at the second division of the spermatocyte, it passed undivided into one of the daughter nuclei. Although not realising the significance of this behaviour, Henking's observation of the 'X' chromosome, as he called it, was to have far reaching effect on future studies of sex determination and almost 70 years later the studies of the 'X' chromosomes in man and animals.

In 1903 Sutton put forward a hypothesis, later known as the Sutton-Boveri hypothesis which speculated on a chromosomal basis for the hereditary transmission of characters

originally reported by Mendel (1865). This speculation is now a cornerstone of the connection between genetics and the field now known as cytogenetics.

Although much work was carried out on chromosomes and genetics, one of the most surprising elements of cytogenetics was the delay in discovering the true chromosome complement in man. Prior to 1956, it was accepted that the human had 48 chromosomes (Painter 1923 and Hsu 1952) with the sex elements X and Y, although Painter reported that, prior to his work, the estimates of the diploid number ranged from 16 - 47 and the sex determining mechanism was thought to be X - O.

In 1956 Tjio and Levan published a paper on the chromosome number of man which was to have far reaching effects on human genetics. In this report, Tjio and Levan found that man had 46 chromosomes, two less than were thought to be present. This discovery was the start of the modern intensive study of cytogenetics in which it has been realised that the abnormal can only be determined by extensive study of the normal, the ideal example of this being the work of Tjio and Levan as a forerunner in the field. The basic reason for this breakthrough was the development of tissue culture techniques, a great improvement on the previously used tissue sections. From this determination of the normal, has come floods of reports on the abnormalities which exist in the chromosomes of both man and animals, which in many cases have been complementary to one another.

Because of the vast amount of literature published in the last decade, it is considered worth while to present a brief review of the literature in both man and the domestic animals. As the volume of work published is so large, it is possible only to mention findings which are of basic importance by their explanation of various mechanisms, their frequency or, in the case of certain animal conditions, their economic importance or finally, by their help in throwing light on comparative interspecies cytogenetics.

Human Cytogenetics

Soon after Tjio and Levan's startling discovery of the true number of human chromosomes, Ford Jacobs and Lajtha, (1958) developed a culturing technique involving the use of bone marrow cells to obtain well spread metaphase chromosomes. Two years later a further technique was developed, this time using white cells obtained from peripheral blood (Moorehead, Nowell, Melman, Battips and Hungerford, 1960). These three developments together with the discovery of sexual dimorphism in cells, (Barr and Bertram, 1949), were the main reasons for the sudden increase in interest in human chromosomes and from these has sprung the vast knowledge now available.

Down's Syndrome

In 1866, Langdon-Down while examining a group of mental defectives found that certain of the patients had similar abnormalities, the main one of these being oriental features. Because of this finding he named the condition Mongolism. Since then many other features have been noted in this condition which, besides mental retardation include various facial disorders, skeletal abnormalities, muscular hypotonicity, congenital heart disease and leukemia. The incidence of Down's syndrome in the live born population has been variously reported as being between 1 in 600 and 1 in 700, (Penrose, 1961).

Over a hundred years after Langdon-Down's report, the etiology of the condition was explained by Lejeune, Gautier and Turpin, (1959), who found that an extra small autosome was present in these patients. This finding was soon confirmed by Jacobs, Baikie, Court Brown and Strong (1959), although the identity of the supernumerary chromosome was disputed. With the attempt to standardise human chromosomes (Denver report, 1960), it was accepted that the extra chromosome was a member of the G group and therefore number 21 or 22.

It became customary to call the condition Trisomy 21, but doubts have been expressed concerning this nomenclature due to the fact that chromosomes 21 and 22 are structurally very similar, (Patau, 1960). Similarly, the use of autoradiography, which appeared very promising for identification (Schmid, 1963) has since had doubts expressed on its usefulness, (Back, Dormer, Baumann and Olbrich, 1967, and Fraccaro, Lindsten and Tiepolo, 1967). At present therefore, the identity of the extra chromosome is still in doubt, but in any case no difficulty exists in the cytogenetic diagnosis of Down's syndrome, when trisomy is present.

However, it was noted soon after the discovery of trisomy mongolism that certain cases of Mongoloid children had 46 chromosomes. Polani, Briggs, Ford, Clarke and Berg, (1960) reported a young mongoloid girl with the normal number of chromosomes, but an abnormal chromosome similar to a C group was present and one D group chromosome was missing. This was interpreted as being a 15/22 translocation, thus making the child effectively trisomic for 22, as 4 G group chromosomes were also present. Fraccaro, Kaijser, and Lindsten, (1960) simultaneously found a child with an apparent 21/22 translocation although they were unable to positively identify it because the father possessed aneuploidy of an equivocal nature. Polani, (1963) in a review of the literature found that in some cases of 13 - 15/21 translocations, the abnormality was transmitted through generations, whereas in other cases it appeared spontaneously. It has been well known for a long time that Down's syndrome is maternal age dependent, (Shuttleworth, 1909; Penrose, 1933 and 1934). However, Sergovitch, Soltan and Carr, (1962) carried out a study of 12 unrelated translocated mongols and found that maternal age did not differ from controls. These findings bear out the results of Penrose, (1951) who recognised that the maternal age frequency distribution curve for Mongolism suggested two groups of patients, the larger of which was associated with advancing age and the smaller group independent

of this effect.

In addition to the phenotypic abnormalities commonly seen in mongoloid children at birth, in later life it has been found that these children die young, the life expectancy being about 10 years, (Knudsen, 1965), the main causes of this early death being cardiac defects and infection. A third factor is leukemia which developed in Mongoloid children at a frequency much higher than in the control population. Stewart, Webb and Hewitt, (1958) in a survey of childhood malignancies, found that mongoloids died of leukemia almost 20 times more frequently than that of normal children. Carter, (1956) had similarly suggested this correlation, but his sample was rather small.

A final interesting correlation between mongolism and neoplasia, is the fact that although difficulty is encountered in identifying the human 21 or 22 chromosomes, it would appear that the chromosome trisomic in Down's syndrome and that deleted in chronic Myeloid Leukemia (Nowell and Hungerford, 1960) is probably the same one.

Klinefelter's Syndrome

Klinefelter, Reifenstein and Albright, (1942) originally described a syndrome which appears during adolescence and whose features included gynecomastia, small testes, azoospermia, reduced function of Leydig cells and increased output of follicle stimulating hormone. The syndrome was later modified slightly to include patients lacking gynecomastia, (Heller and Nelson, 1945). By the newly developed sex chromatin technique it was shown that a number of patients with Klinefelter's syndrome, were sex chromatin positive (Bradbury, Bunge and Boccabella, 1956; Plunkett and Barr, 1956). This was interpreted as meaning that these patients were genetic females. Ferguson-Smith (1959) found that prepubertal boys had deficiencies of germ cells and at puberty hyalinisation occurred to give the classical histological picture of Klinefelter's syndrome. In the same year, the true nature of Klinefelter's

syndrome was demonstrated by Jacobs and Strong, (1959) and Ford, Jones, Miller, Mittwoch, Penrose, Ridler and Shapiro, (1959). Both groups demonstrated that an extra chromosome was present and the chromosome complement was interpreted as being 47,XXY. With these new findings, more groups started to investigate the problem of Klinefelter's syndrome and many chromosomal variants were found. These included XXXY (Barr and Carr, 1960; Ferguson-Smith, Johnstone and Handmaker, 1960), XXXXY (Fraccaro and Lindsten 1960, Miller, Breg, Schmickel and Tretter, 1961), and XXYY (Muldal and Ockey, 1960 and Carr, Barr and Plunkett, 1961). From the clinical findings of these cases it was soon realised that the presence of extra chromosomes led to more severe phenotypic abnormalities. It was noted early in the studies of sex chromatin positive males that the condition was associated with mental deficiency. Early surveys showed that among patients with "lower mentalities" a high frequency of sex chromatin males was found (Ferguson-Smith 1958), this being confirmed by later surveys (Mosier, Scott and Cotter, 1960; McLean, Mitchell, Harnden, Williams, Jacobs, Buckton, Baikie, Court Brown, McBride, Strong, Close and Jones, 1962). A recent analysis of surveys among the mentally subnormal and normal populations (Court Brown, 1969) has shown that the frequencies of sex chromatin positive males were 9.4 per thousand and 2.4 per thousand respectively. In no case in the survey of non-institutionalised males was any patient found with more than two X chromosomes.

As well as the findings that the degree of abnormality increased with the number of X chromosomes, it has been found that patients with mosaicism which includes a normal XY cell line are less severely affected than non-mosaics. In cases of XY/XXY, Ferguson-Smith (1966) reported that the frequency of micro-orchidism and azoospermia was lower than that found in XXY patients and with the exception of those patients selected for subnormal intelligence, the XY/XXY patients were not mentally deficient. From this it appears that the normal XY cell line has a modifying effect on the abnormal line.

XYY Syndrome

In 1962, Hauschka, Hasson, Goldstein, Koepf, and Sandberg reported the first case of a male with a sex chromosome constitution 47,XYY. He was of average intelligence and of normal fertility, although 2 pregnancies had terminated in abortion and a third died three days after birth, being a "blue baby". A further two pregnancies produced offspring with chromosomal abnormalities, one 45,X and the other 47XX,G+. Following this report, four more cases were found, from which no clear picture emerged (Jacobs, Brunton, Melville, Brittain and McClermont, 1965). However studies of the chromosome complements of patients in institutes for the criminal and "hard-to-manage" males of subnormal intelligence revealed that a high frequency possessed an extra Y chromosome (Jacobs etal, 1965 and Casey, Segall, Street and Blank, 1966), Jacobs findings 3.5% of the studied population to have an XYY sex chromosome complement, the majority of these being over 6 foot in height. Jacob's correlation of XYY chromosomes and height was used by Casey, Blank, Street, Segall, McDougall, McGrath and Skinner, (1966) in a study of mentally subnormal patients with a history of antisocial behaviour. It was found that 12 out of 50 patients in this category were XYY. These, together with a later survey, (Jacobs, Price, Court Brown, Brittain and Whatmore, 1968), appear to indicate that a connection exists between this type of patient and the presence of an extra Y chromosome. Although the incidence of XYY patients among the mentally subnormal, and hard-to-manage criminals appears to be significantly high, difficulty is encountered in comparing it with a control population. As the Y chromosome is not detectable by any screening technique the large sex chromatin surveys which have been carried out cannot be used. Only small surveys involving chromosome analysis have been carried out. Out of 550 live born male children, only one XYY baby was found and a survey of entrants of Borstals revealed the present of only one XYY out of 607 (Court Brown, 1969).

From the findings so far obtained it appears possible that an extra Y chromosome in man could be associated with behavioural disorders, but much more work is required before categorical statements can be made concerning the natural history of the XYY man.

Turner's Syndrome.

Turner's syndrome was first described by Turner (1938) as a disorder of woman, consisting of sexual infantilism, webbing of the neck and cubitus valgus. The basic cause of the sexual disturbances was shown to be ovarian abnormality by Wilkins and Fleischmann (1944). These workers described the absence of ovaries with replacement by 'streak' gonads lacking follicles.

In 1954, with development of the sex chromatin technique, these patients were shown to be lacking sex chromatin (Polani, Hunter and Lennox 1954, and Wilkins, Grumbach and Van Wyk, 1954). These two sets of workers found that in 11 cases of Turner's syndrome, 9 had the male type of sex chromatin and 2 were chromatin positive. This was interpreted as meaning that some of Turner's syndrome were genetic males whose testes had somehow failed in utero, thus resulting in female development. However, it was later shown that the chromosome complement of these patients was not in fact male but 45,X, being female but lacking one X chromosome, (Ford, Jones, Polani, de Almeida and Briggs, 1959 and Fraccaro, Kaijser and Lindsten, 1959). Although XO has been found to be the most common chromosome complement in gonadal dysgenesis (Ferguson-Smith, 1965), many other karyotypes have been found including XY (Harnden and Stewart, 1959; de Grouchy, Cottin, Lamy, Netter, Netter-Lambert, Trevoux and Delzant, 1960); and XX (Jacobs, Harnden, Buckton, Court Brown, King, McBride, Macgregor and McLean . Jacobs and her co-workers further found a large number of varying karyotypes in their study, including XO/XY, XO/XX, Xp-X, XO/XXX, XigX and XO/XYY. With such a range of karyotypes published on patients

with widely varying phenotypes, Ferguson-Smith, (1965) analysed the karyotype-phenotype correlations to investigate whether variation in phenotype could be associated with deficiencies in sex chromosomes of various types. From this study, Ferguson-Smith concluded that for patients to show the complete Turner's syndrome it was necessary for the patient to be monosomic for the short arm of the X. He also found that there appeared to be a range of phenotypes varying from normal female to Turner's and between normal male and Turner's depending on the degree of sex chromosome deficiency between XX and XO, and between XY and XO respectively. In the mosaic cases, it was found that the presence of normal XX cells or XXX cells appeared to modify the effects of the XO cell lines.

The frequency of Turner's syndrome has been repeatedly found to be lower than that of Klinefelter's syndrome. Court Brown (1969) found that only 14 out of over 40,000 live born female babies were sex chromatin negative compared with 71 sex chromatin positive male babies in approximately 42,000 live born male babies. One possible explanation for this has been the findings in the chromosomal analysis of abortions. Carr, (1965) examined the karyotypes of 200 spontaneous abortions and found that 11 were XO or monosomy for one member of the C group. Further studies have confirmed Carr's findings, the Geneva Conference, (1966), reporting that 4.1% of all spontaneous abortions had an XO or C group monosomy and approximately 20% of the spontaneous abortions associated with chromosomal abnormalities had this karyotype. It would appear therefore, that XO fetuses are less viable than XXY fetuses, as yet none of the latter having been associated with spontaneous abortions.

Abortions

The first estimate of the significance of chromosomal abnormality in spontaneous abortions in human was made by Carr, (1963). Prior to this, it has been noted, however, that various aberrations such as triploidy existed in aborted material,

(Delhanty, Ellis and Rowley, 1961; Penrose and Delhanty, 1961). Carr studied 54 spontaneous abortions and found 12 to have chromosomal abnormalities. Three of these were X monosomy, six were trisomies, and of the remaining three, two were triploid and the other tetraploid. Carr concluded from his results that this might be a possible explanation of the low incidence of XO births compared with XXY. Soon after Carr's publication, Clendenin and Benirschke (1963) reported their findings that 3 out of 10 spontaneous abortions were associated with chromosome aberrations. Of these, one was XO, one trisomy E and the last a translocation. Following these reports many more similar studies were published (Inhorn, Therman and Pätau 1964, Hall and Källën, 1964, and Thiede and Salm, 1964) all groups finding chromosomal abnormalities to be present in fetuses which were spontaneously ~~aborted~~ aborted.

The Geneva Conference (1966) summarised all the work carried out up to and including 1966. Of 788 spontaneous abortions, 155 were found to have chromosomal aberrations, a frequency just under 20% The most common single abnormality was monosomy X with the majority of the remainder being either trisomies of various groups or triploidy. From this report it is obvious that in the human, chromosomal abnormalities are associated with a relatively high percentage of pregnancy failures. It has been estimated that 15% of pregnancies terminate in spontaneous abortion (Roth, 1963; Warburton and Fraser, 1964). As 20% of these are associated with chromosomal abnormalities, approximately 3% of all conceptions end in chromosomally associated abortions. Furthermore, as the incidence of 20% of abortions which have chromosomal abnormalities is more than 50 times the frequency of chromosomal abnormalities in live born infants (Carr 1967), the mechanism of rejection of abnormal pregnancies in the human appears to be a very efficient one.

Intersexuality

Intersexuality, or the study of individuals possessing both male and female features, whether gonads, reproduction tract, genetic sex or behaviour, has in the last decade developed into a most complicated and in some cases confusing subject. It has warranted entire books, (Ashley, 1962, Overzier 1963, Armstrong and Marshall, 1964), and numerous classifications (Ashley, 1962, Lennox, 1966). Because of this, no attempt will be made to cover comprehensively this complex field.

True Hermaphrodites possess both ovarian and testicular tissue, the remainder of the reproductive tract and the patients' phenotype being very variable from almost normal male, (Hungerford, Donnelly, Nowell and Beck, 1959), tending towards female (Harnden and Armstrong, 1959). Overzier, (1963) listed 171 patients and found that although well over half were reared as males, as many as 25% were brought up as females, thus demonstrating the extreme variation found in this condition.

The first true hermaphrodite patients whose chromosomes were studied, were found to have a 46,XX constitution, (Hungerford etal. 1959, Harnden and Armstrong, 1959, Ferguson-Smith, Johnstone and Weinberg, 1960). There was however, a possibility that one of the cases of Ferguson-Smith etal might have been a mosaic XX/XXX although certainly in no cells was a Y chromosome observed. The majority of true hermaphrodites were reported to have the sex chromosome constitution XX (Shearman, Singh, Lee, Hudson and Ilbery, 1964). However, various other complements were found such as XY (Sandberg, Koepf, Crosswhite and Hauschka, 1960; and Shearman etal 1964), XX/XXY/XXYYY (Fraccaro, Taylor, Bodian and Newns, 1962); XO/XY (Hirschhorn, Decker and Cooper, 1960). Fraccaro and his co-workers questioned the findings of earlier workers who were unable to identify Y chromosomes in their

cases and postulated that for testicular development to occur it was necessary to have a Y chromosome present. Both Fraccaro and Brögger and Aagenaes (1965) have suggested that in those cases in which no Y chromosome was detected it was possible that mosaicism was present but was undetected due to the failure of the investigations to examine sufficient numbers of tissues. Brogger and his co-workers, in their study of a true hermaphrodite, examined 4 tissues, finding only XX cells and it was only in a second testicular biopsy that XY cells were detected. Bain and Scott, (1965) similarly found XX/XY in a hermaphrodite and postulated a double fertilization origin, based on blood groupings. Solomon, Hamm and Green, (1964) reported studies on a non-hermaphrodite boy in whom previously a Y had not been found, but on re-examination this was found, thus showing the difficulty of detecting mosaicism. From the experience in these two cases it seemed possible that previous work in which an XY cell was not present had failed to determine mosaicism. Hungerford, Donelly and Nowell, (1965), while admitting that mosaicism was possible in their case in which skin from four sites as well as blood had been examined, without a Y being found, stated that no adult multicellular organism could be demonstrated not to be a mosaic, and if sufficient tissues had been examined without finding a second cell line, it was necessary to look for another explanation. Hungerford, therefore, put forward the suggestion that in those cases in which mosaicism could not be determined, a possible explanation for the presence of testicular tissue in the apparent absence of a Y chromosome, was that part of the Y had been translocated to a larger chromosome in such a way that it was not detectable microscopically. Ferguson-Smith, (1966a) put forward a similar hypothesis to explain XX true hermaphrodites suggesting that it seemed unlikely that in all such cases a Y chromosome had gone undetected. He postulated crossing over between the X and the Y could occur at prophase of the first meiotic division of the

primary spermatocyte. If this happened, one of the X chromosomes in the XX hermaphrodites would bear some male determining genes and therefore testicular development could occur in the apparent absence of a Y. At the same time it would be expected that some X materials would be reciprocally translocated to the Y and that would explain unusual Xg blood group results obtained by Sanger, Race, Tippett, Gavin and Hardisty, (1964). Recent studies (Ferguson-Smith personal communication) have made this possibility appear even more likely, again on the grounds of Xg inheritance. By random inactivation of the normal X and the abnormal X with the translocated Y, either ovarian or testicular tissue would develop, depending on the type of X which was mainly active. Thus in true hermaphroditism, the chromosome complement XX/XY has been found, explaining the presence of both testes and ovaries, the original cause of this being possible double fertilization of ovum and polar body (Zeutler, Beattie and Reisman, 1964). XX has been found very frequently in such cases and as it seems unlikely that mosaicism has been undetected in all cases, a X - Y interchange postulated by Ferguson-Smith seems a possible explanation. An alternative hypothesis that testicular material may be formed in the absence of Y chromosome (Mittwoch, 1967) is still possible, although by some as yet unknown mechanism.

Pseudohermaphroditism

Prior to the characterisation of various conditions such as Klinefelter's and Turner's syndromes, the broad term Pseudohermaphroditism covered a large number of conditions, one of these, testicular feminisation which was previously classified as one of the pseudohermaphrodites, is now regarded and discussed here as a separate entity.

Morris (1953), first coined the name 'syndrome of testicular feminisation' for a syndrome distinct from the general term male pseudohermaphrodite. In reporting

the syndrome, Morris stated that the condition warranted separation due to the fact that the old term usually implied that the external genitalia were such that confusion of the sex might arise. Patients with the condition have a typical female appearance with breast development, but have only sparce pubic and axillary hair. The vagina is blind ending, menstruation does not occur and the women are sterile. Morris found that instead of being ovaries the gonads had the appearance of undescended testes, which produced both oestrogens and androgens. The etiological agent behind the condition has been shown to be a familial one being transmitted through the mother (Grumbach and Barr 1958), although McKusick, (1962), was unable to determine whether the linkage was X- or autosomal borne. A chromosomal basis was postulated by Danon and Sachs, (1957) who suggested that these patients were probably XXY. However, Jacobs, Baikie, Court Brown, Forrest, Roy, Stewart and Lennox, (1959) found that all four cases which they studied had an XY karyotype, a finding in agreement with that of Grumbach and Barr, (1958) who had found a number of cases to be sex chromatin negative.

Hauser, (1963) stated that the cause of the syndrome might be in one of three places, the target organs, the hypophysis or in the testes themselves, and came to the conclusion that it was the result of early intra-uterine testicular insufficiency. Lennox, (1966) came to a similar conclusion on the evidence of Griffiths, Grant and Whyte, (1963). These works showed by <u>in vitro</u> studies on the testes that it could produce testesterone, but not oestrogen. However, an excess of 16 hydroxy-progesterone was produced and this might act as an antiandrogen (Lennox, 1966). This theory is as yet unproven and the alternative explanation of an autosomalgene causing failure of the target organ to respond to testosterone seems a more attractive possibility, (Hamerton, 1968).

Neoplasia

In human malignant tumours and acute leukemias, abnormal cell division occurs with resulting chromosomal aberrations, (Yunis 1965). The only intensively studied neoplastic condition in man which has shown a constant chromosomal abnormality, however, has been chronic myeloid leukaemia. Nowell and Hungerford, (1960) examined the chromosomes of two males with the condition and found that an abnormal chromosome was present in peripheral blood cultures and suggested that the Y was affected. In two cases of acute leukemia, however, the abnormality was not detected. Further studies revealed that the chromosome involved was not the Y but a deleted member of the 21 - 22 group (Nowell and Hungerford 1960a; Baikie, Court Brown, Buckton, Harnden, Jacobs and Tough 1960). This deleted 21 - 22, as well as being present in blood, was found in bone marrow, but attempts to find it in skin cultures were unsuccessful, (Baikie etal 1960). Subsequent investigations have detected the abnormality (Nowell and Hungerford, 1961; Tough, Court Brown, Baikie, Buckton, Harnden, Jacobs, King and McBride, 1961). It was further found that during treatment for the condition, the abnormality, now generally known as the Philadelphia chromosome, Ph' after the city in which it was discovered, persisted in bone marrow cultures. There now seems to be no doubt that the Ph' chromosome is a primary change associated with chronic myeloid leukemia but only with this condition.

With the suggestion that the Ph' chromosome was a deletion of the long arm of a G chromosome, (Schmid 1963), the interesting hypothesis was put forward that the G_1 chromosome contained genes related to leukopoiesis and leukaemogenesis (Yunis, 1965). Krivit and Good, (1957) and Stewart, Webb and Hewitt, (1958) had presented results which indicated that acute leukaemia amongst mongol children occurred at a much higher frequency than would be expected by chance. In 1959

it was shown that the condition of Mongolism was very commonly associated with trisomy of a chromosome which ~~had~~ has since become known as number 21 or G1. This discovery of Lejeune, Gautier and Turpin, (1959) and Jacobs, Baikie, Court Brown and Strong, (1959), together with that of Nowell and Hungerford, (1960) appeared to indicate that the same chromosome was involved in both Mongolism with associated acute leukaemia, and chronic myeloid leukaemia. As yet this hypothesis is unproved.

VETERINARY CYTOGENETICS

Compared with the knowledge which has accrued in the last decade concerning the cytogenetics of human abnormalities, the amount of information which is available on domestic animals is very sparse. However, enough work has been carried out on a number of conditions for it to be realised that veterinary cytogenetics is potentially a very fruitful field and one which, in time may answer problems which have arisen from the human studies.

Freemartinism

In three species, namely cattle, sheep and pigs, freemartinism has been studied, and of the three by far the most work has been carried out on cattle. Tandler and Keller, (1911) and Lillie, (1917), independently suggested that for freemartinism to occur, cattle twins must be of unlike sexes and vascular anastomosis must develop. They both suggested that transplacental passage of hormones from male to female foetus occurred and because of this, the gonads of the female were modified, thus resulting in a freemartin condition. The conclusion that the freemartins were genetic females was borne out by the studies of Moore, Graham and Barr, (1957) who studied the sex chromatin in neurones and found that they were sex chromatin positive.

With the development of modern cytogenetic techniques, Ohno, Trujillo, Stenius, Christian and Teplitz, (1962) found that both members of unlike pairs of twins possessed XX and XY cell lines in the bone marrow. Confirmation of this chimaerism was reported by Fechheimer, Herschler and Gilmore, (1963), and Goodfellow and Strong, (1965), the conclusion being drawn that lymphocytes must pass across the transplacental anastomosis as well as the hormones previously hypothesised. Previous studies in freemartinism had similarly shown that substances, other than hormones, might cross the anastomosis such as red cell precursors, (Owen, 1945; Owen, Davis

and Morgan, 1946), and skin antigens (Anderson, Billingham, Lampkin and Medawar, (1951).

With these discoveries an alternative theory to that of the hormone transfer was put forward by Ohno etal (1962), and Fechheimer etal, (1963) who hypothesised that the sterilisation of the freemartin was brought about by the effect of migrating germ cells from the male to the female. Ohno etal, (1962) demonstrated that XX cells were present in the testes of one male co-twin, but failed to demonstrate XY cells in the freemartin gonad, although these workers did not expect to find any, due to the probable elimination of the germ cells by the effect of androgens.

However, work carried out on the Marmoset Monkey has tended to raise doubts about the germ cell theory. This species invariably produces two dizygotic young, and early in pregnancy placental anastomosis occurs, (Wislocki, 1939). However, Wislocki examined three pairs of unlike twins embryos, and was unable to demonstrate any abnormality of the genital tract, postulating that compared with cattle, some difference in hormone production must occur. As in cattle, marrow cell chimaerism XX/XY has been demonstrated, (Benirschke, Anderson and Brownhill, 1962; Benirschke and Brownhill 1963), thus showing that cellular exchanges between the foetuses had occurred. Furthermore, Benirschke & Brownhill, (1963), showed that XX/XY chimerism was also present in the gonads. Thus, although male cells are present in the gonads of the female co-twin, no freemartin-like condition developed. One possible explanation of the difference between cattle and the marmoset monkey is an enzymatic property of the marmoset placenta. In the latter species Ryan, Benirschke and Smith, (1961) showed that the placenta can convert androstenedione -4-C^{14} into oestrone, whereas this did not appear to occur in bovine placenta. Thus, in cattle at present, there is no indisputable theory as to the mechanism which causes the female co-twin embryo to be made sterile and its internal reproductive tract modified into a state which can vary from almost male to that of undeveloped female, although

the external genitalia is essentially female, (personal observation).

In sheep, the incidence of freemartinism is much lower than that in cattle. Bruere, (1966) studied six cases and quoted a further seven from the literature. In his six cases, he found the cytogenetic findings to be identical to those in the cow in that blood cell chimerism XX/XY was present. Other tissues examined were XX and the animals were sex chromatin female type. As in the cow, the external genitalia were female in appearance, although an enlarged clitoris was observed in two of his cases. From the literature, it appears that the incidence of freemartinism in sheep is very low, but Slee, (1963) found that vascular anastomosis is more frequent if the pregnancy consists of three or more young, whereas with twin pregnancies vascular anastomosis occurs relatively infrequently, (Biggers and McFeely, 1966).

In the pig, the condition is possibly even rarer than in the sheep. Hughes, (1929) examined 400 to 500 uteri in order to find unlike twins in which vascular connections were present. A total of four embryos were found with some degree of genital modification, as is found in cattle freemartinism, although Hughes found that the suppression of female differentiation was not as complete as in cattle. Recently two reports have been published concerning pigs with anatomical features similar to those found in freemartinism. McFee, Knight and Banner, (1966) found a white cell mosaicism XX/XY to be present in an intersex pig, and classed it as a male pseudohermaphrodite due to its testicular - like gonads, but neither the chromosome constitution of other tissues or the genital sex of neuromes were studied. A similar case was studied by Bruere, Fielden and Hutchings in a pig with descended testes, and a vulva but no scrotum. As with McFee's case, the blood cultures were a mixture of XX and XY cells and in this case neurones were sex chromatin positive. The internal genital tract in both cases was similar to the male-type freemartin found in cattle and sheep, having testes, vas deferens, prostate, seminal vesicles,

vulva and vagina.

Thus in three species, cattle, sheep and pigs, freemartinism is found, although in the latter two species, the incidence is much lower than in cattle. As yet, the mechanism behind this intriguing condition is not known and the subject still warrants much more research.

The XXY Syndrome

As the term Klinefelter's syndrome was coined by Klinefelter, Reifenstein and Albright, (1942) to describe a condition in the human, it is not valid to use the description when referring to animals. For this reason, the term XXY animals will be used as an alternative.

The first report of presumed XXY animals was by Thuline and Norby, (1961) who used the sex-linked coat colours in cats to detect two phenotypic male animals with 39 chromosomes. Although neither the karyotype nor the sex chromosome complement was reported, the fact that they were sex chromatin positive, had 39 chromosomes and appeared male was considered strong enough evidence to presume that they were XXY. In one animal, the testes showed abnormal meiosis with spermatogonia present, but no spermatids nor sperm. In the other cat, no gonads could be detected. Soon after this report, further cases were detected, one 39, XXY (Jones 1963) and a second with the interesting karyotype, 39,XXY/57,XXY (Chu, Thuline and Norby, 1964). The gonadal histology of Chu's case was not dissimilar to that of Thuline and Norby in that spermatogenesis was incomplete, only small numbers of Type A spermatogonia being present and no late stages of development.

One further case of an XXY male cat was reported by McFeely, Hare and Biggers, (1967) who used the unusual tortoiseshell markings to detect it. On fibroblast culturing, the animal was found to have a karyotype 38, XY/39, XXY, being phenotypically a normal male with the exception of testicular development. A few isolated spermatozoa

were found and an occasional spermatid, but there was a complete absence of sperm. Thus, in this case, as in the others with karyotypes of XXY and its variants, no abnormality of the genital tract was seen but there was lack of spermatogonia with subsequent virtual absence of spermatogenesis. The only exception to this rule has been that of one case of Thuline and Norby, (1961).

In cattle, one brief report by Scott and Gregory, (1965) concerned an intersex animal. This was found to have the sex chromosome XXY and the normal complement of autosomes. However, only a small number of cells were counted and no indication of the nature of the abnormal genitalia was reported.

Breeuwsma, (1968) reported the presence of an XXY sex chromosome ~~contribution~~ constitution in a sex chromatin-positive pig with small testes, uterus - like organ and a small penis. The testicular histology revealed complete absence of spermatogenesis but Sertoli cells were present.

Entirely different anatomical findings were reported by Gluhouschi, Bistriceanu, Rosu, and Bratu, (1968) who detected 3 pigs with 39, XXY karyotypes out of 12 intersexes whose chromosomes they studied. In every case, a vagina, uterus and oviducts were present, but the report did not make clear the macroscopic or histological appearance of the gonads. In addition the number of cells counted and analysed was not reported. No illustrations were presented and these findings await confirmation.

In domestic animals the developmental abnormalities associated with an XXY karyotype are still confused. In the cat and the sheep (Bruere, Marshall and Ward, 1969), an apparently normal male phenotype develops, but the spermatogonial population is greatly reduced, although in no case has hyalinisation of seminiferous tubules been observed, this being typical of the human condition, (Klinefelter etal, 1942). In the pig and the cow, the reports are confused and lacking in detail and until further studies

are carried out on these species, no firm conclusions can be drawn.

Intersexuality

In domestic animals, due to the small numbers of cases so far reported, the classification of intersexes is still confused, no syndromes such as testicular feminisation, (Morris, 1953) having been determined. Furthermore, there exists such a species difference in certain conditions that compartmentalisation is virtually impossible. For this reason, intersexuality in this introduction will continue to be described under the headings true hermaphrodites and pseudo-hermaphrodites. Only those conditions in which the number of cases reported make it possible to construct a picture of the abnormalities will be discussed in any detail. The classification used in this description is that adopted by Biggers and McFeely, (1966).

True Hermaphroditism.

Although true hermaphroditism has been reported in all species of domestic animals, (Bigger and McFeely, 1966), in very few cases has the genetic constitution been determined, and most of these cases in which it has been studied, the information available is unsatisfactory. In the pig, Cantwell, Johnston and Zeller, (1958) examined two cases with both ovarian and testicular tissue and found both to be sex chromatin positive. In the cat, Thuline and Norby, (1966) examined a case which was phenotypically male, with a scrotal testis and an intra-abdominal juvenile ovary finding it to be sex chromatin positive but with an XX/XY mosaicism. Similar chromosome findings were reported by Dunn, Kennet and Lein, (1968) in a bovine true hermaphrodite.

In the dog, one case has been reported, (McFeely, Hare and Biggers, 1967). Only 8 cells were examined and although they were all 78, XX no conclusion can be drawn from this study as to the chromosome complement, it being possible that a second

cell was present and which went undetected.

Thus in domestic animals, 2 species have been shown to possess XX/XY sex chromosomes, in true hermaphroditism and a third, XX, although mosaicism was possibly undetected, in this latter case. The mechanism behind the mosaicism has been suggested to be dispermic fertilization of an ovum and its polar body (Dunn etal, 1968), this being similar to that hypothesised in human cases by Zuetler, Beattie and Reisman, (1964), and by Bain and Scott, (1965). However, until many more cases have been studied, the nature of true hermaphroditism in animals remains obscure.

Pseudo Hermaphroditism

With the exception of male pseudo hermaphrodites in the pig and goat, very few cases of pseudohermaphroditism whether male with testes or female with ovaries, have been studied.

McFeely, Hare and Biggers, (1967) examined four pseudo hermaphrodites in cattle, finding one to be XY, two XX/XY and the fourth a mosaic XX/X?. In the latter case, a large acrocentric was postulated to be a re-arrangement of the second X, but positive identification was not possible. Although the anatomical details were very brief, there is reason to believe that the two XX/XY mosaics were in fact freemartins. The one case with a presumed XY constitution, in which the possibility of mosaicism could not be excluded, only 14 cells being counted, had testes and external female genitalia, thus being very similar to the situation found in the canine. In this species male pseudohermaphrodites have mainly been found to be genetically male, by the use of sex chromatin studies and chromosome analysis, (Schultz, 1962; Brown, Swanton and Brinkhaus, 1963; McFeely, Hare and Biggers, 1967, and Gerneke, 1968). However, with the exception of the presence of testes, the anatomical findings in these cases have been so variable that no typical picture has emerged. Recently, Edols and Allan, (1968) and Hare and McFeely, (1969) have

reported cases of male pseudohermaphrodites apparently with the chromosome constitution 78, XX.

In the horse, two male pseuodohermaphrodites have similarly been found to have XX sex chromosomes, (Bornstein, 1967). Both had testes and uterus, but one was phenotypically female, while the other had a penis - like organ and male behaviour.

The two species in which intersexuality has been most fully studied, the pig and the goat have a number of features in common, although anatomically they can be quite distinct. In the pig, a typical picture has emerged of the condition. Externally, the animals are essentially female, but with an enlarged elitoris. Internally, testes are present together with uterus and male accessory glands, (Makino, Sasaku, Sofuni and Ishikawa, 1962; Gerneke, 1964 and 1967), although occasionally descended testes are observed, (McFeely, Hare and Biggers, 1967). The analysis of the anatomical findings in intersex pigs ~~are~~ is complicated by the reports on animals claimed to be intersexes but lacking bisexual features, (Vogt, 1968). In the goat, the anatomical features are much more varied. Phenotypically, they can be female, (Basrur and Coubrough, 1964; Short, Hamerton, Grieves and Pollard, 1968), or male, (Short R.V. personal communication). Internally, goat intersexes are very variable, having a well developed uteri, (Basrur etal, 1964), or an intersex state, (Short etal, 1968). In some cases, testicular function appears to be reasonably well developed with meiosis proceeding, (Basrur etal) and producing testosterone, (Short etal).

The one common finding in the pig and goat is that all male pseudohermaphrodites so far found have been genetic females whether by sex chromatin analysis, (Johnston, Zeller and Cantwell, 1958), or by chromosome analysis, (Makino etal, 1962; Basrur etal, 1964; McFeely etal, 1966). One explanation for the development of male

features in genetic female goats has been put forward by Soller and Angel, (1964) who found that in breeding trials involving the gene for polledness, only females homozygous for the polled factor were affected. Eaton, (1945) had noted the association of hermaphrodites with polledness, but Soller's study further noted the homozygous nature of the condition. In Eaton's original work he had postulated a pleiomorphic effect for the hornlessness gene, this explanation being accepted by Soller and Angel. Unfortunately, in the pig, no such genetic marker exists, but a hereditary nature of the condition has been postulated, (Johnston, Zeller and Cantwell, 1958), the effects being due to an autosomal recessive gene.

Recently, two theories have been put forward in a further attempt to explain the development of testes in XX animal. Hamerton, (1968) postulated that the Y chromosome acts as a controlling centre for a structural gene or its operator which can initiate medullary stimulation and cortical inhibition, thus bringing about a male development. Hamerton further suggested that the polledness gene in the goat and a similar gene in the pig could act in a similar way to the Y controlling gene in the male. McFeely, Hare and Biggers, (1967) put forward a not dissimilar theory to explain the anomalous development of testes in XX goats and pigs. They hypothesised that the X and Y chromosome contain homologous segments and therefore the X will bear male determining genes which could be repressed or derepressed. If the Y is absent, these genes stay repressed. In the presence of the autosonal gene in the goat and the pig, the male genes on the X may be switched on to give testicular development.

Whatever the mechanism is behind the testicular development in XX animals of all species, much more work will have to be done to categorise intersexes in order that there might be some understanding of these basic mechanisms of aberrant sexual development.

Neoplasia

In domestic animals only one neoplastic condition has been found to be associated with cell lines displaying a consistent chromosomal change. Transmissible canine venereal sarcoma is a poorly understood condition which can affect both sexes. It is found on the penis and in the submucosa of the vagina, taking the form of a papular or papillary proliferation. Natural transmission is largely by coitus and requires the implantation of intact cells, (Jubb and Kennedy, 1963). Makino, (1963), studied seventeen spontaneous tumours obtained from dogs in Japan at widely separated areas and at different times and found each case to have a very similar stemline. The cells usually contained 59 chromosomes made up of 42 acrocentric chromosomes and 17 non-acrocentrics. A nearly identical cell line was found in America by Weber, Nowell and Hare, (1965) in primary sarcomas from various sites of metastases and in the transmitted tumours in both fourth and fifteenth passages. Again 59 chromosomes were found, although in this case two extra acrocentric and two less metacentrics were found. From this finding Weber and his coworkers postulated that the tumour probably had a common origin in a single dog as proposed by Makino and that it is a naturally transplanted tumour spread by coitus.

Basrur and Gilman, (1966) studied the chromosome complement of cells obtained from lymph nodes of five cases of canine lymphosarcoma. In four of the animals, alterations in chromosome numbers were detected in cells examined without resort to culture. No common abnormality, however, was found. In the peripheral blood cultures and lymph nodes cultures a modal number of 78 was found in all cases.

In lymphosarcoma in cattle, similar observations have been reported in that abnormal karyotypes have been found, but no constant picture has emerged. Basrur, Gilman and McSherry, (1964) found that in one cow an extra chromosome was present but it was not known whether the triple X cells were the progenitors of the malignant

lymphocytes or whether it was abnormal karyotype localised in the one lymph node studied. Hare, McFeely, Abt and Feierman, (1964) found that eight out of nine cattle studied had changes in the chromosomal constitution of certain of their cells. However, no constant changes were detected and four of the cases which had abnormalities in lymph node cells had normal karyotypes from periferal blood cultures. In a larger survey, Hare, Yang and McFeely, (1969) found that 34 out of 47 cases of bovine lymphosarcoma had chromosome changes in lymph node cells. However, again no constant change was detected, although in all 15 animals from which more than one node was studied, the same cell line was observed from each node. In 10 cases, one of the X chromosomes was missing, this being a neoplastic change as all the cases had normal karyotype from fibroblast cultures.

Thus in domestic animals, although chromosome abnormalities are observed, in neoplasia, with the exception of canine transmissible venereal sarcoma, no constant chromosomal feature of a tumour has yet been found.

Prenatal Mortality

In domestic animals spontaneous abortion is uncommonly observed, due to the outdoor nature of the cow and the multiparous nature of the pig. Because of this, the only sign usually seen of abortion is a return to service in the cow, or a lowered litter size in the pig. In the cow and the pig, prenatal mortality during the first half of pregnancy is estimated at about 18% and 32% respectively, (Hanly, 1961), many factors having been shown to play a part in this mortality, (Boyd, 1965).

Bomsel-Helmreich, (1961) first showed that in the pig, by delaying service a number of abnormal blastocysts with triploidy and tetraploidy were obtained. The significance of this was not realised until the work of Carr, (1963) on spontaneous abortions. McFeely, (1966 and 1967) found that by examining 10 day old blastocysts directly, after a short incubation with colcemide, good chromosomal preparations were obtained.

Seven gilts were used in the study and of 88 blastocysts collected, 9 were found to be chromosomally abnormal and a further 2 were degenerating, the cause of which could not be determined. Of the 9 abnormal blastocysts, 4 were triploid, one a diploid/triploid mosaic, 3 were tetraploid and the other one contained a deletion. McFeely suggested that as none of these abnormalities had been seen in live cattle and as Carr had seen similar defects in an abortion series, it seemed likely that some of the abnormal blastocysts would result in death probably due to the chromosomal aberration.

In the cow, McFeely, and Rajakoski, (1968) similarly found that some blastocysts probably contain chromosomal aberrations. Out of eight blastocysts examined, McFeely found that one was triploid and would probably end in embryonic death.

Thus, in embryonic death studies in domestic animals, it would appear very likely that chromosomal abnormalities should be searched for embryonic death. Using the figures of McFeely, (1967) it would be expected that approximately 28 of the 88 blastocysts would have died, (Hanly, 1962). Even assuming that the degeneration of the two blastocysts was not chromosomally induced, it could be hypothesised that the 9 blastocysts with aberrations would have died. If this had happened approximately 32% of the embryonic deaths in the series would be due to chromosomal abnormality. It must be remembered that assumption has played a part in the calculations, and the sample is small, but even allowing for this, it would seem quite clear that the chromosomal abnormality is associated with embryonic death.

Section II

Chromosome Studies in the Pig.

Peripheral Blood Culturing

Introduction

Prior to 1960, the techniques used for obtaining human metaphase chromosomes for morphological examination were of two types. Firstly, the long term culturing of biopsy material, mainly that of skin, (Tjio and Puck, 1958) and secondly the short term culture of bone-marrow by puncture methods, (Ford, Jacobs and Lajtha, 1958). Although both these methods produced metaphase spreads in reasonably adequate numbers they involved a number of disadvantages. The main one was the interference with the patient which was required to obtain the biopsy and secondly, a limitation was placed on the number of samples which could be obtained in a given time without the aid of assistants.

In 1960, a technique was developed by Moorehead, Nowell, Mellman, Battips and Hungerford which used blood as a source of potential metaphase chromosomes. This method combined a number of techniques which had been developed by other workers. It had been reported just prior to Moorehead's publication that phytohaemagglutin, which was used originally to separate white cells from whole blood, was a specific initiator of mitotic activity, (Nowell, 1960), and without this substance no division of white cells occurred. Moorehead combined this finding with the pre-treatment of mitotic cells using hypotonic solution (Hsu and Pomerat, 1953), and the air-drying method of Rothfels and Siminovitch, (1958), the latter technique aiding in providing well-spread metaphase chromosomes. The development of Moorehead's technique, coming as it did so quickly after the discovery that chromosomal abnormalities were associated with Mongolism, (Lejeune, Gautier and Turpin, 1959), Klinefelter's syndrome, (Jacobs and Strong, 1959) and Turner's syndrome, (Ford, Jones, Polani, de Almeida and Briggs 1959), provided a means whereby this renewed interest in human cytogenetics could be explored to its fullest extent. The importance of the blood culturing technique cannot be over-estimated and can be

judged by the number of chromosomal abnormalities detected with its use.

However, it was not until 1962 that the first attempts were made to obtain pig chromosomes from blood cultures. Makino, Sasaki, Sofuni and Ishikawa (1962) used a blood culturing technique involving trypsin digestion of the cells, followed by coverslip squashing, this being a modification of the method of Hungerford, Donnelly, Nowell and Beck, (1959).

McConnell, Fechheimer and Gilmore, (1963) used a technique similar to that of Moorehead etal (1960) in that Bactophytohaemagglutin was added to whole blood in order to hasten white cell separation. These workers used the same hypotonic treatment as was used in the human technique but for about 2 - 3 times longer i.e. 20 - 30 mins. Stone, (1963) although using basically the same technique made a number of modifications. Separation of the white cells was achieved by allowing the blood to stand at room temperature for 90 mins. Phytohaemagglutin was not added to whole blood but to the culture medium to which was added the supernatent plasma and cells. A similar method of separation of white cells to that of Stone, (1963) was used by McFeely and Hare, (1966). These workers used 15% Fetal Calf Serum as a substitute for autologous plasma which was used in all previous methods.

A number of papers were published in the following years utilising peripheral blood cultures, (Henricson and Backstrom 1964; and Hard and Eisen, 1965), but details of the techniques were not reported.

An entirely different method of innoculating white cells into tissue culture medium was reported by McFee, Banner and Rary, (1966) who used a technique originally developed for the growth of sheep chromosomes. In this, they innoculated whole blood into the medium and found it to be entirely satisfactory. However, Srivastava and Lasley, (1968) were unable to obtain chromosomes with this method as mats of blood were formed when whole blood was used as the innoculum. A further method has been used to obtain chromosome spreads (Breeuwsma, 1968), using a microtechnique involving the innoculation

f small amounts of whole blood into tissue culture medium, this being a modification f Arakaki and Sparkes, (1963).

rom a study of the literature, therefore, it appears that no standard method of ulturing pig lymphocytes has evolved and virtually everybody who carries out this ork has a different technique for obtaining chromosomes.

terials and Method

llection of Blood

enous blood collected from either the Anterior Vena Cava or an ear vein was used throughout the study. The former was used under two circumstances. Firstly, at ie Glasgow Slaughter House, in all ages of pigs, after electrical stunning, the vena ava was sectioned. Secondly, in young pigs which were under 100 lb. in weight, the echnique of Dr. P. Imlah of the Department of Animal Health, University of Edinburgh, as used. The pigs were placed on their backs, the site of insertion sterilised with lcohol and a 20G, 2 inch needle attached to a 20 ml. sterile syringe was pushed through ie skin at a point about 1 inch to the right of the sternum and directed downwards, awards and backwards to the full extent of its length. Slight negative pressure was pplied by slightly withdrawing the barrel of the syringe and the needle was then radually pulled back along its original path, until blood was seen to flow into the arrel. 20 ml. of blood was collected as quickly as possible, without causing frothing nd immediately emptied into a sterile 1 oz. Universal bottle containing 1000 inter-ational units, (1000 i.u.) of Heparin (Boots Pure Drug Co. Ltd.).

n pigs over about 100 lb. in weight, blood was obtained from the large ear veins. he pig was securely restrained in a standing position and a suitable ear vein chosen. he area over the vein was cleaned with alcohol and allowed to dry before the underside f the ear was heated with warm water in order to dilate the vein. An elastic band as placed round the base of the ear and once the veins were sufficiently dilated, a 0G, 1½ inch needle on a 20 ml. syringe was inserted below the skin over the vein and hen pushed in the direction of the heart until the needle was securely in place. light negative pressure was applied with the syringe until 20 ml. of blood had been ollected. This was then emptied into a sterile Universal bottle containing 1000 i.u. f Heparin.

eparin containing 1000 i.u./ml. (Boots Pure Drug Co. Ltd.) was used throughout
ıe study. The amount used was varied in a number of trial cultures. In equal
ımbers of cultures, 0.5 ml, 1.0 ml. and 2.0 ml. of Heparin / 20 ml. of blood was used,
.ving final concentrations of 25,50 and 100 i.u. Heparin per ml. of blood, respectively.
ıe lowest concentration was found to result in a higher frequency of culture clotting
_vitro whereas the highest concentration gave poorer growth rates. 50 units/ml.
ıole blood collected gave adequate growth rates and also eliminated clotting of the
.ltures except in those cases in which the amount of blood in the plasma layer was
.cessively high.

paration of Blood

was found early in the study that the use of whole blood as a source of lymphocytes
s not feasible as whole blood added to cultures containing Phytohaemagglutinin
sulted in large blood clots being formed within 12 hours, independent of the amount
heparin used. It was found necessary, therefore, to separate white cells from the
d cells. Three methods were used to bring about the separation.

(1) The addition of 1 ml. Phytohaemagglutinin (Burroughs Welcome Ltd.) to 20 ml. of whole blood with subsequent refrigeration for 1 hour before spinning, resulted in very good separation of three layers. The top layer consisted mainly of cell-free plasma, the middle layer was a buffy coat and the bottom layer agglutinated red cells. However it was found difficult to draw off the buffy coat without disturbing the red-cell layer and as the plasma layer was almost free of white cells the fluid which was removed had a very low cell count. This tended to result in very low numbers of lymphocytes at the end of the culture period.

(2) Immediately after collection, the blood was spun at about 750 r.p.m. for 5 minutes. At the end of this time the blood was examined and if sedimentation had occurred sufficiently to allow a red-cell-free supernatant to form, the latter was

removed together with as much buffy coat as was possible without disturbing the red cells. In most cases however, it was necessary to respin the blood to obtain adequate separation of red cells and white-cell-rich supernatant.

(3) Much better separation was obtained if, as soon after collection as possible, the blood was placed in a refrigerator at $+4^{\circ}C$ for about 1 hour. At the end of this period, the majority of blood samples separated out into the required layers after only 5 mins. spinning at 750 r.p.m. and it was this latter method of separation of white cells which was adopted for routine use in the study.

White Cell Concentration

After the separation of the white cells in plasma, a cell count was carried out on the sample to determine the range within which adequate cell growth occurred. Sufficient numbers of ~~divided~~ dividing lymphocytes were found in cultures to which had been added white cells at a final concentration of from 0.2×10^6 cells to 1.8×10^6 white cells/ml. culture. It was found however that outwith these extremes, cell growth was less than adequate. If the count was less than the lower extreme, there were too few cells present to supply adequate numbers of metaphases and if the concentration was above 1.8×10^6 cells/ml. of culture, there appeared to be an inhibitory effect on the dividing cells.

In the vast majority of blood samples taken and subsequently separated, the addition of 1 ml. of white-cell-rich plasma gave a final concentration of white cells within the range required for adequate growth.

Plasma Concentration

To determine the amount of plasma or serum required in the culture medium, multiple culture trials were carried out. Autologous plasma produced by centrifuging whole blood after removal of white-cell-rich supernatant, foetal calf serum (Flow laboratories)

ıd autologous serum, produced by allowing whole blood to clot, storing overnight
: +4°C and subsequently centrifuging the sample, were used in the trial. To identical
ıltures without serum the three materials were added to give final concentrations of
ıch of 10%, 20% and 25%. A subjective judgement based on mitotic index was carried
ıt and it was found that the poorest growth rate was obtained using foetal calf serum
ıd that the results obtained with autologous plasma and autologous serum were equally
›od. Because of the difficulty in obtaining large enough samples of whole blood by
enepuncture of ear veins to give adequate amounts of serum as well as white-cell-rich
.asma, it was decided to concentrate on using autologous plasma.

ırther multiple cultures were set up using final plasma concentrations of 0%, 5%,
)%, 15%, 20%, 25%, 30%. Efficiency of these amounts was again judged by a visual
›praisal of mitotic index. It was found that using 0% and 5% plasma the growth rates
ere very poor. Best growth rates were obtained using 10%, 15% and 20%, with 15%
ing the optimum concentration. Growth rate was poorer when concentrations of over
)% were used. A final concentration of 15% plasma in cultures was used routinely, as
ıe amount of plasma obtained from a 20 ml. sample of whole blood averaged about 6 ml.
'ter removal of white cells, and using this amount four cultures could be set up
›nveniently.

.lture Medium

›o culture media were used during the study. The original medium used was T.C. 199
'laxo Ltd.) containing both bicarbonate and antibiotics. Growth rates were reasonably
tisfactory but were not consistently good. Weymouth's Medium (Burroughs Wellcome
d.), was then used and in comparative trials between this and T.C. 199, more consistently
tisfactory growth rates were obtained with Weymouth's medium, the latter being adopted
the medium for use.

is was made according to the manufacturer's instructions. Weymouth's medium M.B. 752/1,

the form of a 10 x concentrate, was divided into 10 ml. aliquots in sterile 1 oz. iversal bottles and stored at -10°C. until required for use. When required this ɔunt was thawed and added to 90 ml. of sterile de-ionised water in a 250 ml. medical at. To this was added 5 mls. of sterile 4.4% Sodium Bicarbonate, (Wellcome T.C.) which was also stored at -10°C. in sterile 6 ml. bijoux bottles. Antibiotics re added in the form of crystamycin (Glaxo Ltd.). One vial of crystamycin containg 500,000 units of penicillin G. and 0.5 gms. streptomycin base was dissolved in ml. sterile Hanks solution, (Burroughs Wellcome Ltd.) and distributed in 0.5 ml. ɔunts in bijoux bottles and stored at - 10°C. 0.5 ml. of the antibiotics was added the medium to give final concentration of 250 units Penicillin G. and 0.25 mg. reptomycin/ml. of complete medium.

tohaemagglutinin

this study, dried Phytohaemagglutinin, (Burroughs Wellcome Ltd.) in vials was used. s was reconstituted with 5 ml. sterile Hanks solution and stored at +4°C until dy for use. Multiple cultures were prepared and to these, Phytohaemagglutinin was ed, the amounts being 0 ml. 0.1 ml. 0.2 ml. 0.3 ml. 0.4 ml. 0.5 ml. No growth obtained in those cultures to which had been added none or 0.1 ml. Adequate wth was obtained using 0.2 ml., even better results being obtained with 0.3 ml. and ml. Although no counts were done, the impression was gained that a slight decrease growth occurred with the use of 0.5 ml. phytohaemagglutinin. However, it was found t by using 0.2 ml, clotting occurred less frequently ~~that~~ than with higher concentrations for this reason 0.2 ml was added routinely to the cultures.

gth of Culture Period

liminarly adopting a fixed time of colchicine treatment of 2.5 hours and a concentration ug/ml culture incubation times, before the addition of colchicine, of 68, 69, 70, 72 and 73 hours were used to find the optimum incubation time, all being carried out

at 37°C. In those cultures to which the colchicine was added from 68 to 70 hours, the chromosome spreads included a higher percentage of early metaphase spreads than was seen in the 71 and 72 hour cultures. As might be expected in the 73 hour cultures, later metaphase spreads, which appeared more contracted and therefore more difficult to analyse, were seen with a higher frequency and therefore it was judged that 73 hour cultures were too long. From the results it was decided that 71 hours was the optimum time for colchicine to be added, and 2.5 hours colchicine treatment was also considered to be satisfactory.

Colchicine Treatment

Colcemid (Deacetylmethylcolchicine, C.I.B.A. Laboratories Ltd.) was used. 80 mgs. of pure substance was dissolved in 100 mls. of sterile Hanks and stored in 10 mls. aliquots at -10°C. 10 mls. of the stock solution was diluted with 90 mls. sterile Hanks, and this was stored in 1 ml. aliquots at -10°C. The working solution contained 80 ug/ml and to each 10 ml culture was added 0.2 ml giving a final concentration of 1.6 ug/ml. culture. The amount added was kept constant and the length of time for which the colcemid was in contact with the growing cultures was varied.

After 71 hours of culturing, colchicine was added for 1.5, 2.0, 2.5, 3.0 and 3.5 hours to multiple cultures. After these periods, the cultures were harvested normally. Examination of the slides prepared, revealed that a higher frequency of cells which had had colcemid added for 3 or 3.5 hours were excessively contracted when compared with those from the shorter period cultures. Conversely the 1.5 and 2.0 hour cultures tended to have a higher proportion of excessively stretched chromosomes and thus it was decided to leave the colcemid in contact with the growing cells for a 2.5 hour period.

Hypotonic Treatment

At the end of the colcemid treatment time, i.e. a total of 73.5 hours, the culture medium was transferred to pre-warmed (37°C) 10 mls. centrifuge tubes for the subsequent

procedure. The cultures were centrifuged for 5 minutes at 1000 r.p.m. after which time the supernatant medium was pipetted off gently, leaving only a button of cells and about 1 ml. of medium. 9 ml of the prewarmed 37°C hypotonic solution was gently added to the button and this broken up by gentle pipetting. The pipetting was continued for 5 minutes to break up any aggregations of cells. At the end of the 5 minutes, stoppers were placed in the tubes and the cultures left, without disturbing, for the rest of the hypotonic treatment in a water bath at 37°C.

The hypotonic solution used was aqueous sodium citrate. Trials were run using different combinations of hypotonic concentrations and times. The concentrations used were 0.1, 0.3, 0.5, 0.7, 0.9, and 1.1%, the times being 15, 20, 25, 30 minutes. The combination which gave the best-spread chromosomes was 0.3% sodium citrate for 25 minutes. It was found that using either stronger solutions or shorter times, more contracted metaphases were seen, in which it was more difficult to determine the exact morphology of the chromosomes. Using 0.1% citrate tended to give higher frequency of broken cells in which chromosomes were lost, even when used for shorter periods.

Fixation

At the end of the hypotonic treatment, the cultures were centrifuged for 5 minutes at 1,000 r.p.m. and the supernatent pipetted off, leaving only the cell button and as little hypotonic solution as possible. The fixative used was Absolute Alcohol and Acetic Acid, made up in the ratio of 3 parts: 1 part. This was made up freshly for each culture and cooled down to +4°C before use. Three mls. of the fixative was gently poured down the side of the centrifuge tube and the cell button gently broken up with the tip of a pipette. The tubes were then replaced in the refrigerator at +4°C for 15 minutes, at the end of which time they were re-centrifuged for 5 mins. at 1,000 r.p.m. The supernatent was again pipetted off down to the button and 1 ml. of fixative poured on to the cells. The cells were resuspended in the fresh fixative

and left at +4°C for a minimum of 20 minutes. This latter stage was in some cases extended to 12 hours without any noticeable detrimental effect.

Prior to a third change of fixative, glass microscope slides which were stored in 1% chromic acid, were placed in a beaker and washed thoroughly in fast cold running water for about 30 minutes. After the second fixation was completed the cultures were again centrifuged, the supernatant removed and the cells resuspended in 0.5 ml. of fixative. About 0.2 ml. of the suspended cells was drawn up into the 5 inch stem of a Pasteur Pipette. Holding a thoroughly clean glass slide at an angle of about 20° to the horizontal, the cells were dropped on to the slides from a height of about 18 inches. The slides were then dried by vigorously waving them over the flame of a Bunsen Burner. They were then placed on a hot plate to finish the drying process.

Staining

2% Orcein in 65% Acetic acid was used for staining the chromosomes. The method of preparing this stain was that of La Cour (1941) with the modification that 2% Orcein was used.

The staining procedure was as follows:-

2% Aceto Orcein	60 mins.
50% Acetic Acid	30 secs.
70% Alcohol	30 secs.
95% Alcohol	30 secs.
Absolute Alcohol	60 secs. 1 change.
Xylene	30 secs.
Xylene	5 mins. or upwards

The slides were then permanently mounted using DePeX (Gurr) and allowed to dry before examination.

Chromosome Number

Introduction

Although it is now accepted that the modal number of chromosomes of the domestic pig (Sus scrofa) is 38, it is only since 1962 that the examination of well-spread chromosomes of good morphology has resulted in accuracy of counting and karyotyping.

The early estimates of chromosome numbers were carried out using gonadal tissue and the chromosomes obtained from the preparations were of a poor standard. The difficulty of obtaining accurate estimates of the chromosome number ~~are~~ is shown by the wide variation in results obtained by various workers. Wodsedalek (1913) reported that the pig modal number was 18 in the male and 20 in the female, whereas Hance (1917) found the number to range from 40 - 58. In both these reports the drawings of the chromosomes showed that they were so condensed that the individual structures were almost unidentifiable. In 1933, Bryden determined the chromosomal number to be 38, but using the same technique Makino (1944) disputed this and stated that the pig had 40 chromosomes. Makino further suggested that the inaccurate results obtained previously were the result of poor and insufficient fixation.

However, Sachs (1954) in a study of the Old Swedish Breed found that 40 chromosomes were present in testicular tissue, although, as in previous work, the preparations were very poor and chromosomal structure was very indistinct. In 1951, Melander, who also studied the Old Swedish Breed, found the chromosomal number to be 30, whereas in seven other species the number was 38. Further confusion was caused by Spalding and Berry (1956) who obtained a modal number of 40. In all these studies, the preparations obtained were such that any degree of accuracy of counting was virtually impossible and it was remarkable that most of the findings, in retrospect, were so near the now accepted value of 38. As stated before, Makino (1944) was of the opinion that the main difficulty encountered was that of inadequate fixation. With the advent of the

odern technique of hypotonic treatment, it also seems likely that the condensation f the chromosomes was partly due to the lack of hypotonic pre-treatment with its esulting spreading action.

he inadequency of the preparations, which had been used prior to 1961, was high-ighted by the report of Ruddle, (1961) which displayed metaphase chromosomes obtained y tissue culturing of kidney cells using colchicine pre-treatment and hypotonic olutions. In this report, Ruddle found that the chromosome number in the pig was 8, the illustrations of the chromosomes showing the great improvement in chromosome orphology obtained by using a tissue culturing technique. For the first time, an ttempt could be made to arrange the chromosomes in homologous pairs with any degree f confidence. Ruddle's arrangement consisted of 8 groups based on morphology, no ttempt being apparently made to use length as a criterion, except within individual roups. As this report was of great importance, in that for the first time the tructure of pig chromosomes could be determined, it is worth describing the individual eatures of the chromosomes in some detail.

roup 1 This group consisted of a single pair of submetacentric chromosomes, being the longest in the complement.

roup 2 A single medium-small submetacentric pair displaying prominent secondary constrictions.

roup 3 Two pairs of large chromosomes with subacrocentric structure which were clearly separable from the remainder of the karyotype by their centromeric position.

roup 4 This was the largest group of chromosomes consisting of six pairs of metacentric and submetacentric chromosomes of varying size, but excluding 2 pairs of small metacentric chromosomes and the Y. However, Ruddle stated that the X chromosome also belonged to the group, concluding that

the female diploid number of the group was thirteen. However, as the illustration of the normal karyotype was that of a male cell, it must be assumed that the statement was a misprint. If this is not the case, Ruddle was apparently claiming that the sex determining mechanism in the female was XO, a most unlikely statement in view of the knowledge at that time.

Group 5 The remaining metacentric chromosomes, excluding the Y, were placed in this group. It thus consisted of two pairs of small metacentric chromosomes which were separable from group 4, on length.

Group 6 This group consisted of a single pair of long acrocentric chromosomes.

Group 7 Two pairs of medium sized acrocentric chromosomes.

Group 8 Three pairs of small acrocentrics.

Y Chromosome. The Y was identified as being a very small, slightly submetacentric chromosome.

As can be seen from this description, Ruddle did not attempt to positively identify any more than four chromosomes, the remainder being placed in groups and only arranged on a length basis within these.

Makino, Sasaki, Sofuni and Ishikawa (1962), using a peripheral blood culturing technique also found the diploid number in the pig to be 38, finding 36 out of 40 cells to have this number. In contrast to Ruddle, (1961), Makino and his co-workers numbered each chromosome individually, although giving no detailed description of the chromosomes with the exception of the X, which was described as a medium-sized submetacentric, and the Y, the smallest of the complement, being metacentric. As far as can be gleaned from the small photographs presented, the karyotypes of both Ruddle and Makino were composed of chromosomes with the same structures, although the arrangements were entirely different. Nevertheless, all the chromosomes which in Ruddle's report had a distinctive appearance were observed to be present in Makino's publication.

he first attempt to assign numerical values to individual chromosomes was arried out by McConnell, Fechheimer and Gilmore, (1963). In this study, these orkers, who also found that 38 was the modal number, measured the chromosomes from cells and from the results constructed an idiogram. From this, McConnell oncluded that most of the chromosomes could be identified with reliability on the asis of size and centromeric position. As in the case of the previous two reports, ifficulty was encountered in attempting to identify individual chromosomes in cConnell's illustrations due to the small size of the photographs. However, as efore, all the chromosomes which were individually identifiable were found to be resent and comparable to those reported by Ruddle, (1961), Makino etal (1962).

ince the first three reports on the chromosomal constitution of the domestic pig, any reports have been published confirming 38 as being the modal number, (Stone, 1963; erneke, 1964; Henricson and Backstron, 1964; Hard and Eisen, 1965; McFee, Knight nd Banner, 1966; Gerneke, 1967; Vogt, 1968; Bruere, Fielden and Hutchings, 1968). owever, all these reports were involved with individual cases and in only two reports ave numerous animals been examined, such that an estimate could be made as to the ercentage frequency with which the modal number is found. Similarly, in most reports, he distribution of chromosome counts in aneuploid cells have been virtually ignored.

cConnell, Fechheimer and Gilmore, (1963) studied six pigs and found that out of 152 ells, 131 or 86% contained the modal number of 38. Of the remaining cells, the ajority were ranged about the mean, varying from less than 37 to greater than 39. A arger survey was carried out by Srivastava and Lasley, (1968) who examined 690 cells om 6 animals and found that 88.5% contained 38 chromosomes. The aneuploid cells re found to have a much smaller scatter than that found by McConnell etal, (1963) that all non-modal cells contained either 37 or 39 chromosomes.

us, although 38 is now accepted as the modal number for domestic pigs, very little formation is available concerning the range of chromosome counts found in normal imals. Unless these facts are determined, animals with mosaicism may well be

ndetected and thus potential valuable experimental material lost. One exception
o the diploid number in pigs was a study on the chromosomes of the European Wild
ig by McFee, Banner and Rary, (1966). It was found that in thirty six wild pigs
tudied, the diploid number was either 36 or 37, no cases possessing 38 chromosomes.
nalysis of cells containing 36 chromosomes showed that two pairs of acrocentrics
ound in the domestic pig were missing and an extra single large pair of submetacentric
hromosomes was present. Cells with 37 chromosomes were found to be lacking a single
arge acrocentric and a single small acrocentric, whereas one large submetacentric
hromosome not seen in domestic pigs was present. McFee postulated that "pure" wild
igs contained 36 chromosomes and from these, derived the domestic pig by a mechanism of
entric splitting of the large submetacentrics. He further suggested that animals
ith 37 chromosomes were intermediate in this evolutionary process, or that they arose
rom cross-breeding of wild and domestic pigs, McFee having found that crosses of 38 x
6 pigs could result in pigs with 37 chromosomes.

cFee and Banner (1969) carried out large-scale breeding trials between animals with
ll combinations of chromosome numbers and found that the chromosome complement in
rogeny of matings between parents with 36, 37 or 38 chromosomes could be predicted on
Mendelian ratio basis. From their work, McFee and Banner found that offspring
ith 37 chromosomes were obtained from matings of 36 x 37, 36 x 38, 37 x 37, and 37 x
8. Moreover, offspring with 38 chromosomes were obtained from 37 x 37 crosses, so
t appears likely that if only one animal underwent a centric split of a large sub-
etacentric, this could eventuate in animals with 38 chromosomes, the complement in
omestic pigs. However, the hypothesis is as yet unproven.

eports such as those of Henricson and Backstrom, (1964); McFee etal (1966) and McFee
nd Banner, (1969) stress the need for standardisation of karyotypes in the pig.
enricson and Backstrom, (1964) studied the chromosome morphology of a boar with reduced
ertility and found that a translocation was present between a large acrocentric and

small metacentric chromosome. However, although in that case and those of the ild pigs, the chromosomes involved were reasonably easily identified, the numbers ssigned to the affected chromosomes in McFee's cases bore no comparison to the ones ssigned to the corresponding chromosomes of Henricson and Backstrom.

imilar difficulty is encountered in attempting to identify chromosomes in reports y different workers. As yet, no standard karyotype has been worked out, yet there re enough identifiable chromosomes in the pig karyotype to form the basis of a standard. ll workers previously quoted as publishing karyotypes have numbered the largest hromosome in the set as number 1. Six acrocentrics have been consistently found, omprising three large pairs and three smaller pairs. However, McFee, Knight and anner, (1966) have grouped all these chromosomes together, whereas McConnell, echheimer and Gilmore, (1963) and Henricson and Backstrom, (1964) separated the two roups, placing the larger set immediately after chromosome 1 and the smaller set at he very end of the complement. In a similar fashion, virtually all pig chromosomes ave been placed in variable positions by different workers, and it would seem that, t a time when many reports on pig chromosomes are being published, a standard arrange- ent is long overdue.

aterials and Methods

hirty five animals, twenty females and fifteen males were examined cytogenetically. hese were selected at random at the Glasgow Slaughter House or at the Veterinary ospital, each animal selected being examined for any phenotypic abnormality or sign f disease. All thirty five appeared normal at inspection.

ach slide was scanned using a X10 objective and a X10 eye piece. Cells which ppeared well spread and unbroken were selected and then examined at X1250. All cells riginally selected at X100 were counted at the highest magnification, the chromosome

content of each cell being counted repeatedly until the same score was achieved twice in succession. The position of the cell on the slide was recorded by its vernier reading and the number of chromosomes in the cell similarly noted. In cells which were found to be aneuploid, attempts were made to identify the chromosomes which were lacking or extra. Both modal and aneuploid cells which were particularly good, were photographed and karyotyped.

The results of the chromosome counts from each animal were analysed, the numerical distribution being calculated together with the percentage spread.

Results

From the thirty five animals studied a total of 1758 cells were counted, tables 1 and 1a showing the distribution of the cell counts. The vast majority (85,32%) contained 38 chromosomes, this being the modal number for the species. Of the 14,68% aneuploid cells, 10,24% contained either 37 or 39 chromosomes. In individual animals the percentage of cells containing the modal number ranged from 73,7% to 100%, twenty five pigs having a modal frequency greater than 80%. The range within which the frequencies of aneuploid cells fell is also presented in table 1.

In 120 cells it was possible to identify the aberrant chromosome in aneuploid cells containing 37 or 39 chromosomes. In 87 of the cells an acrocentric chromosome was involved, being a member of the 17 - 18 group in 69 cells. In the remaining 33 aneuploid cells not involving an acrocentric, no specific chromosomes were involved, it appearing that a random mechanism had led to the development of aneuploidy.

Discussion

This study has shown conclusively that the modal number of chromosomes in the pig is 38, this number being found in the majority of cells in every one of the thirty-five pigs studied. The results obtained have confirmed the findings of Ruddle, (1961),

Makino, Sasaki, Sofuni and Ishikawa, (1962) and McConnell, Fechheimer and Gilmore, (1963), these workers being the first to determine diploid number for the species using the newly developed techniques of colchicine and hypotonic treatment of tissue culture material. As stated previously, this value was first obtained many years ago by workers including Krallinger, (1931), Bryden, (1933) and Crew and Koller, (1939). However, as these reports were concerned with germinal tissue, which was fixed and sectioned in a normal histological manner, the resulting preparations were very condensed and the chromosomal structure very indistinct. This was probably due to poor fixation, lack of hypotonic treatment and the fact that as variable section thicknesses were used, the chromosomes were very liable to be cut through resulting in variable counts as noted by Bryden, (1933). Furthermore, using the same tissues other workers obtained results which varied markedly from 38. Wodsedalek, (1913) found the diploid number in the male to be 18 and in the female 20, whereas Hance, (1917) reported the value to vary from 40 - 58. A diploid number of 40 was reported by Hance, (1917), Makino (1944), Sachs, (1954) and Spalding and Berry, (1956), all these workers using germinal material. These wide variations in findings, together with the poor quality of preparations, tend to give very little weight to the results obtained by the older techniques and for this reason it is considered valid to discuss only the findings obtained by the recently developed techniques.

Although there can be no doubts concerning the modal number for the pig, the majority of studies on the pig chromosomes have been carried out on abnormal animals and as such these cannot be considered a representative sample of the species. Nevertheless, the frequency of cells with the modal number has been remarkably constant over many of the studies, Gerneke, (1964), Hard and Eisen, (1965), Vogt (1968) and Bruere, Fielden and Hutchings, (1968) all finding a frequency of modal cells of between 90% and 96%. However, the majority of reports failed to present distributions of aneuploid cells and in one case no distribution of any description was presented (Henricson and Backstrom, 1964). In one further case, McFee, Knight and Banner, (1966) found only

73% of cells to contain 38 chromosomes, stating the frequency with 37 or 39 chromosomes was higher than found in normal pigs, but presented no details. This latter paper partly explains the reasons for criticism of the above papers and also why their findings cannot be used in comparison with the present results. Firstly, in phenotypically abnormal animals, there is always the possibility of mosaicism which may lead to counts which are not representative of the normal member of the species. In this respect McFee found only 73% of cells to contain the modal number which is markedly lower than the other frequencies reported in phenotypically abnormal animals. Secondly, McFee failed to present the aneuploidy frequency and made no attempt to analyse these aneuploid cells to determine if any constant aberration was present which might represent a second cell line. Similarly, Henricson and Backstrom 1964), Vogt (1968) and Bruere, Fielden and Hutchings, (1968) failed either to present detailed results or to carry out analysis of aneuploid cells. For these reasons, although in most cases there was very close relationship between the modal frequencies, no direct comparison can be made between these results and those of present study.

Two studies have been carried out which examined the distribution of chromosome counts in cells obtained by peripheral blood culture. The smaller of these (McConnell, Sechheimer and Gilmore, 1963) examined six animals, a total of 152 cells being counted. The results of this show a close relationship with the present study. 86.2% of cells were found to contain the modal number of chromosomes, compared with 85.3% in the present study. Similarly, the distribution of aneuploid cells ~~were~~ was very similar, in that McConnell found 4% and 3.3% of cells to contain 37 and 39 chromosomes respectively compared with 5.8% and 4.4%. A larger study was made by Srivastava and Lasley, (1968), who counted 690 cells from 6 animals. Their results showed that the percentages of cells with 37, 38 and 39 chromosomes were 7.2%, 88.5. and 4.3% respectively. Thus in the three studies so far carried out, very close correlation has been found in that from 85.3% to 88.5% of cells contain the modal number and the majority of aneuploid

cells contain either 37 or 39 chromosomes.

The reasons for aneuploid cells being present with peripheral blood culturing can only be a matter of conjecture. The simplest explanation for the loss of chromosomes is that during processing, cells become damaged and in this way chromosomes are lost. This would certainly appear to be the case in some cells, as occasionally they were found to be obviously broken and to contain less than 30 chromosomes. However, this cannot explain the presence of extra chromosomes and the tetraploid cells occasionally seen. Tjio, Puck and Robinson, (1959), in a study of human marrow cells, found that over 99% cells contained the modal number of chromosomes. As this technique involved both hypotonic treatment and a certain amount of agitation of cells, the only difference in the procedure between that technique and that of peripheral blood culturing is the actual culturing period.

It therefore seems not unlikely that divisional errors could occur during in vitro culturing, this leading to aneuploidy. The fact that the majority of the chromosomes which were involved in aneuploidy were small acrocentrics suggests that the loss or gain of chromosomes is not a random process. It could be postulated, therefore, that a mechanism such as anaphase lag or mitotic non-disjunction, if involving a certain chromosome, would result in one daughter cell containing one chromosome less and the other, one extra. If this is the case, this would explain the almost equal number of cells containing 37 and 39 chromosomes. As very little is known about the order in which chromosomes segregate in the cell at metaphase, it is not possible to confirm this theory, but if studies were to show that the small acrocentrics tend to lag behind the others at anaphase, this theory of selective aberrant distribution of the acrocentrics might be substantiated.

ig Karyotype

ntroduction

ne arrangement of the karyotype which was finally adopted was that which most closely llowed the recommendation of the Denver Report (1960) on human chromosomes, the ain guide lines of which were that the chromosomes should be serially numbered as early as possible in descending order of length and be classified into groups between nich, identification could be made relatively easily.

llowing these criteria, six groups of chromosomes were formed, (Figs. 1, 1a and 2). s is done with the human karyotype, each of the groups was allocated a letter from to F, the individual chromosome pairs being numbered 1 - 18 and the sex chromosomes ot being numbered but labelled X or Y.

ne morphological description of metaphase chromosomes as being acrocentric, subacro-entric, submetacentric and metacentric, (Wilson 1928), a system which is generally ccepted at present, has been criticised by Levan, Fredga and Sandberg, (1964). These uthors suggested an alternative system of nomenclature which although more specific, eant that, if it was adopted, two methods of description would exist and would result n greater confusion that at present. Because of this, it was decided to continue to escribe the pig chromosomes in the generally accepted manner.

esults

roup A.

his group consisted of a single pair of submetacentric chromosomes designated number 1. ; was by far the largest chromosome of the set, being easily distinguished by this eature. It was noted occasionally that polymorphism existed in this pair, in that ne member of the pair was markedly smaller than its homologue, (Fig. 3). It is rprising that this difference has not been recorded previously as the difference in ngth was in some cases as long as 25%. Measurements were carried out on 120 randomly

selected cells from 15 animals in order to estimate the frequency at which a detectable difference was present between members of the pair. Any pairs which showed a difference which was visually noticeable were measured using dividers and a ruler marked in millimetres. A total of 14 cells ~~were~~ was found to contain chromosomes number 1 which differed in length by an amount in excess of 10%. This polymorphism was found not to be confined to only a small number of animals, as the 14 cells were distributed among 8 of the 15 pigs studied.

Group B

It was decided to separate this group of acrocentric chromosomes into two subgroups consisting of the largest pair, number 2, and the two smaller pairs, numbers 3 and 4. Chromosome pair number 2 was invariably found to be longer than the others, and, as such, could be identified. The other four chromosomes were found to be more variable in size and it was not considered possible to separate them into distinct pairs. In many cases, it was found that two distinct pairs were indeed formed, but in others this separation was impossible. Because of this, it was decided to continue if possible to number the larger pair, 3, and the small pair 4, but to adopt the system of connecting the two by a line, signifying the inability to distinguish them.

In most cases, a short arm was not visible on the members of group B, thus raising doubts about its presence and therefore about its nomenclature as an acrocentric chromosome. However, a definite short arm was identified in a sufficient number of cases to remove the doubts. The most obvious reason for the apparent absence of the short arm was that when the cells were dropped on the slides, the short arm became situated under the centromere and appeared to be absent. In many cases in which its presence was in doubt, the centromeric area appeared more darkly stained, suggesting that overlapping of the short arm had occurred. There can

ertainly be no doubt that the members of group 2 do possess short arms and thus it s not valid to describe them as telocentric chromosomes.

roup C

he four members of group C had a structure tending towards a subacrocentric configration and as such were morphologically distinguishable from the members of group D. t was considered that although a degree of overlapping of lengths existed between hese groups, it was valid to separate them on centromeric position. Group C, therefore, consisted of 4 pairs of homologues, 2 of which, numbers 5 and 9, were decidedly ore acrocentric than the remaining members and were themselves easily separated by ength. Similarly, the other two pairs, numbers 6 and 7 could be separated on the asis of length, both having approximately the same centromeric position. Thus, the ndividual members of Group C were easily separable from the other members of the group nd were also, as a group, distinguishable from the other groups in the set. It was herefore valid to specifically number each member of the group, the arrangement being n the basis of length, the longest being number 5 and the shortest number 8.

roup D

roup D consisted of 10 chromosomes in the female and 9 in the male. From this finding t was obvious that the group included the X chromosomes.

he identification of the X chromosomes in the pig has been a matter of considerable ontroversy ever since methods were evolved which resulted in chromosomes of adequate uality for accurate analysis. By necessity, the identification of the X is carried ut by a process of elimination. By this process, it has been found that in both exes, four pairs of chromosomes could be identified on the basis of their centromeric osition, all being submetacentric, individual identification of pairs being impossible sing that criterion alone. However, if separation was attempted, based on length, dentification was much more feasible. By this method, one pair (number 9) was learly longer than the other members of the group, this being a consistent finding in

satisfactory preparations. In no such preparations was there any doubt as to the identity of the largest pair. Similarly, one pair (number 12) was distinctly smaller than any other member of the group and again positive identification was possible in the vast majority of good spreads. The remaining two pairs of submetacentrics in this group were less easily separated, but in the majority of cases, it was found that there were two larger and two smaller chromosomes and on this basis, it was considered valid to number these, 10 and 11, the four chromosomes making up the two pairs being intermediate in size between pairs 9 and 12.

As the morphology of these 8 chromosomes in group D was found to be standard, it was assumed that the remaining one or two chromosomes in male and female cells respectively were X chromosomes. The morphology of these chromosomes was found to be quite distinct from the rest of the group. In comparison with the autosomes, the centromeric position of the X was found to be more metacentric, in some cases appearing to be exactly metacentric. On this basis, it was found that the X chromosome could be quite definitely distinguished from the remainder of Group D. More variation was found, however, when positioning of the X was attempted. In most cases, the length of the X was found to be midway between pairs 10 and 11. However, occasionally this varied, sometimes being larger than 10 and on other occasions smaller than 11. Nevertheless, it was considered quite feasible to place the X between numbers 10 and 11, and by keeping this order standard, less confusion would arise.

Thus Group D consists, in both sexes, of 4 pairs of submetacentric autosomes and either one or two nearly metacentric chromosomes, depending on the sex, which were identified as the X chromosomes, on the basis of length being midway between chromosome pairs 10 and 11.

Group E Chromosome 13 - 15 and Y.

Group E consists of the remaining small non-acrocentric chromosomes in the complement,

totalling six in the female and seven in the male. Thus included in the group was the Y chromosome. As well as being distinguishable from Group D on the basis of size, Group E contained mainly metacentric chromosomes, by virtue of whose morphology it can be separated as a group. Within this, identification of one pair and the extra male chromosome was easily achieved, but the separation of the other two pairs of autosomes was more difficult.

The largest pair in the group, number 13, was easily identified by both its size and the long constriction which it bore on the proximal portion of the short arm. As the constriction was consistently found on both members of the pair, no difficulty in identification was met. However, the exact morphology of number 13 was difficult to determine because of the varying length of the constriction, but its appearance in the majority of cells was that of a metacentric.

The single chromosome found only in male cells again presented no difficulty in identification, as it was the smallest chromosome in the set and as such, it was impossible to confuse it with any other chromosome. Morphologically, the Y had the appearance of a submetacentric chromosome, although in some cases, due to its size, the definition of its centromere was indistinct and difficulty was met in determining its exact morphology.

The remaining two pairs of the group, members 14 and 15, were situated in size between pair 13 and the Y. Both were very nearly metacentric and as such were relatively hard to distinguish from each other.

In most cells, however, two of the four chromosomes were larger than the others, although the difference was not great. However, as the feature was reasonably consistent it was valid to claim that a difference did exist, and as such, a definite number can be assigned to each, namely the larger pair being called 14 and the smaller 15.

Group F Chromosomes 16 - 18

This group consisted of three small pairs of acrocentrics, whose size differed greatly from that of group B. As was the case with Group B, one pair, number 16, was markedly larger than the remainder of the group. Of the other four chromosomes in the group, two were consistently found to be larger. Thus the two pairs have been numbered separately, namely 17 and 18 for the large and small pairs respectively, as it was considered that the difference was marked enough and sufficiently consistent to warrant this separation.

In group F, as in group B, the question of terminology arises, as to whether short arms were present, thus justifying the term acrocentric. Short arms were found to be present in sufficient numbers to be certain that short arms do exist on chromosomes 16 - 18. As was the case in Group B, many of the small acrocentrics were found to have very deeply staining centromeric regions, indicating the presence of a short arm which was probably underlying the centromere.

Materials and Methods

One $8\frac{1}{2}$" x $6\frac{1}{2}$" print of the metaphase spread to be karyotyped was selected and using a hot tacking iron (Ademco), dry mounting tissue (Ilford) was attached to the back. The individual chromosomes were cut from the photograph using a scalpel blade. This procedure was found to be much more satisfactory than the use of scissors, in that chromosomes in close proximity to one another were more easily cut out using the scalpel.

Once the individual chromosomes were cut out and placed in a shallow open box, a suitable sized piece of white cardboard was cut. Medium thick white cardboard of size $12\frac{1}{2}$" x $10\frac{1}{2}$" (Millers Drawing Materials, Ltd.) was sectioned into two pieces $10\frac{1}{2}$" x $6\frac{1}{4}$" in size. Having already decided on a standard arrangement for the chromosomes, four thin pencil lines were drawn lengthwise on the card at 1", 3", $4\frac{1}{2}$" and 6" from the top of the card respectively. These lines were positioned such that the centromeres of the chromosomes in each line could be placed on the line, in order that the chromosomes would be lined up in a standard position. The distance between each line was determined by trial and error and the arrangement, finally decided, left enough room between the lines to accommodate the chromosomes from metaphase spreads of most sizes.

Results

The final positioning of the complement can be seen in figures 1 and 2. The procedure for selecting the individual chromosomes was also standard.

1. The two largest chromosomes were placed in position.
2. The twelve acrocentrics were then removed and divided in two groups consisting of the six larger members and six smaller ones. Within each group the acrocentrics were paired in order of size where possible, largest first.

3. In male cells, the Y chromosome was then selected and placed in position.

4. The six small non-acrocentric chromosomes were paired. The largest of the three pairs almost invariably bore a secondary constriction and this was positioned first, the other 4 chromosomes were then paired, the larger pair being placed first. This group was group E.

5. Four chromosome pairs were selected whose centomeres were more acrocentric than any others in the set, (Group C). These were arranged in order of size, the first and fourth pair being the most subacrocentric.

6. The remaining 10 chromosomes in the female or 9 in the male were then arranged in order of length, all having approximately the same centromeric position with the exception of one in the male and two in the female. The exceptions which were found to be the X chromosomes were placed in the middle of the row, this position being based on the usual size of the X's.

When the karyotype was completed, the cardboard with the loose chromosomes on top was carefully placed in a dry mounting press (Kennett Ltd), for approximately one minute to ensure complete attachment. The card was then removed and a photograph of the cell was attached to the karyotype using sellotape. The karyotype was coded and stored until required for analysis.

Discussion

The karyotype arrangement which was finally decided upon was, as stated previously, based on the recommendation of the Denver Report, (1960). Using the criteria of this report, the pig karyotype falls quite naturally into six groups, excluding the Y chromosome in the male. With very few exceptions the karyotype is arranged in order of size and the features of each group are distinguishable.

However, when this arrangement is compared with those of other workers, it is realised that direct comparison is impossible. Virtually the sole point of agreement between

the groups of workers is that the largest chromosome (No. 1 in the present study) should be placed first. There are three main difficulties in comparing the findings of different groups.

Firstly, in only two cases have measurements been presented, secondly, the fact that in some cases no attempt has been made to identify individual chromosomes and thirdly, the size of reproduction of karyotype in journals is such that any slight difference which may exist between chromosomes is greatly reduced.

Nevertheless, it is considered worthwhile to attempt to correlate the findings of various workers in order to determine what common ground there is between them. Two main factors in the arrangement cause the most confusion. Firstly, the arrangement of the acrocentric chromosomes. In about half of published karyotypes, all the acrocentrics are placed in one group, (McFee, Banner and Rary, 1966; Bruere, Fielden and Hutchings, 1968), whereas in the others, the large acrocentrics are placed near the beginning of the set and the smaller ones at the very end, (Henricson and Backstrom, 1964; Hard and Eisen, 1965). By carrying out the latter procedure there is a semblance of decreasing order of length, whereas by placing them all at the beginning the resulting karyotype consists of the second largest and the second smallest chromosome in the same group. If length is to be a main criterion, as it is in most published karyotypes, it would appear more logical to keep to this as far as possible. Similar criticism can be made about the arrangement of the two subacrocentric chromosomes (Numbers 5 and 8). By separating these two pairs and producing a separate group, (McFee, Banner and Rary, 1966; Vogt, 1966 ; Bruere etal, 1968) this leads to chromosomes of different lengths being placed adjacently with resulting imbalance of the karyotype.

McConnell, Fechheimer and Gilmore, (1963) caused further confusion by numbering the sex chromosomes, a system which is contrary to the accepted procedure in the

species studied such as man (Denver Report, 1960), cattle, (Sasaki and Makino, 1962), and sheep (Bruere, 1966). It is to be hoped that this classification which has fortunately not been repeated since McConnell's report, will be abandoned completely.

Although the arranging of chromosomes has no standard method, all the karyotypes so far produced have indicated that chromosome morphology is standard, most chromosomes being identifiable in all karyotypes published. With the exception of the wild pig (McFee etal, 1966) this general agreement concerning the chromosome complement should mean that in time a standard method of arrangement will be agreed.

The final problem which arises in discussion of the pig karyotype is the identity of the sex chromosomes. In the present study the Y chromosome was found to be the smallest chromosome in the complement having a submetacentric morphology. This is in complete agreement with all previous reports concerning the Y, with one exception. Stone, (1963) reported that the Y was larger than X, being a medium-sized chromosome. The illustrated preparations accompanying the report were very poor and as Stone's findings are contrary to every subsequent report, this must be viewed with extreme caution.

However, there is no such universal acceptance of the structure of the X. This has been described as a medium sized almost metacentric (Ruddle, 1964). The present studies bear out the findings of Ruddle, in that the X is the most metacentric of the medium-sized chromosomes of Group D. Without measurements to bear out these results, great difficulty is encountered in attempting to evaluate these varying findings.

In at least two cases, great care must be taken in interpreting the preparations. McFee, Banner and Rary, (1966) presented 3 karyotypes and allowing for the difficulty in judging illustrations which have been reduced for publication, a certain amount of dubiety is present concerning the pairing of homologues which was carried out.

Similarly, in the first karyotype of the pig which was published, (Makino etal, 1962) doubt again must be expressed about the pairing of chromosomes.

Although many varying reports have been published concerning the structure of the X, most workers have found it to be metacentric or nearly metacentric. McConnell etal, (1963), Makino etal, (1962) Ruddle, (1964), Breeuwsma, (1968) and Bruere etal (1968) have all found this to be the case, thus confirming the present study. However, reiterating, in the absence of idiogram results, impressions gained from reduced illustration may be erroneous. Cornefert-Jensen, Hare and Abt, (1968) made a detailed study of the X chromosomes using the late-replicating feature of one X in the female for identification. They found that the X was submetacentric and that there were similar sized chromosomes which were more metacentric than the X. Although this finding appears to contradict the present study and those of other workers, the method used by Cornefert-Jensen to measure the chromosome may possibly have led to inaccuracy. Measuring was carried out by using dividers and a ruler and by adopting this method, it seems possible that curvature of the chromosome arms was not taken into effect. From the illustrations published, certain chromosomes were curved to a greater degree than others and for this reason, the finding will require further confirmation. It is possible however that the X chromosome and especially the late X due to its unique lack of decondensation on forming the sex chromatin body may present different morphologies due to differential contraction of the arms. If the X does adopt different properties of length and morphology, as has been found with the human X, (Bishop, Leese and Blank, 1965) it is possible that erroneous pairings might occur in arranging the karyotypes. However, this possibility will involve much more work than has been possible in this study and it is to be hoped that further study on this problem will be possible in the future. However, the present study and those of other workers indicates that the X is indeed nearly metacentric and at the present state of knowledge, must be considered to be the most likely structure.

Secondary Constrictions.

Introduction

The phenomenon of secondary constrictions has been known for many years from the study of plants, much work having been carried out on the subject, leading to the theory that these regions are often associated with the formation of the nucleolus. (Heitz, 1931). Although Rothfels and Siminovitch (1958a) had used the presence of these constricted areas to aid identification in monkey chromosomes, it was not until 1962 that a quantitative study on the frequency of occurrence of secondary constrictions in human chromosomes was carried out by Ferguson-Smith, Ferguson-Smith, Ellis and Dickson. Prior to this, it had been reported that satellites were present on certain chromosomes of the human karyotype, (Chu and Giles, 1959; Levan and Hsu, 1959) but there was no agreement as to the identity of these chromosomes. An attempt was made to standardise the identification of human chromosomes by the Denver Report (1960) which decided that pairs 13 and 14 could be separated from 15 and similarly 21 from 22 by the presence of satellites. This statement, however, was contradicted by Ferguson-Smith and Handmaker, (1961) who found that as all 5 pairs possessed satellites they could not be separated by the use of this criterion. These authors also reported that about 60% of the cells examined showed a degree of attachment, in that chromosomes appeared to be connected to one another by their satellites. Many other authors reported finding secondary constrictions on various human chromosomes, (De la Chapelle, 1961; Patau, Therman, Inhorn, Smith and Ruess, 1961; Muldal and Ockey, 1961), but in most cases there was a lack of agreement concerning the exact identity of the chromosomes involved. The consensus of opinion was that constrictions were found on chromosomes 1 and also in a number of the 6 - 12 group, as well as the satellited members of 13 - 15 and 21 - 22 groups. Using a modified fixation technique, Saskela and Moorehead, (1962) produced cells with both enhanced

secondary constrictions and higher frequency, the constricted areas having a similar puffy appearance as was found on the sex chromosomes. They therefore interpreted these findings as indication that both the secondary constrictions and the sex chromosomes were composed of heteropyknotic material.

The first large-scale study of secondary constrictions of human chromosomes was carried out by Ferguson-Smith, Ferguson-Smith, Ellis and Dickson, (1962). This work confirmed that all the members of 13 - 15 and 21 - 22 groups possessed satellites on the short arm, the frequency varying from 38 to 62% of available chromosomes. A large proportion of the other chromosomes also bore constrictions, the main one being numbers 1, 9, 6 and 17.

An attempt to standardise the various findings on secondary constrictions in the human was made by the London Conference, (1963). This report suggested that with the exception of the 5 pairs comprising groups 13 - 15 and 21 - 22, there were only four pairs which possessed secondary constrictions with a frequency which warranted the use of them in identification.

A number of workers used modified techniques to obtain higher frequencies of secondary constrictions. Saskela and Moorehead, (1962) had used a modified fixation method, whereas Sasaki and Makino, (1963) obtained a very high incidence by the use of calcium-free medium for varying lengths of time in their culture of aborted material. They also obtained up to 13% of cells demonstrating satellite association, suggesting that the calcium-free medium might have affected the nucleolus, leaving the chromosomes with associated satellites, Ferguson-Smith and Handmaker, (1961) having postulated that the satellited chromosomes were involved in nucleolar organisation. By the use of variations in colchicine concentration, Palmer and Funderbunk, (1965) obtained higher incidences of secondary constrictions when lower concentrations were used. Similarly, by using 5 - Bromodeoxyuridine, they were able to obtain a higher frequency

of constrictions.

Using the data obtained by Saskela and Moorehead, (1962) and Sasaki and Makino, (1963), Schmid (1963) found a suggestive relationship between the areas which possessed secondary constrictions and those which were found to be late replicating by the use of autoradiography, but was, however, unable to find a similar correlation in studies of the satellited chromosomes.

Ferguson-Smith, (1964) in a study of human pachytene chromosomes found that the principal nucleoli were terminally associated with bivalents which appeared to be the satellited chromosomes 13 - 15 and 21 - 22. This work confirmed an earlier postulate by Ferguson-Smith and Handmaker, (1961) that there was a possible association between satellites and nucleoli. Although this apparently contradicted early work by Schultz and St. Lawrence, (1949) and Yerganian, (1957) who had found that the bivalent primarily associated with the nucleolus was one of the large ones, Ferguson-Smith and Handmaker, (1961) had attempted to explain the discrepancy by postulating that instead of the nucleolus being medianly placed on a large bivalent, it was terminally associated with two smaller chromosomes which gave the appearance of one large chromosome.

In 1963, McConnell, Fechheimer and Gilmore reported the occurrence of secondary constrictions in the pig. These workers found that a long constriction was frequently found on one member of pair number 8 and occasionally on both. However, because they obtained their idiogram by direct measurement of photographs which were at relatively small magnification, the values obtained are not of great value in identifying the chromosome involved. McConnell further found the almost invariable presence of a constriction on one member of chromosome pair 13, this being also frequently seen on the homologue. This latter finding was confirmed by Ruddle, (1964), who found the constriction to be consistently present on both homologues,

but, however, made no mention of any other chromosomes bearing secondary constrictions. The presence of the constriction on chromosome 13 has been noted frequently in published karyotype, (McFeely and Hare, 1966; McFee, Knight and Banner, 1966), but no attempts have been made to determine its exact frequency or the frequency of any other secondary constriction in the pig karyotype.

In the cat, Chu, Thuline and Norby, (1964) recorded that a satellited pair of chromosomes was present, but again no frequency of occurrence was quoted.

In the sheep, Bruere and McLaren, (1967) carried out a detailed study of the distribution of secondary constrictions in cells obtained from peripheral blood cultures. They found that the majority of constrictions were present on the five largest pairs of chromosomes, but concluded that the recorded incidence was not high enough to aid identification. They suggested however, that the three large metacentric pairs were probably the main nucleolar organisers in the sheep. Bruere also found that by using sodium citrate as a hypotonic agent, a higher incidence of constrictions was obtained than when hypotonic Hank's balanced salt solution was used.

Thus it has been found that in certain species studied, the presence of secondary constrictions can be of great use as an aid to identification. It has further been noted that by the use of various modifications of the techniques used, much higher frequencies of the constrictions can be obtained.

Materials and Method

In order to assess the total incidence of secondary constrictions in metaphases obtained from pig lymphocytes 300 karyotypes were examined and analysed. This study was carried out for two reasons, firstly to determine how often the secondary constriction on chromosome 13 occurred, as it was noted that the vast majority of these chromosomes possessed constrictionsof varying lengths at the most proximal

region of the short arm. Secondly, it was hoped that in other chromosomes, similar markers might be found and by using these, more positive identification of the chromosomes might be possible.

Examination of 300 of the best karyotypes was carried out, the criteria for selection of a secondary constriction being that, in the same position on both chromatids, a constricted area was present in which there was some evidence of continuity between the non-constricted parts of the arm, no disalignment being present. (Figure 5). In some cases, it was not possible to observe the fine chromatin of the constriction, because of the poor staining quality of the area, but if alignment appeared to be normal, it was classed a secondary constriction.

A sketch idiogram was made and copies made from this on a Roneo Duplicator. One such copy was allocated to each photographic karyotype and onto this was marked each secondary constriction observed. The position of a constriction was carefully measured with dividers and a ruler marked in millimetres. The centromere-constriction distance, was converted into distance units on the sketch idiogram, the constriction carefully drawn into the latter. This procedure was repeated for each constriction examined.

When all the cells had been analysed, every constriction which had been found was transferred onto a master sketch idiogram. In order to give a composite picture of the freqeuncy and position of constrictions in the pig karyotype, all those constrictions found were drawn onto a copy of the accurately constructed idiogram, the relative positions of each constriction being kept constant by calculating the size relationship between the sketch and the accurate idiogram, and using this to produce the final constriction picture.

The freqeuncy with which chromosomes were associated was studied by examining the

metaphase spreads of the 300 cells used in the above study.

Results

The distribution of the secondary constrictions in the 300 cells examined is presented in Fig. 6. A total of 694 secondary constrictions were found, giving a frequency of 2.31 per cell. Of this total, 570 or 82.13% were seen to be on the proximal third of the short arm of 13, it being so close to the centromere as to make measurement of the constriction-centromere distance impossible. Table 5 presents the frequency with which constrictions were seen, expressed as a percentage of the total possible, which is 600 in the case of the autosomes. Using the criterion of Ferguson-Smith etal, (1962) only those chromosomal areas which had a frequency of 3% or higher were included in this table. Finally, table 6 demonstrates the observed and expected frequencies of the constrictions on each chromosome.

Group A.

Only 3 secondary constrictions were seen on chromosome 1, these being observed on the distal third of the long arm. This represents 0.43% of the total constrictions and 1.5% of the available chromosomes.

Group B.

3 secondary constrictions were seen in this group, two of which were in the middle third of chromosome 2. As in group A, the frequencies were so low as to be ignored.

Group C.

17 constrictions were observed, the majority of these being found on the proximal thirds of the short arms of numbers 6 and 8. However, the frequencies again were very low, representing only 1.5% and 0.83% respectively of the available chromosomes.

roup D.

he highest incidence of constrictions (25) was seen on the proximal third of he short arm of chromosome number 9. However, this was only 4.03% of the total onstrictions and was observed only in 4.1% of all chromosomes. Of the remainder f the group, the proximal thirds of the short arms of the X, numbers 11 and 12 ore the most secondary constrictions, but again the frequencies were very low 1.59, 1.59 and 2.59% of all constriction, respectively).

roup E.

n this group, the vast majority of all secondary constrictions were found. As an be seen from table 6, it is almost entirely due to the very high frequency found n the proximal third of the short arm of number 13. In this position, 570 onstrictions were found, this representing 82.13% of all secondary constrictions ound. Thus in 95% of all chromosomes 13's examined, a constriction was present. ineteen secondary constrictions were observed on chromosomes 14 - 15, eight being ound in the proximal and middle thirds of the short arm of 14 and 11 on the proximal ird of the short arm of chromosome 15. The frequency with which constrictions ere seen on 14 and 15 were only 1.33% and 1.83% respectively of the possible total.

roup F.

ly 5 constrictions were seen in Group F, all of these being on the distal third 16. As for many chromosomes in the set this represents a frequency of less than .

Chromosomes.

Secondary constrictions were seen in the 100 male cells examined.

none of the 300 cells examined was there any evidence of chromosome association. ile this must be a subjective assessment, in no cells were any chromosomes situated such a way as to indicate that any mechanism was holding them in association.

Discussion

The results from the present study show that, generally speaking, the frequency of secondary constrictions is so low as to make this marker useless for chromosomal identification, (Table 5 and figure 6). However, a number of observations can be made from the findings. Firstly, the distribution of the constrictions is not a random matter. If this was the case, the distribution would be expected to bear some relationship to chromosomal length and this can clearly be seen not to be the case, (Table 6). By applying the X^2 test, P was less than 0.01, this being very highly significant. Secondly, although chromosomes 9 and 12 had a frequency of 3% and over, the frequency with which they were found makes it unlikely that they can be used with any confidence for identification purposes. Thus, the only constriction which can be used as a marker is the one found consistently on the proximal short arm of number 13. This is in agreement with previous work, (McConnell etal, 1963 and Ruddle, 1964), but is the first estimate of the exact frequency of this marker. Finally, in this study, no chromosome was seen corresponding to the one observed by McConnell and his co-workers to bear a secondary constriction frequently, and which he numbered pair 8. Although that chromosome is within the size range of chromosome number 9 in the present study, the incidence with which a constriction was seen (4.16%) does not agree with McConnell's finding.

It would appear likely, therefore, that technical differences could account for this discrepancy. Differences due to technique are discussed more fully below, but it is worth noting that McConnell and his co-workers used hypotonic Hank's solution instead of sodium citrate as in the present study. Bruere and McLaren, (1967) however found that an increased frequency of secondary constrictions in the sheep was seen when sodium citrate was used instead of Hank's solution, possibly because of the removal of calcium. It is possible that this might not be the case in the pig, but as McConnell did not carry out a quantitative study, the relative frequency of

constrictions between the two methods cannot be determined.

It appears likely that the frequency of secondary constrictions in the pig is low when compared with the incidence in human lymphocytes cultured under basically the same circumstances, (Ferguson-Smith etal, 1962). However, using modifications of the fixation technique, (Saskela and Moorehead, 1962), the culture medium, (Sasaki and Makino, 1963) colchicine concentration and 5 - Bromodeoxyuridine levels (Palmer and Funderbunk, 1965), it has been found possible to increase the frequency of secondary constrictions markedly. It must be noted, nevertheless, that although Saskela, (1962) and Sasaki, (1963) used technique modification, their work was carried out on tissues other than blood, and even when these modifications were used on human lymphocyte cultures, (Palmer and Funderbunk, 1965) the frequency with which secondary constrictions were found, was still markedly lower than in the embryonic tissue used by Saskela and Sasaki.

It would therefore appear that it may be possible to increase the frequency of constrictions by technique modifications. By using these methods, secondary constrictions may become potentially more useful as an identification tool. However, it would appear that this is more likely to come about by using different tissues, it having been shown that embryonic tissues possess a higher frequency of secondary constrictions than do blood cells. However, it is not known whether this increased incidence is due to the embryonic nature of the tissue or to the nature of the tissue itself, as so far, no work has been carried out non-embryonic fibroblast cultures.

In the only published report of quantitative studies on secondary constrictions in domestic animals, Bruere and McLaren, (1967) found a low frequency in metaphases derived from blood. By using sodium citrate instead of Hanks, they obtained a higher incidence which they suggested might have been due to calcium removal. Thus, in the sheep as in the pig, (present study), the incidence of secondary constrictions

is lower than in human chromosomes.

The reason for the difference in frequency between the pig and the human is not known. However, it has been shown by Ferguson-Smith, (1964) that the satellited human chromosomes at pachytene are physically associated with the nucleolus and are probably the nucleolar organisers. In the same paper it was noted that 68% of primary spermatocytes contained one nucleolus and 27% a double nucleolus, the remaining 5% having either 3 or 4. Ferguson-Smith reported that in some cases, the principal nucleolus was associated with five bivalents and interpreted these as being the 5 satellited pairs. He also found that in some cases a bivalent was found with the nucleoli associated with chromomeres situated at the centre of a bivalent, the nucleolus in question not being the principal nucleolus. Thus from this work, it could be postulated that the principal nucleolus in man is organised by the satellited chromosomes and the other nucleoli are associated with the non-satellited chromosomes which bear other secondary constrictions. In the pig, no work has been carried out on nucleolar numbers at pachytene and therefore any hypothesis concerning them has to be pure conjecture. However, it would seem to be a reasonable hypothesis that as in the pig, virtually only one chromosome possesses a secondary constriction (number 13) and its frequency is 95% of available chromosomes, this is the principal nucleolar organiser and that the vast majority of primary spermatocytes at pachytene will be expected to possess only one nucleolus. Obviously, this hypothesis cannot be substantiated until such work on pachytene chromosomes is carried out.

The absence of chromosome associations in the present study is rather surprising. In the human, association of the satellited chromosomes is a common observation, (Ferguson-Smith and Handmaker, 1961) and this feature has also been seen in other species such as the sheep, (Bruere and McLaren, 1967) and the pig, (Ruddle, personal communication by Ferguson-Smith).

Although the reason for this discrepancy can only be hypothesised, Ruddle's studies were carried out on embryonic tissues which, in the human, appears to contain a higher frequency of secondary constrictions. If this is also the case in the pig, the resultant higher frequency may result in the increased associations observed.

Further work will have to be carried out on various tissues of the pig before this apparent tissue difference is clarified.

This present study has shown conclusively that the secondary constriction on the proximal short arm of number 13 is a marker which can be used as an aid to identification. Although the constriction was lacking in 5% of the chromosomes, in only 3 out of the 300 cells analysed did both members lack the constriction. By using one of the modification techniques for obtaining higher frequencies of constrictions, it might be expected to obtain a frequency for pair 13 of 100%. With the exception of chromosome pair 13, secondary constrictions appear to be of no value as morphological markers in metaphases obtained from pig lymphocytes. However, by using either modifications of the technique or different tissues, it is possible that higher frequencies might be obtained and as a result, there may emerge further markers as further aids to identification.

Idiogram

Materials and Method

Ten cells, five male and five female., were selected,these being chosen using two criteria. No overlapping had to be present and the chromosomes had not to be in very early or very late metaphase, that is excessively stretched or contracted. The actual degree of contraction of the chromosomes was not considered important as all lengths were later reduced to a common factor. The karyotypes used in the study represented 9 different pigs, each autosome of every cell being identified as a number from 1 - 36 using the same order for each cell. All 10 cells were treated in exactly the same manner throughout the whole procedure.

The negatives of the cells were projected onto a sheet of white paper attached to a flat surface and the distance of the projector from the wall adjusted until a magnification of X10,000 was obtained. This was achieved by using a 35 m.m. negative of a 1 m.m. microscope grid which was divided into 100 equal divisions. The negative which was photographed at the same magnification as the cells (X 625), was projected onto the wall until the distance between two adjacent spaces was 100 m.m. As the true distance was 0.01 m.m. this represented a magnification of X10,000. The position of the projector in relation to the wall was noted and kept constant throughout the tracing of all 10 cells. Care was taken to ensure that the angle between the line of direction of the centre of the beam and the wall was exactly 90° in order that there would be no optical abberation of the peripheral chromosomes with resulting inaccuracies in measurement.

A large sheet of plain white paper was firmly attached to the wall and the negative of the cell to be measured, projected onto it using an Aldis Gnome projector with a negative holder. Using a sharp pencil, each chromosome was then treated in an identical manner. Each arm was traced by a line down the centre of the arm, carefully

following any bend in the chromatid. The end of the chromosome arm was usually distinct and easily definable. A line at right angles to the direction of the arm was drawn to mark the end of the arm and the line tracing of the chromosome was continued until the two lines intersected. This procedure was carried out for each arm of every chromosome.

The exact marking of the centre of the centromere was more complicated and the method used depended on whether the chromosome was acrocentric or non-acrocentric. Although short arms were seen on the acrocentric chromosomes of the pig, none were present on those used in the idiogram construction, Thus, to determine the centromeric position a procedure similar to that used for the marking of the end of the chromatid arms was used. A line at right angles to the direction of the arms was drawn to mark the end of the chromosome at the point which was presumably nearly exactly the centromere. The tracing lines of the chromatid arms were continued until they both met this terminal line.

A different procedure was adopted for non-acrocentric chromosomes. In those chromosomes in which the centromere was small and easily definable, a small cross, whose arms pointed towards the points of insertion of the chromatid arms, was drawn over the centromere and the lines marking the chromatid arms were extended to meet the corresponding arms of the cross. In those chromosomes whose centromeres were large and difficult to define, the points of insertion into the centromere of the long and short arms of the left chromatid were joined and the right chromatids treated similarly. A third line parallel to these two lines was drawn midway between them. Two further lines were drawn joining the points of insertion of the two short arms and the two long arms into the centromere.

A final line parallel to the 4th and 5th lines and midway between them was then drawn. The point of intersection of the final line and the line midway between

the first two was considered to be the centromere. The points of insertion of the four arms into the centromere were then extended to the centre point of the centromere.

Cells containing secondary constrictions were avoided completely with the exception of the constrictions of pair No. 13 which were present in both homologues in all ten cells. Secondary constrictions were included in their entirity in the arm tracings. Although by including the constrictions in the measurement false lengths and ratios were obtained, it was considered that as virtually every chromosome of pair No 13 bears this constriction, an even more erroneous picture would have been obtained by excluding the area from the measurements or by only giving it a fraction of its actual length.

A map measurer graduated in $\frac{1}{8}$ inch units was used for measuring all the arms, an $\frac{1}{8}$" being used as the basic unit. All lengths were measured to the nearest 0.5 unit. Each chromatid arm was measured repeatedly until the same reading was obtained three times in succession. This was considered important because variations as large as 1 unit were obtained if the instrument was not used in exactly the same way for each reading. The map reader was zeroed carefully, held vertically, and run at a constant speed from the centromere to the end of the chromatid arm. When this procedure was carried out exactly, repeated measurements were usually constant.

All the arms of every chromosome were measured and the total length of each chromatid of the non-acrocentric chromosomes was obtained by adding the measurements for the long and short arms, the total length of the acrocentrics being the same as the long arm measurement. It was considered that it was more accurate to add the lengths of the long and short arms to obtain the total length than to attempt to measure the whole chromatid.

Once all the measurements were completed, the measurements in map reader units were

converted into their corresponding millimetre values. A straight line graph was constructed of millimetre length against map reader units. The map reader was run along a ruler for known lengths i.e. 10 m.m., 20 m.m., 30 m.m. and so on up to 100 m.m., the number of map reader units corresponding to those lengths noted and, from these, the graph constructed.

Using the graph all lengths were recalculated as millimetre readings. This transposition was carried out in order that an idea of the actual sizes of the chromosomes could be gained. For each chromosome pair, the total lengths of the members of the pair were meaned to give a total length for each of the 18 autosomes of the haploid set. In the female cells a similar procedure was carried out for the X so that male and female cells both had the same weight, i.e. a set of nineteen values representing the haploid set of autosomesplus one X, the Y at this stage being left out of the calculations. The sum total of the haploid set was taken and from this a factor was calculated by which each measurement was multiplied in order that the total haploid length of the cell was 1000.

The factor was arrived at using the equation:-

$$F = \frac{1000}{T}$$ where T is the total haploid chromosome length in m.m.

Each chromosome measurement was multiplied by this factor, the resulting chromosome length being expressed as a fraction, expressed in parts per thousand of the total haploid chromosome length (Haploid T.C.L.). The Y chromosome which had not been included, was at this stage multiplied by the same factor.

Each cell was treated in a similar manner. As each chromosome in every cell was expressed similarly it was valid at this stage to calculate mean values for each of the 19 chromosomes of the ten haploid sets. Table 2 shows the chromosome lengths of all 10 cells together with the means for these chromosomes.

From the basic arm measurements two indices were calculated. These were:-

1. The arm ratio:- long arm/short arm.
2. The centromeric index:- Short arm x 100/total length.

Both ratios were calculated for each homologue and the values for each homologue were meaned to give the ratios for each chromosome. Finally, the ratios of all 10 cells were averaged to give the final values for the average haploid cell. Thus each chromosome of the haploid set had three values assigned to it.

1. Length relative to the total length of the normal X-containing haploid set expressed per thousand.
2. Arm Ratio
3. Centromeric Index.

In order to construct the idiogram, it was necessary to re-calculate the lengths of the short and long arms. Using two of the indices which had been calculated for each chromosome, namely the total length and centromeric index it was possible to calculate the required values. By multiplying the total length by the centromeric index and dividing the result by 100, the value for the short arm was obtained. The length of the long arm was simply calculated by subtracting the value of the short arm from the total length.

Using the lengths of the two arms and the total length of each chromosome the idiogram was constructed. Although the short arms of the acrocentric chromosomes were not always visible, it was decided to represent them in the idiogram, as they were quite frequently seen and there is no doubt that the acrocentrics do possess very small short arms.

Results

As the preliminary calculations leading up to the final indices for the chromosomes

in each cell were very long and complicated it has been decided to present only the final values, for each cell, of the three indices used in the idiogram construction.

(1) Total length (2) Arm ratio (3) Centromeric index

Table 2 presents the total length of each chromosome expressed as parts per thousand of the total chromosome length of the haploid set (T.C.L.). To arrive at this calculation, the total length of the haploid chromosomesin millimetres was recalculated to be 100 m.m.s. long and each chromosome was then similarly recalculated to give the values in the table.

A simpler calculation was involved in calculating the arm ratio. This index was arrived at by dividing the length of the long arm of each chromosome by its short arm. Thus two values for each chromosome pair resulted and these were meaned to arrive at the final calculation in table 3.

Similarly, the centromeric index was calculated by the formula:-

Short arm x 100/long arm.

Again the values for each pair of chromosomes ~~was~~ were meaned to give results for the haploid set (table 4). Table 4a displays all three indices together with the standard deviation of each. As described in another section, the length of Y was not included in the calculation, but each one was multiplied by the conversion factor by which the total lengths were converted into parts per 1000 of the total haploid length of the set.

Using the total chromosome length and the arm ratio of the mean haploid set, the lengths of the long arm and short arm of each chromosome were re-calculated and from these values the idiogram was constructed (fig. 4).

Discussion

The results obtained in the study show that the chromosomal arrangement which was decided upon, on the grounds of visual comparison, is justified by the mathematical findings. Using a combination of total length and the arm ratio it is quite clear that the chromosomes of the pig do, in fact, fall quite naturally into 6 distinct groups as described elsewhere.

Only two publications have reported a construction of a pig idiogram and direct comparison between these reports and the present findings is not possible for a number of reasons. The first idiogram of the pig was produced by McConnell, Fechheimer and Gilmore, (1963), who failed to describe the methods used in its construction, but gave the impression that measurements were taken directly from photographs, in which case it must be assumed that the magnification was no greater than X3000 or X4000. If this is the case it would be expected that the possible error was greater than would be the case if much larger magnifications had been used. Further it was not stated whether both male and female cells were given the same weight by omitting the Y in the male cells and counting the X twice.

Similarly, the work of Ruddle, (1964) does not appear to bear direct comparison with the present results. As with the idiogram of McConnell etal, it was not stated how the sex chromosomes were involved in the calculations. Ruddle did not attempt to separate the majority of the chromosomes and as such it is only possible to take from the work, 6 chromosomes which were given individual status. With these reservations in mind, the idiogram from the present work will be analysed and where possible, comparison made with the work of Ruddle and McConnell.

Group A.

Chromosome 1. This chromosome is clearly larger than any other one in the set,

being almost twice the length of the next largest non-acrocentric chromosome. Although the range in length appears large, from 104 to 127, the standard deviation is only about 6% about the mean of 112,36, this being quite small considering the possible errors in measuring a chromosome of such a length. The calculated arm ratio of 2.08 gives the chromosome very close co-relation with chromosome M-1 of Ruddle, which had a length of approximately 113 after recalculation to bring the unit used by him into line with present study, and an arm ratio of 1.98. The chromosome in McConnell's work corresponding this chromosome had a length of 106 and a centric index of 1.5 compared with 1.48 in the present study. There can certainly be no doubt concerning the identity of chromosome 1 and very close correlation exists between the findings in the various studies.

Group B.

Chromosomes 2 - 4. The largest chromosome in the group of acrocentrics is clearly longer than the other two. However, although the smaller pairs appear to be separable on the basis of their length, it is erroneous to positively separate the two pairs as overlapping nearly occurs when one standard deviation is applied. Certainly in all the cells studied, two chromosomes were longer than the others, in some cases there being a distinct difference in length between the pairs. However, great caution must be taken in selecting chromosomes on the basis of length alone and subsequently grouping them using the same criterion. Furthermore, although separation of the two pairs at large magnification was possible in 8 out of the 10 cells it is not always possible to do the same at the magnifications normally used for karyotyping (X3000) and thus it is considered more practicable to group chromosome pairs 3 and 4 as a single sub-group, although continuing to number the larger pairs as number 3 and the smaller as 4. Again the results of Ruddle and McConnell bear very close resemblance to the present values. Both these workers found that the largest pairs could be separated from the others, Ruddle finding the length to

be 82 and McConnell 80 as compared to 84.5. Ruddle did not separate the two smaller pairs but gave an average value of 58 for the sub-group, whilst McConnell's values were 60 and 58, both very close to the values of 62.8 and 56.11 obtained here.

In groups C and D, great difficulty occurs in attempting to co-relate the findings of McConnell, Ruddle and the present study, in that Ruddle placed most of the chromosome pairs into one group, and the size and quality of McConnell's illustrations make the task very difficult. In both cases the only chromosomes which can be positively identified for comparison are pairs number 5 and 8 and in the case of McConnell's work the X chromosome. With the exception of the chromosomes 8 and 9 which are placed in their respective groups on the basis of their arm ratios, all the chromosomes in group 5 - 8 and 9 - X - 12 are placed in descending order of size.

Group C

Chromosomes 5 - 8 form a group consisting of four of the largest chromosomes, all having arm ratios over 1.8. Within group C there are two distinct sub-groups which overlap on the criterion of length, but judged by arm ratio are very different. Chromosomes 5 and 8 with arm ratios of 2.85 and 3.02 have by far the most terminal centromeres with the exception of the acrocentrics. Pair 5 with a length of 66.7 are much larger than pair 8 (52.1) and as such are easily separated from each other as well as from the rest of the complement. Ruddle similarly found these two pairs to be separable from the rest of the complement, the length of number 5 being 67.6 (cf. 66.7) but its arm ratio to be 3.27 whereas McConnell's centric index was 1.5 corresponding to an arm ratio of 2.0.

Again, although discrepancies between the published idiograms and the present study exist in the case of pair number 8, all workers have found that it was, as in the case of number 5, easily distinguished. The length as given by Ruddle and McConnell was 55.8 and 50.0 respectively, (compared with 52.1) the arm ratio being 2.92 by Ruddle (3.02 in the present work) and the centric index 1.4 (McConnell) compared

with 1.33. Thus in the case of pairs number 5 and 8 although there is agreement as to the positive identity of the chromosomes, the indices calculated by the three pieces of work do vary quite markedly, especially when the work of McConnell is compared to that of Ruddle and the present study.

The two other chromosomes in group C are easily distinguished from the two pairs already described in that their arm ratios differ greatly, pairs 6 and 7 being much more metacentric. As pairs 6 and 7 have virtually the same arm ratio (1.89 and 1.83 respectively) they are separable only by their lengths. It can be seen there is clear difference between the lengths of 6 and 7, a difference which existed in every cell. However, although in every cell there were two larger and two smaller chromosomes comprising pairs 6 and 7, it cannot be certain that in fact the two larger ones are a pair.

Nevertheless in five cells, when the length alone was used to separate the pairs, number 6 had a greater arm ratio and in only three cells was the reverse the case. From this evidence, although slight, it would appear to be more valid to pair the 4 chromosomes on the basis of length, rather than by using arm ratios, a much clearer differentiation being obtained by the former method.

Group D

This group consists of 5 homologous pairs of chromosomes in the female and 4 pairs plus a single chromosome in the male. Thus the chromosomes making up the group are four pairs of autosomes and the X chromosomes. Using the convention of the Denver Report, by not numbering the X chromosomes, the group consists of pairs 9 -12 including the X.

On measurement the group consists of eight chromosomes, all with virtually the same arm ratios of about 1.40, the remaining one or two chromosomes, depending on the sex,

having much more median centromeres and arm ratios distinctly different from the remaining members of the group. On the basis of chromosomal length four reasonably distinct pairs can be formed. The largest pair is markedly longer than the other pairs and as such is considered to be a pair distinct from the rest. On similar grounds, another pair is formed by the two smallest chromosomes. A less clear differentiation exists between the other 2 pairs formed. Although this difference does exist, in some cases it is not large enough to make differentiation definite. However, it is considered that, in the majority of cells, the difference is large enough to be able to make distinction possible between the two pairs, 10 and 11. No such difficulty exists in the identification of the X chromosome. By virtue of its almost median centromeric position it is clearly differentiable from any other chromosome of similar size. In every cell examined, the X had an arm ratio lower than any other chromosome of comparable size in group and as such it is considered that the identification of the X is unequivocal.

The wide range in arm ratios in all the chromosome pairs in group D poses the question of to what extent the arm ratios can be used to identify chromosomes. Firstly in any biological study it is to be expected that a variation will be found and therefore the spread of arm ratios is not unexpected. Secondly it is possible that differential contraction of the long and short arms of some chromosomes may occur and if this is the case, the arm ratios will be of less significance. Another variable present is the measuring technique. In many cases it was impossible to be certain as to the exact point of the end of the chromatid arm or the exact centre of the centromere. Furthermore, with smaller chromosomes a slight error in judging the centromeric position would result in disproportionate errors in the arm ratios. A final explanation of the wide range and overlaps present in the arm ratios is the possibility that pairing chromosomes on the basis of length is not a valid method. However, when a chromosome with known identity, such as chromosome 1,

is examined it is found that both the variation and the range of the arm ratios are as great as those found in group D.

Thus it would appear that unless the pairs of chromosomes under investigation have arm ratios which are reasonably distinct, it is erroneous to attempt to identify them using this criterion. However, where identification is carried out using length as the criterion, each pair of autosomes in group D differs from its nearest neighbour by at least one standard deviation.

Therefore, although positive identification of pairs 9, 10 and 11 cannot be certain, from the figures presented it is possible to separate all the members of Group D on the basis of length with a certain degree of confidence.

Group E.

Chromosomes 13 - 15. As was the case with Group D, the members of group E can only be separated by length. It was found that the range and spread of arm ratios was such that separation was impossible using this criterion although group E can be separated from the autosomes of group D by their arm ratios. As was the case previously, the lack of uniformity of arm ratios between homologous pairs in different cells either means that the measuring of small chromosomes is not sufficiently accurate, leading to errors which are disproportionately larger due to the short length of chromatid or that the chromosomes which were being paired were not in fact homologous. However, as the lengths of the homologous pairs show quite clear correlation it appears less likely that the pairings are wrong than that the measuring technique was at fault.

It is quite clear that pair number 13 is clearly separable from the other two pairs on the basis of length. A less clear distinction exists between pairs 14 and 15,

but on the basis of the measurements presented, a marked difference is present and, as such, it appears to be valid to separate the two smaller pairs of group E. However, it would appear that chromosomes12 and 13 were similar in size, although differing in arm ratio. This similarity only arose because of the presence of the secondary constrictions found on pair 13. As these constrictions are seen in the vast majority of cells it was considered that a more realistic evaluation of the length of 13 would be obtained if the constricted area was included in the total measurement. Thus, no confusion can arise in identification between 12 and 13, because of the constriction.

Group F

Chromosomes 16 - 18. From the results obtained for this group of acrocentric chromosomes it appears that 3 distinct homologous pairs are formed. A clear difference in length exists between pairs 16 and 17, no overlap occurring when two standard deviations are applied. Using this criterion, pairs 17 and 18 are less easy to separate, overlapping occurring with two standard deviations applied, although when one is added to each, no such overlap occurs.

Thus, for the small acrocentric chromosomes, one large pair is present, clearly larger than the other two, and between the latter there is a distinct length difference although less than between 16 and 17.

Y chromosome

The Y is easily distinguishable from the rest of the chromosome set by virtue of its size, it being the smallest in the complement. Although the difference in the length between it and the smallest acrocentric is not very great, when compared with the next larger non-acrocentric chromosome, number 15, it is clear that there is a distinct difference in length. (17.76 ± 3.22 compared with 27.91 ± 2.41). As there can be no doubt about the identity of the Y, the large standard deviation

found must be due to a combination of the small sample (5 counted) and the possible error in measuring a chromosome of the size of the Y.

Summary

It has been shown that, by using a combination of total chromosomal length and arm ratio, with the exception of pairs 3 and 4 which are considered not to be separable, all chromosomes in the pig karyotype can be positively identified.

Section III

Sex Chromatin Studies.

Sex Chromatin

The sex chromatin body is a small body, about 1μ in diameter which can be detected in female somatic tissues but not in male cells. The frequency observed varies according to species and tissue. It stains very deeply with Cresyl Violet and contains D.N.A. as shown by its property of being both Feulgen and methyl green positive. The body's shape can vary from circular to triangular depending on its position in the nucleus. The present theory concerning the body is that all or part of each X chromosome in excess of one, is inactivated and condensed during interphase, thus forming the sex chromatin body.

Introduction to Sex Chromatin

In 1949, Barr and Bertram published a paper concerning the "nucleolar satellite" in cat neurones. 30% - 40% of the neurones of mature female cats were found to contain the nucleolar satellite whereas in nerve cells of male cats a poorly developed body was observed only infrequently. This report also mentioned that a similar sex difference existed in humans. Barr and his co-worker postulated that as in the human the nucleolar chromosomes were said to be frequently the sex chromosomes, (Gates 1942), the same could apply in the cat. They therefore suggested that the heterochromatic portion of the nucleolar chromosome gave rise to the nucleolar satellite, and because the female had a double complement of this material, the body was more easily visibly than in the male cells, Barr, Bertram and Lindsay (1950) reported their findings in a more complete study of sexual dimorphism in cat neurones, finding that in different regions of the nervous system, the incidence of the satellite varied from 56% to 87%, whereas the smaller body in the male was seen in a frequency ranging from 2% to 6%. Barr and his co-workers gained the impression that in all neurones of both sexes a nucleolar satellite was present.

In this latter paper it was stated that the nucleolar satellite contained D.N.A.,

whereas the nucleolus contained R.N.A. This fact suggested that the association between the satellite and the nucleolus was not as close as had first been thought (Barr and Bertram, 1949). Barr, Bertram and Lindsay further confirmed the original finding that sexual dimorphism was found in human cells. They also suggested that the original hypothesis by Barr and Bertram was erroneous in that the nucleolar satellite was a product of the X chromosome, the two X's of the female fusing to form a visible body, whereas in the male, the product of the single X was too small to be observed.

Graham and Barr, (1952) examined feline non-nervous tissues and found sexual dimorphism to be present in many of these. They described the body as having a clear area indicative of a bipartite structure endorsing the theory of Barr, Bertram and Lindsay concerning the derivation of the body.

However, Brusa, (1952) was unable to detect sexual dimorphism in the neurones of pigeons, and although he confirmed that the sex chromatin body was present in the female cat neurones, he was unwilling to associate this body with the sex chromosomes as the sex difference had only been seen in cats. This proposition however, had been invalidated by the findings of sexual dimorphism in eight other mammals by Moore, Graham and Barr, (1951). Brusa further claimed that the body which Barr, Bertram and Lindsay had called the nucleolarsatellite was in fact the accessory body of Cajal or the Basophil clot of Levi. Coidan, (1952) described paranucleolar bodies in the spinal cord neurones of the male and female mole and cotton rat, as well as in the males of cat, monkey, gorilla and humans. However, she was unable to detect sexual dimorphism in any of these species. In the cat and the primate species studied, Coidan failed to examine the female tissues and, thus the observation of absence of sexual dimorphism in these species is entirely invalid.

Churman, (1954) investigated a large number of regions of the nervous system of

cats for the presence of accessory body of Cajal and the sex chromatin. She rejected Brusa and Coidan's suggestions that accessory body and sex chromatin body were identical showing that there was no sex difference in the frequency of the accessory body, finding its incidence to vary between difference of regions of the nervous system. Further studies of the accessory body of Cajal and the S.C. body by Lindsay and Barr, (1955) confirmed the findings of Schurman, in that there was no difference in the incidence of the accessory body of Cajal between male and female. Lindsay and Barr were able to demonstrate in female cells both the sex chromatin body and the accessory body in the same cell, by staining with methyl green following protargol staining.

Graham, (1954) continued her work in the cat by examining cell nuclei of cat embryos for 19 days gestation to maturity. She found that sexual dimorphism was already present in both nervous and non-nervous tissues at day 19 and that a similar situation was present at all stages of gestation as well as in mature animals. Austin and Amoroso, (1957) extended the work of Graham, (1954) to earlier stages of cat embryos. They examined implanted embryos between 15 and 20 days, and in these sex chromatin masses were found in about half of the embryos, in the remainder very few nuclei contained objects resembling the body. As the embryos were too early to be sexed, it was assumed that the embryos positive for sex chromatin were females and that sexual dimorphism exists from the 15th day. Of twelve 6 - 14 day old blastocysts only one of the oldest exhibited the body. In none of large numbers of earlier blastocysts was the body seen, and it would appear that in the cat, sex chromatin is not found before the 13th or 14th days of gestation. Similar work on sex chromatin in human and monkey embryos by Park, (1957) produced similar results to those of Austin and Amoroso. Park found that in the human trophoblast sex chromatin was seen at 12 days and in the embryo itself at about 16 days. In the macaque the corresponding times being 10 days and 19 days respectively.

Although most of the early studies on the sex chromatin body were carried out on cat cells, human tissues had been examined, although not as fully as those of the cat. In their original paper of 1949, Barr and Bertram briefly mentioned that sex difference existed in human sympathetic ganglion cells. A year later, Barr, Bertram and Lindsay confirmed these findings, but failed to show dimorphism in Purkinje cells, both sexes having approximately the same incidence of sex chromatin bodies.

Moore, (1962) in a description of the early stages of sex chromatin studies explained the reason for the three year period between discovery of sexual dimorphism in human cells and its routine demonstration in tissues. Difficulty was found in obtaining tissues which were fixed suitably for examination of cytological detail. Once this problem was overcome, by the use of fresh autopsy specimens and modified Davidson's solution, studies of adrenal gland and skin were initiated and in 1953, Moore, Graham and Barr reported that dimorphism was present in skin cells of the human. They studied skin biopsies from 50 male and 50 females, the mean incidence of sex chromatin bodies in females being 69% and in the male 5%, the body in the latter being smaller than the body in females.

The fact that marked sexual dimorphism was present in skin, made Moore and his co-workers realise that for the first time a human tissue was conveniently available for sex determination and that this could be used as a diagnostic aid. Two patients with ambiguous sexual development were studied by means of sex chromatin examination of skin. One female pseudohermaphrodite with adrenogenital syndrome was shown to be sex chromatin positive, thus being a genetic female, and a male pseudohermaphrodite was studied being found to be chromatin negative with a presumed XY genotype. The importance of this clinical test was quickly realised in the medical world and numerous reports were published on findings derived from the use of the skin biopsy techniques, (Hunter and Lennox, 1954; Hunter, Lennox and Pearson, 1954; Marberger

and Nelson, 1954).

Although the reliability of the skin biopsy technique was never in doubt, it was realised that the method was not perfect in that the biopsies could be distressing to certain patients, such as young children and the mentally sick. Other tissues were studied in an attempt to find a method which would make the determination of sex much simpler. Davidson and Smith, (1954) examining blood smears found that there was a sex difference in the polymorphonuclear neutrophil leucocytes. A small body, 1.5u in diameter, with a solid round head which Davidson and Smith called the drumstick was found as an accessory body to the neutrophils. In female smears they found that by counting 500 polymorphs, 6 drumsticks were invariably found, on average in 227 such cells. Such frequencies were never observed in males, any accessory body which was found being a primitive lobe or small club. From these findings Davidson and Smith suggested that the drumstick examination as a test of the genetic sex was valid but had the disadvantage of requiring skilled interpretation. Moore and Barr, (1955) and Marberger, Boccabella and Nelson, (1955) almost simultaneously published the technique of buccal muscosa scrapings for detection of sex chromatin detection almost simultaneously, both showing that sexual dimorphism was present in buccal smears. Moore and Barr found that 40 - 60% of cells were postive in females, while Marberger reported a range of 20 - 79% with an average of 46%. In the male Moore and Barr found one or two irregular clumps of chromatin in males while Marberger and his co-workers reported an incidence of only 0.6%. The technique of detection of sex chromatin by the use of buccal smears is now universal and the claim of Moore and Barr that the technique had a great future has been amply justified.

Two of the first reports using buccal smear technique were of studies on Klinefelter's Syndrome. Bunge and Bradbury, (1956) examined five cases of Klinefelter's Syndrome

finding all five patients to be sex chromatin positive. In the gonads of the three cases whose testicular histology was studied, the typical Klinefelter's picture of sclerosed tubules and clumped interstitial cells was seen, no ovarian tissue being recognisable. A laparotomy was carried out on one patient and no structures resembling internal female genitalia were found. Bunge and Bradbury concluded that further evidence was needed before the contradiction of apparently female somatic cells and male-type genitalia could be resolved.

Similar findings to these of Bunge and Bradbury were made by Plunkett and Barr, 1956) who examined two cases of testicular dysgenesis and found both to be sex chromatin positive, postulating that their cases had either an XXY sex chromosome constitution, or more likely, XX, derived from a mutation or a crossing over.

The field in which the importance of the development of the Buccal smear has been most striking, is ~~in~~ however in large scale screening of various types of populations. The first large scale estimate of chromatin-positive males in general population was made by Moore, (1959) who examined about 2000 males and found 5 chromatin positive cases. Later surveys by McLean, Harnden, Court-Brown, Bond and Mantle, (1964) screened the enormous total of 10,725 patients. Such surveys would have been impossible by any other method other than the Buccal smear method.

The exciting possibility of prenatal determination of sex by the study of sex chromatin amniotic cells was reported almost simultaneously by four groups of workers. Nettles, (1956) reported that by using properly prepared slides of amniotic cells no difficulty was met in diagnosing the sex of the unborn infant. Shortly after this report, Fuchs and Riis, (1956), Makowski, Prem, and Kaiser, (1956) and Sachs, Serr and Danon, (1956) all reported similar findings but Fuchs and his co-workers stressed that this technique should never be carried out for curiosity and that in the human, its functional value was limited. However, both Fuchs and Sachs, Serr and Danon

considered that in domestic animals it was possible that the method was applicable to prenatal sex diagnosis. As well as being present in the cells from amniotic fluid, it was found that the sex chromatin body was present in both amnion and chorion of female embryos and newborn children, (Klinger, 1957). In tissue from females, Klinger found that the body was present in an average of 91% of the cells, but in male tissue the frequency was only 21%.

In 1959, Ohno, Kaplan and Kinosita, on evidence obtained from liver cells of the Norwegian rat, postulated that the sex chromatin body was formed by only one X chromosome in the female cells. This was the first contradiction of the theory of the origin of the body postulated by Barr, Bertram and Lindsay, (1950). Ohno and his co-workers studied the prophase cells of regenerating rat liver cells and demonstrated that in cells of females, one chromosome stood out by virtue of its heteropyknosis. They claimed as only the X in the female is almost entirely made up of heterochromatin, the heteropyknotic chromosome was almost certainly an X. This feature was never seen in male prophase cells.

Similar observations in man were reported by Ohno and Makino, (1961) using liver cells from foetuses obtained after theraputic abortions. In liver parenchyma cells the prophase cells of the male contained one very small chromosome which demonstrated precocious condensation, this element having the size of a Y chromosome. The X chromosome in the male did not display positive heteropyknosis whereas in female cells one chromosome at prophase was heavily condensed and in size corresponded to the sex chromatin as well as being in the same size-group as the X chromosome. In the mouse also, it was found that one chromosome was heteropyknotic, (Ohno and Haushka, 1960), and it was thus apparent that in all three species heteropyknotic X formed the sex chromatin body.

Based on this evidence, as well as on the studies carried out by Welshons and

Russell, (1959) who found that female mice with only one X chromosome were fertile, Lyon, (1961) put forward a theory to explain mosaic effects observed in mice heterozygous for a variety of coat types. This theory hypothesised that; (1) the heteropyknotic X chromosome could be either the paternal-derived X or the maternal-derived X, in different cells of the same animal, (2) it was genetically inactivated and (3) this inactivation occurred early in embryonic life.

Although this hypothesis explained a number of apparently anomolous occurrences in the genetics of mouse coat types, a number of findings in the human did not agree with the predictions of the theory, and these led Lyon to modify her postulate. Tjio, Puck and Robinson, (1959) had carried out chromosomal analysis on a female patient with gonadal dysgenesis, some of the diagnostic features being shortness of stature, primary amenorrhea, small uterus and impalpable ovaries. They found that the patient who was sex chromatin negative had only 45 chromosomes and only one X chromosome. Thus, although XO mice were fertile females, in man the lack of an X chromosome led to infertility as well as other phenotypic abnormalities, although as predicted by Lyon the XO patient did not have a sex chromatin body. As well as the case of Tjio and his co-workers, which had an X chromosome missing, a number of patients had been found in which extra X chromosomes were present.

Jacobs, Baikie, Court Brown, McGregor, MacLean and Harnden, (1959) examined a female with secondary amenhorroea and infertile genitalia, finding that an extra X chromosome was present with a resulting karyotype 47,XXX. Sex chromatin studies on this patient revealed, as well as the presence of a single sex body in the majority of cells, two sex chromatin bodies in 14% of Buccal Mucosa cells. A sex chromatin positive male patient examined by Jacobs and Strong, (1959) was found to have, by the use of bone marrow culture, a 47,XXY chromosome complement. This patient had small testes, a feature which was also found in two patients with an XXXY sex chromosome complement and a group of cells with two sex chromatin bodies. (Ferguson-Smith,

Johnstone and Handmaker, 1960). However, as well as micro-orchidism the patients of Ferguson-Smith were mentally retarded to a degree not noted in XXY patients. Ferguson-Smith and his co-workers pointed out that as two sex chromatin bodies were present, the theory that the heteropyknotic regions of the X chromosomes in the female formed the sex chromatin was not valid and if this theory was to be correct, two sex chromatin bodies should represent four chromosomes. A further variation of the sex chromosome complement in man was reported by Fraccaro and Lindsten, (1960) who found a male child, mentally retarded and with abnormalities of the genitalia, heart and kidneys. Three sex chromatin bodies were found and a presumed 49,XXXXY sex chromosome complement.

On the basis of these findings of numerical aberrations of the X chromosomes, Lyon (1962), extended her hypothesis to take account of these cases. She postulated that, because the cases with extra X chromosomes had a maximum number of sex chromatin bodies one less than the number of X chromosomes, all X chromosomes in excess of one must be inactivated. Lyon suggested that this extension would explain how one Y chromosome could produce a male phenotype in the face of four X chromosomes, as in the case of Fraccaro and Lindsten, (1960).

German, (1962) while investigating the asynchrony of replication of human chromosomes found that one chromosome in the 6 - 12 group in the female replicated out of phase with the rest of the complement. As this late replicating chromosome was not seen in male cells, he suggested that the chromosome was an X chromosome, and pointed out that one X in the female had the common property of (1) heteropyknosis, (2) genetic inactivity, (3) asynchrony and late replication. He stressed that it was not known, however, whether they were related. Similar findings supporting the connection between late replication and sex chromatin formation were reported by Morishima, Grumbach and Taylor, (1962) who found that in sex chromatin negative XO females, no late-replication X was seen, whereas in 47,XXX cells from a mosaic female, who had

a proportion of cells with two sex chromatin bodies, two late-replicating X's were seen.

Further evidence as to the connection between the single X nature of the sex chromatin body and the suggested late replicating nature of the same X chromosome, came from studies on structural abnormalities of the X chromosome in human females. In a female with an isochromosome of the long arm of the X (Jacobs, Harnden, Buckton, Court Brown, King, McBride, Macgregor and MacLean, 1961) in which the abnormal X was larger than normal, the sex chromatin body was found to be similarly larger. Muldal, Gilbert, Lajtha, Lindsten, Rowley and Fraccaro, (1963) studied the replication pattern of the X chromosomes in a similar case with an enlarged sex chromatin body. They found that in all the 25 selected cells the abnormal X was late-replicating and they postulated that in this case, the sex chromatin was formed by only one X chromosome, this being late-replicating and that the whole of this abnormal chromosome was involved in the formation of the sex chromatin body. Similar results to these of Muldal and his co-workers were found by Gianelli, (1963).

The Lyon hypothesis, (Lyon 1962) as modified in the light of the findings of humans with supernumerary X chromosomes therefore appeared to be valid in that the number of chromatin bodies was, in most cases, one less than the number of X chromosomes. In patients, in which one of the X chromosomes was abnormal in size, for example, isochromosomes and deletions, (Jacobs etal,1961), the sex chromatin size reflected the chromosomal abnormality thus apparently confirming Lyon's theory.

However, from the findings of Muldal and his co-workers, (1963) and Jacobs and her co-workers, (1963) it appeared that, in the human, the abnormal X chromosomes did not obey the rule of random inactivation of the X's. Thus, in such cases, by this non-random inactivation, the normal X always appeared to be the active one.

Genetic evidence from studies of skin types of the mouse was used by Lyon, (1961) in her original hypothesis and in man, similar results were obtained in experiments on the X-borne gene, Glucose-6-phosphate dehydrogenase (G-6-PD). Davidson, Nitowsky and Childs, (1963) made single cell clones of cultivated fibroblasts from female patients heterozygous for the gene for G-6-PD. They found that some clones had normal levels of enzymatic activity and some were deficient, but in no case were they able to demonstrate clones which had intermediate levels. Grumbach, Marks and Morishima, (1962) found that in both male and females the levels of the enzyme was the same, and they suggested that this bore out the Lyon hypothesis in that in females, the gene for G-6-PD on one X was inactivated and so the female was in this case, effectively monosomic for the X, as males are. They further examined individuals with supernumerary X chromosomes and found, with only one exception, that the enzyme levels were in the same range as normal males and females, indicating that all X's in excess of one had been inactivated.

Although the Lyon hypothesis explains many facts concerning X inactivation and the single X derivation of the sex chromatin body, it fails to explain a number of points. Firstly, why in the human, females with Turner's Syndrome and the karyotype 45,XO are abnormal, why males with Klinefelter's syndrome and the sex chromosomes XXY are infertile and in some cases show abnormalities, (Ferguson-Smith, 1958), and why the abnormalities in males with an extra chromosome get more marked as the number of X's increase (Fraccaro, Kaijser and Lindsten 1960). Secondly, why in studies involving the X-borne gene Xg, red blood cell antigen mosaicism in heterozygous women is not seen. Mann, Cahan, Gelb, Fisher, Hamper, Tippett, Sanger and Race, (1962) found in a male patient, a red cell antigen which behaved as an X-borne dominant antigen. It was expected that this antigen would obey the Lyon hypothesis, as done in the G-6-PD locus, but Reed, Simpson and Chown, (1963)

Gorman, Re, Treacy and Cahan, (1963) and Cohen, Zuelzer and Evans, (1964) were unable to demonstrate mosaicism. The third finding which did not appear to bear out the Lyon hypothesis, concerned X-autosome translocations in the mouse, (Russell 1963). It was found that the translocated autosomal genes did not behave as they should have done, if the Lyon hypothesis of complete genetic inactivation of the X had taken place.

The explanation put forward by Russell to explain the anomolous behaviour of X-borne autosomal genes in the mouse was that inactivation of one of the X's in the female was not complete and that a gradient of inactivation occurred and parts of the X were not inactivated. A similar conclusion was drawn by Ferguson-Smith, Alexander, Bowen, Goodman, Kaufmann, Jones and Heller, (1964) in studies of females with Turner's syndrome. They found that the "streak" gonads in this condition were the result of a large deficiency of any part of the X, whereas the short stature and other stigmata were observed only when the deficiency involved the short arm of the X, such as an iso-chromosome of the long arm of the X.

These two theories, essentially similar, also explained the failure to observe mosaicism in heterozygotes for Xg antigen, (Reed etal 1963, Gorman etal 1963, Cohen etal 1964). Lindsten, Fraccaro, Polani, Hamerton, Sanger and Race, (1963) studied two cases of ovarian dysgenesis associated with iso-chromosomes for the long arm of the X and from Xg studies postulated that the Xg locus was on the short arm of the X.

These findings led Ferguson-Smith etal (1964) to postulate that inactivation might not affect a portion of the short arm of the inactive X, i.e. that part bearing the Xg locus and the genes for stature. This explanation had already been put forward by Lyon, (1963) to explain the anomolies of Xg non-mosaicism, abnormalities of patients with supernumerary X's and X monosomy. She explained these phenomena

by either (a) wrong dosage of the X chromosomes early in embryonic life before inactivation takes place, Park (1956) having found that in human embryos the sex-chromatin body was not seen until the second week, (b) incomplete inactivation of the X in either (1) some tissues, (2) at some stage of mitotic stage, (3) at some locus e.g. Xg locus. Ohno, (1961) had found that in both rat and human female germ cells heteropyknosis was absent and, therefore presumably inactivation of the X did not occur. However, as Lyon (1966) points out, this might explain the sterility of patients with X chromosomes aberrations, but would not explain the other abnormalities such as shortness in XO females and severe phenotypical abnormalities in XXXXY males.

Ferguson-Smith's (1964) suggestion that the genes for stature are on the short arm of the X and that they are not inactivated could explain the abnormalities, but so also could non-inactivation of the X in the embryo before two weeks of age. To date, it is still not known which theory is correct, if indeed either so.

Sexchromatin in the Pig

In comparison to the vast number of reports on sex chromatin in man and the cat, there is very little published concerning the pig. Cantwell, Johnstone and Zeller, (1958) first reported the presence of the sex chromatin body in nervous tissue, finding that in the spinal cord, 95.5% of female cells contained a Feulgen-positive body, whereas a similar body was found in only 5.5% of male cells. Attempts to detect a sex-specific body in non-nervous tissue were unsuccessful. Hoshino and Toryu, (1958) confirmed the presence of sexual dimorphism in nervous tissue finding that an average of 82% of female and 5.8% of male cells in nine regions of the nervous system possessed the sex chromatin body. However, they found that they could identify sex chromatin in eleven out of the twelve non-nervous tissues which they studied, the only exception being ovarian follicular cells. Hay and Moore, (1961) also found that sexual dimorphism could be detected in various regions of the nervous system, 92% of female cells and 6.5% of male cells having the body. However, McFee, Knight and Banner, (1966) reported they were unable to identify the sex chromatin body in nervous tissue due to the presence of coarse chromatin particles in the cells. However, this is the only study which has failed to find sex chromatin in nervous tissue.

Two recent reports on chromosomal abnormalities have used sex chromatin examination as a means of determining the X chromosome complement of the cases. Bruere, Fielden and Hutchings, (1968) found that in a probable freemartin pig with a chromosome complement 38,XX/38,XY, 80% of ventral horns cells were chromatin positive. Bruere and his co-workers also found that of the 164 cells which contained a chromatin body, 3 possessed a second body which was very similar to sex chromatin, a frequency which was similar to the frequency with which Bruere found sex chromatin bodies in male cells. Axelson, (1968) carried out the first study on sex chromatin in early pig embryos aged 2 - 7 days. She found that sex chromatin was present from the 45 cell

stage onward in approximately half the embryos, these being assumed to be female. It was found in this study that double chromatin threads were found protruding from the chromocentre and Axelson postulated that this might indicate that in the pig the sex chromatin body was comprised of only one arm of the X chromosome.

Porcine Nervous Tissue and Liver

Materials and Methods

For the study of pig neurones, it was decided to concentrate on one region of the nervous system in order to standardise findings as much as possible. For these reasons and for ease of collection, thoracic cord from the area of the first to fourth thoracic vertebrae was collected. Cord was chosen in preference to brain tissue because at slaughter, for economic reasons, the skull is not split and so brain tissue is impossible to obtain at commercial abattoirs. Furthermore, spinal cord was found to be much simpler to handle being less friable than brain tissue.

Two inch lengths of entire spinal cord were carefully dissected from the sectioned spinal column, and placed in buffered neutral formaldehyde. The fixative used was 4% formaldehyde, made up by the method of Lillie, (1965) with a slight modification.

Commercial 4% Formaldehyde		100 ml.
NaH_2PO_4	(Anhydrous)	3.5G
K_2HPO_4	(Anhydrous)	6.5G
Water	(distilled)	900 mls.

Fixation time for the cord ranged from 2 days to 2 weeks, no apparent deterioration of the specimens being noted with the longer time. After fixation was complete the cord was trimmed and cut into 5 m.m. long sections before being dehydrated, cleared and infiltrated with Paraplast in the standard way, all these steps being carried out in an automatic tissue processor, (Shandon Ltd.). The infiltrated tissue sections were then blocked out in Tissue-Tek blocks, (Shandon, Elliot Ltd.), using Paraplast (Sherwood Medical Industries Inc.). When cool, the blocked-out tissues were sectioned at 10u and 6 - 8 sections placed on each slide. Two staining

techniques were carried out. Cresyl Violet was used as a routine stain and the Feulgen method with counterstain was used in some cases as a specific D.N.A. stain for chromatin.

Cresyl Violet Method

(a) Bring to water through descending alcohol strengths.

(b) 0.5% aqueous Cresyl Violet, (British Drug Houses Ltd.). 5 mins.

(c) 95% Alcohol.

(d) Acidified 95% Alcohol, (0.3% Acetic Acid in 95% Alcohol). Differentiated under dissecting microscope until nuclear structure was distinct.

(e) 95% Alcohol. Rinse

(f) Absolute Alcohol. 2 changes

(g) Carboxylol (5% Phenol in Xylol). Rinse

(h) Xylol. 2 changes.

(i) Mount in D.P.X.

Using this staining method the nuclear chromatin material stained a deep purple as did the nucleolus, whereas the nucleus was stained a lighter shade. Liver sections were also studied using an identical method to that described above.

The Feulgen Method

(1) Bring sections to water.

(2) Place in normal HCl at 60°C and leave for 8 mins. This was found to be the optimum time for formalin fixed sections.

(3) Wash well in water.

(4) Transfer to Schiffs reagent for 60 - 90 mins.

(5) Transfer to sulphite rinses 3 changes for 2 mins. each.

10% sodium metabisulphite	6 ml.
1N HCl	5 mins.
Distilled Water	Made to 100 mls.

(6) Rinse in distilled water.

These Feulgen stained sections were then counterstained in order that structures other than D.N.A. could be seen.

(7) Stain in a saturated solution of Tartrazine in Cellosolve for 5 mins.

(8) Rinse off excess dye in water.

(9) Stain in 0.1% Toluidine blue in 3% alcohol for 1 min.

10) Rinse quickly in water.

11) Blot dry and place slides in 37°C oven until dry.

12) Clear in xylol and mount in DePeX.

fter staining in Toluidine blue the slides were not dehydrated in alcohols as these emoved the stain very quickly.

.N.A. stained magenta while all other tissues stained various shades of green.

00 neurones were examined from each staining technique from each animal. Each of he cells was scored for the presence of the sex chromatin body and the position of he body in the neurone, this being in one of three positions.

(1) Against the nucleolar membrane. In this position the body was roughly a triangular shape, one side being flattened against the membrane. When the body was seen in ~~the~~ this position it tended to appear larger than when found free in the nucleoplasm. Very strict criteria as to shape and size were adopted for the scoring in this position as smaller bodies were often seen in the region.

(2) Free in the nucleoplasm. When found in position 2, the sex chromatin body was almost circular and the criterion for selection could only be on size. It was only with experience that positive identification could be made, as chromatin bodies larger and smaller than the sex chromatin were

ccasionally observed.

(3) Against the nuclear membrane. The sex chromatin body found in this position adopted a shape similar to that found in position 1, being triangular in shape.

ıe staining characteristics of the sex chromatin body were also very important riteria. In Cresyl Violet-stained sections the body was a very deep purple gainst the lighter nucleoplasm and in the Feulgen sections it stained a magenta shade gainst the green of the rest of the cell. In both cases, by using these criteria,) difficulty was encountered in identification.

; well as staining liver sections with Cresyl Violet, a Schiff-type reaction was .so carried out in order to remove the coarse chromatin present in such cells as ırried out by Lang and Hansel, (1959). Small sections of liver were taken at the .asgow Slaughter House and placed in one of three fixatives, namely 4% Balanced ›rmal Saline, 90% Alcohol and A.F.A., this being modified Davidson's solution. It .s subsequently found that the sections fixed in 4% B.N.F. withstood the prolonged drolysis best and thus this fixation was used routinely.

nventional Schiff's reagent was used in the study as well as a Schiff-type reagent corporating Cresyl Violet. The latter chemical was prepared by adding 1 gram of dium hydrosulphite to 100 ml. of 0.01% solution of Cresyl echt Violet, (Matheson, leman and Bell). Loss of colour was noted immediately and a layer of liquid :affin was poured on to the surface of the fluid which was stored in a 100 ml. agent bottle, which was then placed in the dark for 24 hours. At the end of this ne, a dense precipitate was present which when filtered left the filtrate completely lourless. The bottle was then stored at +4°C in the dark with a thin layer of quid paraffin on top.

e procedure of staining was as described for the Feulgen staining of nerve tissue

lescribed previously. The acid hydrolysis in the technique exposes reactive aldehyde groups by cleavage of the Purine-Deoxyribose bond. These aldehydes are then readily demonstrated with Schiff's reagent. As a result, only D.N.A. is stained after such a procedure, and thus by carrying out the technique coarse chromatin is removed by the hydrolysis and the only material stained is D.N.A.

Although the amount of hydrolysis in the Feulgen technique, previously described, was easily determined, the amount necessary for the removal of chromatin was more difficult to determine. In order to arrive at the optimal amount of hydrolysis, variations were used in the strength of the Hydrochloric Acid, the temperature at which the procedure was carried out and the time of hydrolysis. The strengths of acid used were 4N, 5N and 7N, the times 5, 8, 10, 12, 14, 16 and 18 minutes and the temperatures room heat (20 - 22°C) and 56°C. From all the combinations available 7N at 56°C was found to be the optimal, all times mentioned above being used in combination with that temperature and acid strength.

However, although the staining and removal of chromatin was not unsatisfactory, at no combinations used was it possible to obtain optimal results. When hydrolysis times of less than 14 minutes ~~was~~ were used, the amount of chromatin removed was not sufficient to make a sex chromatin body obvious in female tissues, whereas when the chromatin was cleared satisfactorily, the staining quality of the material dropped severely, probably due to the small amount of chromatin remaining.

From these results, it was decided that further experiments along these lines ~~was~~ were not going to be fruitful. It was thus concluded that the technique of Lang and Hansel was not transferable to the pig, the original work having been carried out on cattle.

Results

Tables 7&8 presents the results obtained from cell counts from cresyl violet-stained

sections of adult spinal cord. 100 cells were counted from each of ten male and ten female cords, every cell being scored for the presence of the sex chromatin body, which if present was judged to be in one of the three positions in the nucleus. In female tissues 61.8% of the ventral horn cells were sex chromatin positive and a further 0.4% contained two bodies. From table 7 it can be seen that the distribution of the body in the nucleus was almost equal between positions 1 and 2, that is flattened against the nucleolus and free in the nucleoplasm, whereas the body was found against the nuclear membrane in only about 3% of the cells in which it was found, (figures 7, 8 and 9).

In the male nervous tissue, the frequency of sex chromatin positive cells was found to be 1.4%, as many bodies being found against the nucleolus as were found in the nucleoplasm, although the numbers were so low as to preclude taking any interpretation from the positions. (Figure 10).

For technical reasons it was only possible to carry out the Feulgen staining method on five female and five male adult spinal cords. Tables 9 and 10 presents the results obtained by this staining method. As before, 100 cells were counted from each specimen, the presence of the sex chromatin body noted, position recorded and finally the presence of Feulgen-positive clumps which might be confused with the sex chromatin body, if the criteria used were less strict than in the present study. This latter scoring was adopted after analysis of the cell counts from the Cresyl Violet sections were carried out. From this it was noted that, as well as a low percentage of sex chromatin positive cells in the male, the frequency of positive cells in the female was almost identical to that obtained by Cresyl Violet staining. (Figure 11).

A mean frequency of 61.4% positive cells was found in female cells with a further 1% containing two sex chromatin bodies. Again the positives of the vast majority of bodies were distributed almost equally between those flattened against the nucleolus

nd those free in the nucleoplasm. In 9.2% of the female cells a single D.N.A. positive clump of chromatin was found which was considered not to have the morphology of the sex chromatin body. Of 46 such clumps found in the 500 cells counted, 23 were in position 1 and 20 in position 2.

In male spinal cord stained with Feulgen a similar frequency of D.N.A. positive clumps were found, (8.4%), the distribution again being almost equal between position 1 and 2. A mean frequency of 2.4% of male cells were found to contain the sex chromatin body, 50% being flattened against the nucleolus and 50% free in the nucleolus. (Figure 12).

amples of spinal cord taken from Foetal pigs, whose amniotic membranes had been reviously used were treated in the same way as the adult cords, that is, male and emale tissues were stained using both Cresyl Violet and the Feulgen methods.

ections from the spinal cords of eight males and six females were examined and 200 ells from each cord examined, 100 from the Cresyl Violet method and 100 from the eulgen stained sections. From table 11 it can be seen in the Cresyl Violet sections, 3.7% of female cells contained a single body and a further 1.5% had two such bodies. he distribution of the sex chromatin bodies is also shown in table 11. Of he 400 bodies observed, 384 (96%) were flattened against the nucleolus and the maining 16 (4%) were found in almost equal numbers free in the nucleoplasm and gainst the nuclear membrane. The number of single non-specific chromatid clumps ich could have been confused with the sex chromatin body was 95 (15.8%) and of these (94.8%) were in position 1, and the remainder (5.2%) in position 2. As can be en from table 11, the single non-specific clump was observed in both sex chromatin sitive and negative cells.

e results from Feulgen-stained sections of five of the six females described above e presented in table 12. In this study 61.4% of the cells were sex chromatin

positive and 1.8% contained two sex chromatin bodies, and of the 325 bodies observed, 95.7% were flattened against the nucleolar membrane. Non-specific clumps of chromatin were seen in 67 cells, (13.4%) and of these 64 (95.5%) were seen in position 1.

Studies on male fetal spinal cord were similarly carried out using both Cresyl Violet and the Feulgen technique, the number of specimens used in each method being 3 and 5 respectively. The results using Cresyl Violet are shown in table 13 which shows that 3.25% of the male cells were sex chromatin positive, 96.1% of the bodies being found in position 1. A further 4.12% of the cells contained a single non-specific chromatin mass and 87.9% of these were found flattened against the nucleolus. The corresponding results using the Feulgen method showed that 6.4% of cells were sex chromatin positive with a further 5.6% showing a body which might be confused with sex chromatin. Both the sex chromatin bodies and the non-specific chromatin showed a similar distribution to that found in the Cresyl Violet sections in that 94.4% and 89.3% respectively, were found against the nucleolar membrane, (table 14).

The attempts to detect sexual dimorphism in liver section, whether stained by Cresyl Violet or after a Schiff's reaction were unsuccessful. In the untreated sections, large amounts of coarse chromatin was present in both sexes which made detection of the sex chromatin impossible. In sections treated by acid hydrolysis, the coarse chromatin was gradually removed until after 18 minutes treatment, the majority was removed but unfortunately at this stage, the staining reaction was so weak that identification was impossible. (Figures 13 and 14).

mnion Preparations

aterials and Methods

regnant uteri at various stages of the gestation period were collected at the lasgow Slaughter House and taken to the laboratory for processing. Dissection f the uteri was started as soon after slaughter as possible, usually within three ours.

sing a new scalpel blade an incision was made directly over the Foetus at the apex f one horn, and the wall of the uterus and the placenta were carefully cut through nd reflected until the innermost layer of the placenta, the amnion was reached. he uterus and outermost layers of the placenta were completely peeled away, leaving he fetus free, surrounded only by amnion and amniotic fluid. Alcohol-cleaned icroscopic slides were marked using a diamond marker before applying the amnion. n incision, the length of which approximated the width of the slide, was made in the mniotic membrane. The microscope slide was grasped by the marked end, the other nd carefully inserted between the fetus and its amniotic membrane and eased in ntil about three quarters of the slide was covered by amnion. The slide was lifted o gently and the amnion was cut by a sharp pair of scissors allowing an overlap of bout 2 cms. at both sides, and at the free end of the slide. The amnion was raightened, any wrinkles smoothed out and the overlaps were then applied to the dersurface of the slide, for better application of the membrane to the slide. The lides were immediately placed in equal parts of Ether and 95% Ethanol (Papanicolau's xative) in Coplin jars and the lids sealed with vaseline.

e foetus was then drawn through the incision in the amnion and sexed according to e external genitalia if it was advanced far enough for this to be done. In the se of the foetuses in which the sexing by observation of the external genitalia was possible, the gonads were removed and fixed in 5% Formal-Saline for histology.

The gonads were obtained by one of two methods. In the male it was found to be easier to obtain the testes by traction on the gubernaculum if the foetus was old enough. An incision was made in each scrotum and the gubernaculum grasped by a pair of rat-toothed forceps. Gentle traction was applied until the testes were drawn through the incision. They were cut clear of the gubernaculum and placed in 5% Formal Saline. In female foetuses and early male foetuses the gonads were isolated following laporotomy, cut free using fine scissors, and placed in fixative.

As well as comparing the sex, as found by amniotic preparation method, with both the external genitalia and the gonads, a sample of spinal cord was obtained, in order to confirm genetic sex and also to carry out studies on frequency of sex chromatin in foetal neurons. Ageing of the foetus was carried out by the use of the Crown-Rump length measurement following the procedure of Marrable and Ashdown, (1967). When working with the youngest embryo, i.e., those of 30 days, which measured only 2.5 cms., the amniotic sac was submerged in isotonic saline while carrying out the dissection. At that stage of embryonic development the volume of the amniotic sac is small and the internal pressure per unit area of amnion is much higher than is found later in gestation and great difficulty was encountered, in mounting the amnion on to the slides. By submerging the sac, the fluid pressure was partially equalised and the amnion was much more easily manipulated onto the slide.

Mounting of the amnion was carried out for all the foetuses in the uterus, the only difference with the rest of the foetuses being the technique for obtaining the foetus in its amniotic sac. The placentation in the pig is epithelio-chorial and the connection between placenta and endometrium is purely one of aprosition, the chorionic villi fitting into corresponding pits in the uterine mucosa (Arey, 1965). With such a placentation, it is very simple to peel the chorion from the uterus without damaging any structures.

Once the foetus and amnion at the apex of each horn had been processed, it was a

simple matter to pull the next chorionic sac through the hole in the uterus left by the previous foetus. An incision was made in the extremity of the chorionic sac and the amniotic sac was eased out from the incision. The procedure of making a mount of the amnion was identical to that previously described.

The advantage of the latter method was the fact that less incisions were required, thus resulting in less risk of accidentally cutting the amnion and losing the amniotic fluid. If loss of fluid occurred in early foetuses, it was extremely unlikely that a useable amnion preparation could be made.

The staining technique used was that of Moore and Barr, (1955) with slight modification. The slides and their applied amnion preparations were left in fixative for at least 24 hours, the time varying between 24 and 48 hours. The slides were then stained in the following way.

70% Alcohol	5 mins.
50% Alcohol	5 mins.
Distilled Water (1 change)	5 mins. each
1% Aqueous sol. Cresyl Violet	5 mins.
Decolourisation in 95% Alcohol using staining Microscope.	
95% Alcohol	2 - 3 dips.
Absolute Alcohol (1 change)	Until nuclear detail was clear.
Zylol (1 change)	5 mins. each

When clearing was complete, the slides were mounted with a coverslip and D.P.X. When the D.P.X. hardened, the excess amnion was stripped off with a sharp scalpel blade.

Because the amniotic membrane was not always completely flat and free from overlap the staining intensity varied over the slide. The slides were scanned under a 10x objective and a suitably stained area chosen for viewing. Selection of the cells for sex chromatin counting was done using a X50 oil immersion objective together with

a X15 wide angled eyepiece.

In certain cells in the amnion of female foetuses a darkly staining body was found flattened against the nuclear membrane, whereas in male cells this did not appear to be present. In good preparations of amnion, four layers of cells were found, each containing a quite distinctive arrangement and type of cell.

(1) The most superficial layer of cells as seen under the microscope corresponded to the cell layer of the amnion furthest from the foetus. This layer was pavement-type epithelium. The nuclei were large, relatively deeply staining and contained multiple chromocentres, an average of about 4 in each cell. No sex specific structure could be detected in this layer.

(2) The layer of cells immediately deeper to (1), contained very large round, lightly staining cells which did not form any regular pattern. The nuclei of both sexes contained multiple chromocentres.

(3) The ~~middle~~ third of the ~~five~~ four layers of cells demonstrated sexual dimorphism. Medium sized ovoid nuclei containing only about 1 or 2 chromocentres, showed in the female the presence of a deeply staining small body flattened against the nuclear membrane. This was very seldom seen in the male.

(4) The innermost layer of the amnion consisted of large lightly staining cells, not dissimilar to layer number 2. The cells were often observed in clumps of 3 or 4, and ~~displayed multiplied~~ multiple chromocentres were present. All coding numbers were covered, and re-coded, so that the observer did not know the identity of the slide. The cell layer which demonstrated the sex chromatin body was focused under 100 x Oil objective and the 5 cells closest to the centre of the field were chosen for evaluation of the body. Only cells completely free of overlapping were chosen, and the criteria for identification of the sex chromatin body were that it was darkly staining,

flattened against the periphery of the nuclear membrane, and was within a certain size range. These criteria meant that the incidence of the body was probably recorded as lower, than if less severe criteria had been chosen. 20 such fields were examined and the final percentage frequency noted against the slide code number. All the preparations from one litter were similarly evaluated and the results noted before the slides were re-identified by removing the covering.

In those foetuses in which gonadal tissue had to be removed in order to sex them, the gonadal tissue was allowed to fix in 5% Formal-Saline for at least 3 days before being embedded, sectioned and stained with Haematoxylin and Eosin. This procedure was carried out in the standard way.

As an extension of the examination of the amniotic membranes, samples of amniotic fluid were aspirated in order to study free cells. Prior to the incision of the membrane, 10 ml. of fluid was removed using syringe and a 20G. needle. The fluid was transferred to a 10 ml. centrifuge tube and the sample centrifuged for 5 minutes at 750 r.p.m. The supernatent fluid was then removed, leaving only the button of cells and debris. 3 ml, of Papanicolaou's fixative was gently poured on to the cells and allowed to remain in contact for approximately 30 minutes. At the end of this time, the sample was again centrifuged, and the supernatent removed, being replaced by 0.5 ml. of fresh fixative. The button was then suspended in the fixative and the suspension dropped on to clean grease-free slides. The slides were then processed in an identical manner to that carried out on the amniotic membranes, being finally mounted with DePeX. prior to examination.

Results

A total of 63 Amnion preparations were made from 8 uteri at different stages of gestation, ranging from 30 to 95 days. Thus the preparations obtained were representative of the last three quarters of development of the foetuses, the gestation

period of the pig being just less than 120 days. Of the 63 amnions processed, 54 were suitable for sex chromatin examination, the remainder lost being mainly from young embryos in whose amniotic sacs the fluid pressure was high, resulting in difficulty in obtaining the membrane without damage.

Table 15 shows the results obtained together with the data obtained from the anatomical features of the foetuses. 100 cells were counted from each preparation and scored for the presence or absence of the sex chromatin body. From the incidence of the body, a diagnosis was made of the genetic sex of the foetus, (figs. 15&16). After the diagnosis of the sex had been made, the slide was decoded and other details of foetus noted. These details were, firstly, the sex of the foetus as diagnosed by external morphology, if this was possible, Secondly, in those cases in which this was not possible and in addition in some cases in which the foetus could be sexed morphologically, the sex according to the ~~histology~~ histological structure of the gonads was noted.

The crown-rump length of most of the foetuses was noted and for each litter a mean length was obtained. From this value the litter was aged using the formula of Marrable and Ashdown, (1967). In the case of the one litter whose measurements were not carried out, foetuses 1 - 3, the exact age was known from the history of the sow.

Of the 54 foetuses sexed by examination of the amniotic ~~membraine~~ membrane, 24 were diagnosed to be female and 30 to be males. The range of incidence of the females was 25% to 63%. The mean for the females was calculated to be 42,87% with a standard deviation of +9.5% whereas in the male foetuses the mean incidence was 0.4% + 1.6%. An attempt was made to correlate the incidence of the body with the age of the foetus but no such correlation was possible as the numbers were small and any conclusions would have been of dubious value.

The sex of the membrane in all 54 cases was the same as that diagnosed by either

external morphology or histological appearance. In no case did the incidence approach overlapping, the lowest frequency found in the female being over seven times that of the highest male frequency, (25% and 3% respectively).

The attempt to display sexual dimorphism by the examination of free amnion cells failed due to the lack of such cells in the preparations. Five such samples were studied and in all of them, no more than 5 or 6 cells were obtained.

Discussion

The present study on amniotic preparations is the first successful attempt to find sexual dimorphism in non-nervous tissue in the pig, with the exception of the study of Hoshino and Toryu, (1958) whose findings will be discussed later. The discovery of sex chromatin in the amnion cells of female foetuses was not an unexpected finding, as in two other species of Artiodactya similar findings have been made. Möller and Neimann-Sörensen, (1957) found that they could diagnose foetal sex using allantoic fluid and both amniotic and allantoic chorion. However, in their study, 11% of the diagnoses made on cells obtained from fluid were incorrect, although the authors attributed the errors to lack of experience and in fact, all the later diagnoses were correct. Using membrane preparations, these authors were able to obtain a 100% accuracy in sexing, although the frequency with which sex chromatin was seen in male preparations was very high, (up to 20%), a finding which could easily confuse sexing in some cases. In the sheep, Bruere (1968a) also succeeded in sexing foetuses using amnion preparations. The results obtained in this study were much more convincing than in the cow, 100% accuracy being obtained and in no male preparations was a chromocentre found which corresponded to the sex chromatin body. However, in the female preparations, the incidence with which Bruere found sex chromatin to be present, (24% - 66%) was lower than that found by Moller, who found it to be 52 - 68%.

Thus the present study on the pig is in agreement with previous work carried out on amnion preparations. The accuracy with which diagnosis of sex can be carried out in pig amnion is equal to that in the sheep (100%), although the frequency with which the body is found is lower than in the sheep being 42% and 50% respectively. However, interspecies comparison cannot be made with any degree of confidence, as for example in the mouse the incidence in female tissue has been found to be almost 80% (Vickers, 1967) and in man 91.5% (Klinger 1957). The possible main explanation is

the criteria used for the identification. In pigsin whose cells chromatin is commonly seen, it is considered necessary to apply very strict criteria for selecting a body as being sex chromatin. It would appear likely that as Moller and Neimann-Sorensen, (1957) found the incidence of sex chromatin in female amnion preparations to be 60% and in the male 12%, they were including in both sexes a number of new non-specific chromocentres. However, this can only be hypotheses, and although it would appear likely, the situation as it stands at present with reference to the domestic animals is that in the pig and sheep, sexing of the foetus is 100% accurate when preparations are made of fetal membranes, whereas the accuracy is more variable when cattle are studied.

The failure to determine the sex of the fetus by examination of free amniotic cells in the present study was very disappointing, this being due to the paucity of cells obtained. In 1956, success was reported in attempts to sex human embryos by aspiration of amniotic fluid and subsequent findings of sex chromatin bodies in female embryos, (Fuchs and Riis, 1956; Sachs, Serr and Danon 1956), the latter group postulating that the finding of sexual dimorphism might be useful in the domestic species. Sach and Danon, (1956) however examined epithelial cells from cattle and sheep fetuses of various ages and were unable to demonstrate sex chromatin bodies, concluding that it was not possible to carry out prenatal diagnosis on these by free amnion cell examination. In contradiction to this finding, Moller and Neimann-Sorensen found that they could diagnose sex by using free amnion and allantoic cells. From their results it would appear unlikely that the technique could be of great practical use. Over 38% of these preparations were unsuitable for diagnosis, either because of poor technique or lack of cells. Of the remainder, 11% were incorrectly diagnosed, and in no case did the authors report the frequency with which the body was found. In their whole membrane preparation of male fetuses, Moller and his co-workers found that up to 20% of cells had sex chromatin bodies, and from this it must be assumed that in some cases diagnosis would be

equivocal if the number of cells were few. As both the present study and that of Bruere, (1966) have shown that the numbers of cells obtained from amniotic fluid is very low, it must be concluded that a diagnosis made using amniotic cells would be a very doubtful one. On these grounds the findings of Moller and Neimann-Sorensen must be viewed with care. It therefore seems unlikely, at present, that antenatal sexing is possible in the domestic animals so far investigated. In the human however, this technique has been used to detect male fetuses which had a 50% chance of having haemophilia due to their mothers being carriers of the condition, (Riis and Fuchs, 1966). Although these authors considered that there was a risk to the well-being of the fetus, they were of the opinion that the procedure was justified. However, in domestic animals even if sexing was possible, there would appear to be no practical reason for carrying out such a procedure, with the possible exception of cattle in which there was twin pregnancy, in which there is a 50% chance of them being unlike. Under these circumstances 95% of the females will develop as free-martins, and if it was possible to demonstrate an unlike twin pregnancy, there might be ground for terminating the pregnancy if the owner was only wanting heifer calves. With this potential exception, the statement of Sachs, Serr and Danon, (1956) that the technique of examination of free amniotic cells in domestic animals might be useful, is unfounded.

One further conclusion which can be made from the present study of amnion preparations is that in the pig foetus, sex chromatin is certainly seen from 30 days onwards, although obviously it could be and most likely is, seen much earlier than this stage. Axelson, (1968) in the only other study of fetal pigs, examined embryos aged 2 - 7 days. She found sex chromatin to be present in blastulas consisting of only 45 cells. Although Axelson did not attempt to age the 45 cell stage, it must be assumed that the embryos were no more than 3 days old, as in the study, embryos of 800 to 900 cells were found, which could have been no older than 7 days. If, as seems very likely, the blastula was only about 3 days old, it indicates that sex chromatin is

formed earlier than in any other species so far studied.

Studies have been carried out on numerous species namely, rat, rabbit, cat, dog, man and macaque monkey. Of these the rabbit displayed the earliest demonstration of sex chromatin, being visible at the 400 cell stage, (Melander 1962). This represented a morula of about 4 days post-mating. In the rat, sex chromatin can be seen in advanced blastocysts at the beginning of implantation, which is about 6 - 7 days, (Austin 1966). In the cat, (Austin and Amoroso, 1957), and the dog, (Austin 1966) sex chromatin was seen at a similar stage to that in the rat. In these species, however, the age at which implantation occurs is about 14 - 15 days. Glenister, (1956) found the chromatin body to be present in the syncytiotrophoblast of an implanting human blastocyst, and it is found to be present in the human embryo itself by the 18th day, (Park 1957). Thus in virtually all the species which have been studied, sex chromatin is seen to be visible about the time of implantation, the exception being the pig. In this species Axelson postulated that the implantation stage was the 900 cell stage. If this is the case it would appear that the sex chromatin body appears about 5 or 6 days pre-implantation. Axelson explained this species difference by the fact that development in the pig appears to be early in various other stages of development. It appears unlikely that in this case the sex chromatin body had been wrongly identified. Although no incidence of the body was given the fact that it was not seen at all until the 45 cell stage and then in approximately 50% of the embryos is strong indication that the body was in fact sex chromatin.

Combining the results of Axelson and those of the present study, it appears that the sex chromatin body can be seen in the pig embryo as early as the 45 cell stage and from then on up to the 900 cell stage, (approx. 7 days). There is no information between 7 and 29 days, but there is no reason to suspect that the body could not be identified. From 30 days to 95 days the sex chromatin body can be seen in the

cells of amniotic preparations. Although the last 20 days approx. have not been studied, it must be expected that sex chromatin will be identifiable in amnion preparations and also as described below, in nervous tissue.

It was decided during the study of the amnion preparations that it would be advantageous to compare the results obtained, with the findings from another fetal tissue. For this reason, spinal cord from 20 foetuses was processed, but due to technical difficulties, only 14 specimens were suitable for Cresyl Violet staining, all of whom were in the age range 79 - 83 days. Great difficulty was encountered with younger foetuses, mainly due to fixation problems and as a result these had to be discarded. Further difficulty occurred in the Feulgen process, due probably to damaging effect of the acid hydrolysis resulting in a final total of five male and 5 female tissues surviving the procedure.

The results obtained by Cresyl Violet and the Feulgen method show remarkable co-relation (Tables 11 - 14). In the female tissues, occasionally difficulty was encountered in that in addition to the sex chromatin body, chromatin clumps were often seen which in some cases resembled the sex chromatin but in most cases could be differentiated from it. This is clearly shown in tables 11 and 12. As can be seen just over 60% of all female cells examined contained a single sex chromatin body and a further 1.5% contained two. In addition, almost 16% of cells stained with Cresyl Violet contained a chromatin clump which although not the sex chromatin could possibly have been scored as such if rigid criteria were not applied. Proof that these extra bodies were in fact chromosomal material comes from the finding of almost identical results with Feulgen staining. In the male tissue a similar picture emerges in that a much smaller frequency of cells displayed chromatin which was not the sex chromatin body, but was D.N.A. positive, (5.6% in Feulgen sections and 4.9% in Cresyl Violet). In addition to this, over 3% of males contained what was identified as sex chromatin in sections of both staining methods.

From the results obtained in this study it is clear that sexual dimorphism can be easily determined in fetal ventral horn cells. In each fetus studied the sex, although known by gonadal structure, appearance of the external genitalia or the results of the amnion preparations and in some cases all three, was not disclosed to the observer until after the scoring was finished. Thus in all cases, the sexing as obtained by nerve cell analysis was the same as that obtained by other methods. The study further shows that two methods are now available for the sexing of foetuses, that of amnion examination and neuron examination. Unfortunately, it was not possible to study the nerve cells of any foetuses younger than 79 days, but from the present study and that of Axelson it must be assumed that sex chromatin will be formed in nerve cells almost as early as the cells themselves are formed.

From the literature it appears that very little work has been carried out on fetal spinal cord and as such, comparisons are extremely difficult to make. In the cat, however, Graham, (1954) carried out a detailed study on fetal nerve tissue. She observed a much higher incidence of sex chromatin in the female than was observed in the present study, (92% and 61.4% respectively), but as she also noted a much higher incidence in amnion preparation from the cat than was found in the present study, (97% and 41% respectively), the discrepancies must be attributed either to a difference in species or in interpretation. It would however appear to be more likely that the discrepancies are due to the former, as in the present study on fetal pig neurones, even if all the chromatin which was classed as confuseable was, in fact, added to the total sex chromatin, the final incidence would still only be about 75%, still well below the incidence found by Graham.

A significant difference was noted in the distribution of the sex chromatin body in the fetal pig neurones as compared with the adult results. In the five female foetuses studied 96.1% of sex chromatin was seen to be flattened against nucleolus whereas in the adult females studied, only 44.1% were seen in this position. Graham

(1954), noted that the position of the sex chromatin varied with the time of gestation, in early gestation the majority of the bodies being seen adjacent to the nuclear membrane whereas in older foetuses and in postnatal life it was most commonly seen against the nucleolar membrane. The findings in the present study are in agreement with those of Graham, although at the corresponding stage of preganancy, two-thirds way to term, only 68% of the cat sex chromatin bodies were against the nucleolus compared with 96% for the pig. However, as Graham studied only one fetus in each sex at this stage, detailed comparison is not valid.

The explanation put forward to explain this apparent movement of the body throughout pregnancy, was that altering cellular environment such as occurs after chromatolysis (Lindsay and Barr, 1953) results in a change of the topography of the sex chromatin. If this can occur with one kind of alteration in the enviroment, Graham suggested that unknown changes in cellular metabolism could lead to a similar result. If this is the case, it could be a possible reason for the altering position of the sex chromatin body in the pig between 80 days old foetuses and adults. There is however, no way of knowing, at present, at which stage the alteration of topography occurs in the pig. Although in the cat, the body appears to progress towards adult position throughout pregnancy, a parallel cannot be assumed, as obvious species differences do occur. This is obvious, as in man, (Bertrand and Girard, 1956), and the monkey, (Prince, 1952) it has been shown that the body is most commonly seen adjacent to the nuclear membrane.

Thus, it appears that a great many factors may play a part in the position of sex chromatin body, among which are stages of gestation, metabolic state of the cells and the species studies. Although many interesting points arise from a study such as the present one, only a few concrete facts can be interpreted. One of these is that sexual dimorphism was easily detected in the ventral horn cells of the pig fetus and that in no case did the male incidence approach the female incidence.

Secondly, it has been very clearly shown that all the bodies interpreted as sex chromatin were indeed D.N.A. material, ~~and~~ thus eliminating the possibility that the body could be misinterpreted. This point will be discussed more fully later. Lastly, it appears very likely that, as there is a certain percentage of cells which contain chromatin material not dissimilar to the sex chromatin body, bodies which in the male are identified as sex chromatin may in fact not be sex specific. Again this point will be more fully discussed when the findings from the adult tissues are considered below.

The results obtained from the studies of adult spinal cord of the pig show very clearly that sexual dimorphism exists in the ventral horn cells. (Tables 7 to 10) The results of the Cresyl Violet stained sections of female tissues are presented in table 7. A mean frequency of 61.6% with an additional 0.4% of cells containing a double body was found. When these results are compared with the corresponding male results, (table 8) it is found that only 1.4% of the cells contained sex chromatin. However, according to the Lyon hypothesis, (Lyon 1962), a sex chromatin body represents an inactivated X chromosome. As this hypothesis is now considered to be valid under many circumstances and generally accepted, this means that a male cell containing a sex chromatin body has effectively the sex chromosomes YO which is considered non-viable, such a cell never having been found. Similarly, a female cell containing two X's would have the genetic constitution OO, which is also considered inviable. It could however, be postulated that XXY and XXX cells would result in the presence of a positive male cell and a double positive female cell respectively. These constitutions however, have never been found in the present study or in any previous study and the hypothesis must be rejected. The third possibility is that the bodies which have been interpreted as sex chromatin are in fact not the sex-specific body. If the theory is correct, the three most likely explanations are (a) the body is the Basophil clot of Levi, (b) the

ccessory body of Cajal, (c) non-specific chromatin. Brusa, (1952) put forward he theory that the sex chromatin body described by Barr and Bertram, (1949) was n fact either the Basophil clot of Levi or the accessory body of Cajal. However, his theory was refuted by Lindsay and Barr, (1955). Nevertheless, in the pig, he possibility still exists that the extra sex chromatin bodies observed were not n fact chromatin. In order to test this hypothesis, sectioned slides were rocessed using the Feulgen techniques. The results obtained by this method tables 9 and 10) show a remarkable correlation with Cresyl Violet sections even onsidering that the sections were from the same animals. From the Feulgen esults it appears that all material which was interpreted as sex chromatin was in act D.N.A. and therefore not either of the bodies suggested by Brusa, (1952). n an attempt to elucidate the problem of the identity of the supernumerary sex hromatin bodies, the incidence of Feulgen positive chromatin bodies which were een in both male and female sections was determined.

he results of this study, (tables 9 and 10) shows that in both sexes, a certain ercentage of cells contain D.N.A. clumps, which vary in morphology from cell to ell and which are not the sex chromatin. The frequency with which these clumps ere seen was the same in male and female sections, (9.2% and 8.4%). This would ppear to indicate that firstly, these clumps are not sex specific. Secondly, it uld appear very likely that as these clumps do vary in morphology, by chance a ertain proportion will closely resemble the sex chromatin body and often may be ored as such. This unfortunate complication in pig neurones, although in no y effecting in the distinct sexual dimorphism which is present, has undoubtedly ayed a part in the wide discrepancies in the reported incidences of sex chromatin the pig. Cantwell, Johnston and Zeller, (1958) reported that both male and male cells contained paranucleolar bodies which were D.N.A. positive, but stated at they could be distinguished from sex chromatin by their much smaller size.

However, as in the present study, approximately 50% of the chromatin clumps were observed to be free in the nucleoplasm, it seems unlikely that they are the bodies described by Cantwell. Furthermore in many cases the chromatin seen was larger than the sex chromatin body. Cantwell and his co-workers further found that in female and male ventral horn cells 95.5% and 5.5% respectively, of cells were sex chromatin positive. No indication, however, was given of the results from individual animals, and therefore range and deviation from the mean is unfortunately unknown. Hoshino and Toryu, (1958) found in three male and three female pigs that 4.5% and 88% respectively of cells contained sex chromatin and agreed with Cantwell and his co-workers that the majority of the bodies were found at the nucleolar membrane. Similar results were obtained by Hay and Moore, (1961) who found 93% and 6% sex chromatin in ventral horn cells of one female and one male animal, again finding the majority of bodies to be present adjacent to the nucleolus. The presence of cells containing two chromatin bodies was reported by Bruere, (1968a). He interpreted this as corresponding to the sex chromatin body in the male and found the shape of both to be more irregular than sex chromatin.

Bruere's report and the present study raises the question of why, if a certain percentage of male cells contain a body which has been scored as sex chromatin, although its structure has been found not to be identical with the sex chromatin body, (Hoshino and Toryu 1958, Cantwell, Johnstone and Zeller, 1958), female cells have not been scored as containing this body in addition to the sex chromatin body. This must either mean that either bodies were not seen in chromatin positive cells and by chance were seen only in cells lacking sex chromatin, thus being scored as chromatin positive, or that if present they were not identified for some reason or lastly, a breed difference in chromatin distribution occurs.

Although impossible to prove on the small samples studied, it is interesting to note that the two studies in which cells have been seen with two sex chromatin bodies present

(Bruere 1968 and the present study), were carried out on the tissues of Landrace, Large White and crosses of these breeds. Work carried out on the other species, (Cantwell etal, Hoshino etal, Hay etal), have failed to demonstrate this feature. Further studies on this possible breed difference would be enlightning.

The discrepancy in the incidence of the sex chromatin body in female neurones between the present study and previous work is also hard to explain. In previous reports the frequency has been found to vary from 85% (Bruere 1968) to 96% (Cantwell etal 1958), compared with 62% obtained in the work presented here. The simplest explanation is that of differences in criteria for selection of the body. As identification must be purely subjective, quite wide variations are not unexpected. Using very rigid guidelines for the body may possibly result in a number of sex chromatin bodies being rejected because of minor variation from the accepted morphology. Similarly, if flexible criteria are used some non-specific chromatin may be identified as sex chromatin. A certain amount of variation from individual to individual is to be expected and unfortunately, no published reports on the pig have presented the scores for each animal studied, giving only the mean. Reporting of these figures would give a better idea of this variation. In the present study the range in the female tissues was found to be 51% - 72% although the majority were grouped very closely about the mean. It is therefore possible that selection of another set of 50 females would give an entirely different mean. To date, although four sets of data have been published, excluding the present report, only 15 animals have been examined, and many more will have to be studied before an accurate estimate of true frequency in the pig is obtained. A further example of variation between either various workers or animals is the topography of the sex chromatin body. Cantwell etal and Hay and Moore found that the majority of the bodies were situated adjacent to the nucleolus while Hoshino and his co-worker and the present work found only about 50% of sex chromatin to be in this position.

From the study of sex chromatin in the spinal cord of the pig, it is obvious that sexual dimorphism is easily recognised, in no case did the incidence of sex chromatin in the male approach that in the female, the highest frequency in the male being 5% and the lowest in the female being 51%. It has been shown that all the bodies scored in Cresyl Violet stained sections are in fact D.N.A. positive using Feulgen and there is no question of other nuclear bodies having confused with chromatin. In both sexes, a small percentage of cells demonstrate an extra sex chromatin body, the most likely explanation of this being non-sex-specific chromatin whose morphology is highly variable.

The attempts to detect sexual dimorphism in adult non-nervous tissue were unfortunately unsuccessful. As stated previously, in untreated tissue, large amounts of coarse chromatin was present throughout the liver cells studied. This was in contrast to the findings of Hoshino and Toryu, (1958) who detected sex chromatin in eleven tissues including liver. However, the illustrations presented were of an equivocal nature, chromatin being observed in both male and female cells. In addition the body which was classified as sex chromatin appeared to be of variable size.

These findings were not confirmed by Cantwell, Johnstone and Zeller, (1958) or Hay and Moore, (1961). Both these sets of workers were in agreement with the present study in that sexual dimorphism is not present in pig tissues, with the exception of nerve material. In the light of these findings, the work of Hoshino and Toryu must be rejected.

In a further attempt to demonstrate sex chromatin in pig liver, the method of Lang and Hansel, (1959) was utilised. By the use of acid hydrolysis of cattle liver, pancreas and adrenal, these workers were able to detect sexual dimorphism. This finding, however, has not been confirmed and in addition the illustrations were not particularly convincing. However, as cattle, like the pig, do not display sex chromatin

in non-nervous tissues, (Moore, 1966), it was considered that the use of Lang's technique might possibly demonstrate sexual dimorphism in the pig. It was found that this was not the case, however, as whenever the coarse chromatin began to be removed the staining reaction became so weak that morphological characteristics were not visible. Although the present study utilised a different stain from that of Lang and Hansel it seems unlikely that this was the cause of the discrepancy, as in Lang's illustrations a considerable amount of chromatin still remained at the stage at which dimorphism was detected. Thus, either Lang's findings are not valid or such a species difference exists as to make the technique inoperable in the pig.

Section IV.

Autoradiographic studies and tritium-induced abnormalities.

AUTORADIOGRAPHY

Introduction

In 1957, Taylor, Woods and Hughes, developed an autoradiographical technique which when later applied to mammalian chromosomes was to be of the utmost importance in the determination of the nature of both normal and abnormal. Taylor and his co-workers used thymidine which was known to be a precursor of deoxyribonucleic acid but not ribonucleic acid (Reichard and Estborn, 1951). However, instead of using ^{15}N or ^{14}C as used previously, Taylor incorporated tritium into the pyrimidine ring of thymidine. As tritium has a low energy (18kev) this meant that for the first time, radioactive thymidine could be incorporated into the DNA molecule and the resultant labelling obtained in the overlying photographic emulsion of such a resolution that only the areas immediately over the radioactivity were exposed, due to the short emission range of the isotope (1u).

In his original paper Taylor studied the replication pattern of Vicia faba and 3 years later made the first study of mammalian chromosomes by this method, (Taylor, 1960). Using embryonic Chinese Hamster cells Taylor found not only that the sex chromosomes replicated later than the autosomes, but also that one of the X chromosomes in female cells replicated in a different manner to its homologue. In male cells both the X and Y were late replicating, the entire Y chromosomes being labelled but only the long arm of X being so. One of the X's in the female was labelled in an identical way to the male X, whereas both arms of its homologue was labelled. Taylor also found that in male cells which had had tritiated thymidine available only in the early stages, the Y was completely free of label and only the short arm of the X had replicated. In female cells one X had labelling only on the short arm as did the male X, whereas its partner was virtually unlabelled. It was also noted that asynchrony exists between pairs, but generally speaking the

replication pattern was similar in homologous pairs. These findings of Taylor were of the utmost importance in leading the way to the study of the replication patterns of human and, to a lesser extent, animal chromosomes.

One of the first reports of autoradiographic studies on man was by Lima de Faria, Reitalu and Bergman (1961). These workers found that by the use of pulse labelling of lymphocytes, asynchrony of replication existed not only between the pairs of chromosomes, but also between the homologues themselves. This finding of asynchrony between pairs was substantiated by German, (1962), who reported that most homologous pairs had similar labelling patterns, although he did report that marked asynchrony within pairs did exist, numbers 1 and 3 amongst others demonstrating this feature. In 3 out of 4 females studied, one chromosome of the 6 - X - 12 group replicated much later than any other chromosome although this was only rarely seen in the 4th patient. This asynchronous chromosome was not seen in male cells, German interpreting it as being an X chromosome although in a few cells its morphology did not fit this interpretation.

Morishima, Grumbach and Taylor, (1962) agreed with German concerning asynchrony of the X chromosome in females and in addition found that the other X chromosome was not conspicuously different from the other autosomes in replication pattern. They examined two patients with numerical abnormalities of the X chromosomes and put forward their findings as being substantiation of the Lyon hypothesis (Lyon, 1961). A sex chromatin-negative female patient with gonadal dysgenesis and a karyotype 45, X was found to have no late replicating chromosome suggesting that it was the late replicating X which normally formed the sex chromatin. It was also noted that the heavily labelled X in normal females was found more often near the periphery of the metaphase spread than was expected by chance, in this feature adopting the position characteristic of the sex chromatin body. The second patient examined by Morishima was a mosaic XO/XX/XXX. No late-replicating X was found in the

majority of the 45,XO cells, whereas in the 46,XX and 47,XXX one and two chromosomes respectively had this feature. In occasional XO cells a late replicating chromosome was seen, whereas in a few XX cells no such chromosome was seen. Morishima's report was the first to identify chromosomal abnormality by the use of autoradiography, showing the value of this technique as a diagnostic aid.

Gilbert, Muldal, Lajtha and Rowley, (1962) carried out a quantitative study of the replication pattern of cells to which had been added tritiated thymidine late in the 'S' period (4 hours before termination of the cultures). In contrast to Lima-de-Faria, (1961), German (1962) and Morishima etal, (1962), they found that only the X chromosomes showed marked homologue asynchrony of replication pattern, no statistical difference being detected in any other pairs. Their findings agreed with previous reports concerning late replicating pairs and they suggested that a very consistent pattern of chromosome replication existed, at least for all cells of the culture studied.

The following year Grumbach, Morishima and Taylor, (1963) studied further patients with numerical and structural abnormalities of the X chromosome. They examined three 47,XXX female and one 48,XXXY patients who had two sex chromatin bodies, and a 49,XXXXX female with multiple bodies, up to four in some cases. In all these cases late replicating X's were found, the number of these being one less than the number of X chromosomes and they noted that in a few cells there was a slight asymmetry between the late X's. Grumbach also studied skin cells from the Penta-X patient and found that when cells were fixed immediately after they had been in contact with tritiated thymidine, a small proportion of the interphase nuclei had 4 peripheral areas with high grain densities. From this finding and from the observations that in cases with super-numerary X chromosomes the number of late replicating X's was the same as the number of sex chromatin bodies. Grumbach

and his co-workers suggested that the X chromosome which was late replicating also formed the sex chromatin body.

The same workers examined one female who had one normal X and one chromosome which was identified as an isochromosome of the long arm of the X i.e., 46,XXqi and a second female patient who was mosaic 45,X/46,XX. In both these cases, the late replicating X chromosome in the XX cells was the iso-X, and as the sex chromatin body was larger than normal, this was put forward as evidence that the late replicating X formed the sex chromatin body, the iso-chromosome in both these cases being larger than the normal X.

Shortly after this report the findings on further cases of female patients with the karyotype 46,XXqi were published. Simultaneously Muldal, Gilbert, Lajtha, Lindsten, Rowley and Fraccaro, (1963) and Gianelli (1963) studied the replication patterns of the X chromosomes. Muldal etal confirmed the finding of Grumbach, (1963) that the X chromosome which invariably replicated late was the iso-X. They also found that the replication pattern over the 2 arms of the suspected iso-X was symmetrical, confirming that the chromosome was indeed an isochromosome. Gianelli's findings supported those of Muldal and his co-workers in that he also found symmetry over both arms of the iso-X which replicated late, and noted that this labelling pattern over both arms was very similar to that of the long arm of the late replicating X in normal females. Gianelli also studied a tripo-X female and his observations were similar to those of Grumbach, (1963) finding 2 late replicating X's and in cells with low cell counts a degree of asynchrony was detected between the 2 late-labelling chromosomes. Similar late replication of the iso-X was reported by Miller, Mukherjee, Bader and Christakos, (1963). They suggested that non-random inactivation of the iso-X chromosome had occurred, with the result that the late replicating and inactive chromosome was always the abnormal X. However, they also accepted the possibility that if random inactivation of one of the

2X chromosomes had occurred, those cells in which the normal X was inactivated may have been inviable.

Schmid, (1963) carried out an extensive autoradiographic study of human chromosomes in an attempt to determine the comparative labelling patterns of chromosomes of similar morphology. Tritiated thymidine was added to growing cells so that it was available during the last 2 hours of the 'S' period. A number of late replicating and early replicating areas were found and these were used as the basis for chromosome identification. Certain of these areas were similar to those reported by Morishima etal, (1962) and German, (1962). Schmid found that in virtually every female cell one X chromosome was heavily labelled at the end of the 'S' period and also that the Y chromosome was, in the early and middle stages of labelling, the most heavily labelled of all chromosomes in the 21-22-Y group of chromosomes. He also reported that the areas of late replication were similar to those areas which demonstrated secondary constrictions and suggested that either late replication of D.N.A. caused a delay in the cycle of despiralization or that delayed replication and a special type of spiralization were both related to structural changes in these regions. This report was the first autoradiographical study to show how morphologically similar chromosomes in the human could be positively identified by their labelling patterns.

Schmid, as well as studying both normal males and females, studied three cases of Down's syndrome, two 21/D translocation mongols and two cases of myeloid leukaemia. It was found in the cases of trisomy Mongolism that three heavily labelled and two lightly labelled chromosomes were found in group 21 - 22, the normal karyotype showing 2 heavily and 2 lightly labelled chromosomes. In the 2 cases of chronic myeloid leukemias, 2 early replicating and one late replicating chromosomes were found in group 21 - 22. The Philadelphia chromosome characteristic for this

condition was found to be late replicating being frequently found to be the latest replicating of the 4 chromosomes. Thus Schmid was able, by replicating patterns, to identify both the extra chromosome in Mongolism and the deleted Ph' chromosome as being the same one. In one of the 21/D translocation cases, by the use of labelling patterns, he was also able to conclude which member of the D group was involved in the translocation.

Rowley, Muldal, Gilbert, Lajtha, Lindsten, Fraccaro and Kaijer, (1963) and Atkins, Böök, Gustavson, Hansson and Hjelm, (1963) confirmed the work of Morishima etal, (1962) who had found that in patients with supernumerary X chromosomes, all but one of these replicated late. Atkins and his co-workers studied an 49,XXXXY infant and found that 3 late replicating X's were present in skin and testes and in addition heavy labelling was present over heterochromatic foci, including the sex chromatin bodies. Similar findings were obtained by Rowley and her co-workers studying a XXXXY male. They found clear evidence of 3 late replicating chromosomes in the C group in virtually all cells examined. By the use of grain counts, they were able to show that the ratio of labelling over the late-replicating X's to the total grain count of the cell was comparable to that found in the normal female. This was taken as evidence that all the late-replicating X's behaved similarly to the normal single heavily labelled X chromosome in females. Rowley, Muldal, Lindsten and Gilbert, (1964) examined a further X chromosome abnormality, a patient with a ring X chromosome (Xr) and the karyotype 45,XO/46,XXr/47XXrXr. They found that in all cells the late labelling X chromosome was the ring chromosome, thus confirming the findings that structurally abnormal X chromosomes were invariably the late replicating X chromosomes.

Using continuous labelling, German, (1964) made a more detailed study of the terminal stage of the 'S' period than in his previous report (German, 1962). His

findings were very similar to those of Schmid, (1963) in that he found differences in labelling patterns between homologous pairs which were structurally similar, notably 2 chromosomes of group 4 - 5 which finished replication earlier than the other two. Similar results were obtained in group 13 - 15 in which 2 replicated early, 2 intermediately and 2 later, and also in group 21-22-Y. In this latter group 2 replicated very early, whereas the other two continued with light radioactivity late in the 'S' period with the Y being the latest replicating of this group. German also noted that homologue asynchrony existed in a few chromosomes, a feature which Gilbert etal, (1962) had been unable to detect.

From the findings of autoradiographic studies on patients with structural abnormalities it was known that the abnormal X chromosome was invariably the late replicating X (Muldal etal, 1963; Rowley etal, 1964). This however, appeared to contradict the Lyon hypothesis, (Lyon 1961), which postulated random inactivation. Two theries were put forward by Rowley etal, (1964) to explain this apparent deviation from the hypothesis. If the hypothesis was correct, the invariable late replication of the abnormal X could be due to random inactivation, with the cells in which the normal X was inactivated, dying due to the imbalance of genetic material. The alternative was that in fact, inactivation as expressed by late-replication was not random in cases of X chromosome abnormalities.

Although it was assumed that random late-replication of the X chromosome did occur in normal mammalian females, there was no way to demonstrate if this was, in fact, the case. However the mule, a hybrid between a female horse and and a male donkey, was found to be ideal for demonstrating the randomness of late-replication, and presumably, therefore, inactivation. In the female mule, one X chromosome is derived from each parent and the two X's are morphologically separable. Mukkerjee and Sinha, (1964) examined 33 cells which showed one late-replicating chromosome. In 17 of these cells the heavily labelled chromosomes had the size and morphology

of the X chromosome of the donkey and the other 16 were identified as being the horse X chromosome. This finding was one of the clearest of pieces of cytological evidence that late-replication of the X chromosome is a random mechanism and therefore, it could be either the maternal or the paternal X which presumably undergoes inactivation.

All autoradiographical studies up to 1964 had been either carried out by continuous labelling to investigate the terminal stages of D.N.A. synthesis or by pulse labelling to show asynchrony. Hsu, (1964) by the use of a DNA synthesis blocking agent fluoredeox yuridine, (FUdR) was able to demonstrate, after relieving the blockage, the late-replicating Y chromosome in the Chinese Hamster was inactive at the commencement of the 'S' period. Mukherjee, (1964) and Mukherjee and Sinha, (1965) studied early D.N.A. synthesis in the human by means of a long pulse labelling technique. Tritiated thymidine was added before the onset of D.N.A. synthesis, removed 20 - 34 hours later and in this way it was certain that the isotope was present at the beginning of the 'S' period. It was found that a marked asynchrony of DNA synthesis existed, in that one chromosome in each of groups C, 13 - 15, 16 - 18 and 21 - 22 appeared to be the last chromosomes to begin replication, thus indicating that homologue asynchrony as well as asynchrony between pairs existed. Neither report attempted to identify the individual chromosomes which were late in initiation.

Hsu and Lockhart, (1965) examined a male infant with the karyotype 49,XXXXY, and studied both the terminal stage of DNA synthesis and the initial stage of the 'S' period, the latter by using the DNA synthesis blocking technique of Hsu, (1964). The Y chromosome and three chromosomes of the C group were found to be late-replicating the latter being interpreted as X chromosomes. These findings were in complete agreement with those of Rowley etal, (1963) and Atkins etal, (1963).

In their study of the pattern of labelling at the beginning of the 'S' phase, they found that three presumed X chromosomes and the Y chromosome were relatively inactive in tritiated thymidine uptake. Thus from this convincing study it would appear that the chromosome in the C group of the female that Mukherjee, (1964) and Mukherjee and Sinha, (1965) had noted to be late in starting replication was probably an X chromosome which presumably was the one which replicates late in the normal female.

Further indication of the extreme usefulness of autoradiographic studies has been in the identification of chromosomal abnormalities as presented by Yunis, Hook and Mayer, (1965). Four females with trisomy Mongolism and four balanced translocation carriers for Down's syndrome, two G/G's and two G/D's translocations were studied. The extra G group chromosome in the trisomies were generally found to be late-replicating, thus agreeing with the findings of Schmid, (1963). The same chromosomes designated G_1 found to be involved in two types of translocations. In the G/G translocation a G_1 chromosome was translocated onto a G_2 chromosome, and similarly, in the case of the D/G translocation, a G_1 chromosome was translocated onto a D_2 chromosome. As well as being very important findings in themselves, the translocation showed that the newly formed chromosome tended to retain the original patterns of the two chromosomes which made it up.

Domestic Animals

In comparison with human chromosomes very few autoradiographical studies have been carried out on domestic animals. There would appear to be two main reasons for this. Firstly lack of money and workers and secondly, the structure of the chromosomes. In the goat, cow, sheep and dog virtually all the autosomes are acrocentric and thus even approximate pairing of homologues is exceedingly difficult and is liable to result in gross inaccuracies. However, there are advantages in

the cow and the dog, in that the sex chromosomes are easily separated from the rest of the karyotype on morphological grounds and it has been in the former species that the majority of radiographical studies have been carried out.

In the cow the sex chromosomes are the only non-acrocentric chromosomes, the X being a large submetacentric and the Y a small metacentric chromosome, (Sasaki and Makino, 1962). Therefore, the sex chromosomes in this species can be unequivocably identified. Mukherjee and Sinha, (1963) and Gartler and Burt, (1964) studied the replication patterns of the sex chromosomes and found that in the female one of the X chromosomes continued its replication later in the 'S' period than its homologue, the short arm of the late X finishing replication later than the long arm. In the male, the Y chromosome was found to be the last to begin D.N.A. replication and was one of the last to finish.

Mukherjee and Sinha (1964) in their study of the replication patterns of mule chromosomes, as well as finding a late replicating X chromosome in both the horse and donkey haploid set, studied the autosomal replication patterns. Although they found that approximately equal numbers of cells contained a late X from the horse set and the donkey set, they noted that there was a difference in the labelling patterns of these two types of X chromosomes. In the donkey late X, the telomeric region of the long arm appeared to terminate replication later than the rest of the chromosome. No such pattern was found in the X chromosome of the horse. Mukherjee and Sinha found that there was no correlation between the size of a particular chromosome and its time of termination of D.N.A. synthesis. They also found that there was a great variation in the rate of D.N.A. synthesis among different chromosomes of the complement and also among different segments of the same chromosome.

Evans, (1965) studied the terminal stage of D.N.A. synthesis in the goat, cow and

pig. His findings in the cow agreed with those of Mukerjee and Sinha, (1963) and Gartler and Burt, (1964) in that one of the X's in the female finished its replication later than its homologue. Evans also found that in both X chromosomes, the long arm replicated before the short arm and that the short arm of one X was the last segment to terminate synthesis, a similar finding to that of Mukherjee and Sinha. In the goat the X chromosomes were found to be the third longest chromosome pair and one of these in the female was found to replicate late. A heavily labelled chromosome was observed in the pig and this conformed to the morphology of the X chromosome, this being confirmed by Cornefert- Jensen, Hare and Abt, (1968). Evans however, did not study the replication pattern of the autosomes in any of these species.

The first report using autoradiography as a diagnostic tool was by Gartler and Burt, (1965). A kidney culture was studied from a male bovine animal in which the Y chromosome was no longer identifiable as a separate structure. One subacrocentric chromosome with a secondary constriction was suspected to be a translocation of the Y and a large acrocentric. The part of the marker chromosome which was suspected to be the Y chromosome was found to be late replicating and also to start replication later than most of the rest of the chromosomes in the chromosome complement. Gartler and Burt, although unable to be certain, concluded that, as the replication pattern was very similar to a normal Y chromosome, the Y was present as a part of the abnormal chromosome.

Although a late replicating X chromosome was seen in bovine females as in other species, it was found that neither X chromosome of the cow replicated out of phase at the beginning of the 'S' period (Mukherjee, Sinha, Mann, Ghosal and Wright, 1967). This difference between the cow, man (Peterson, 1964) and the Chinese Hamster (Hsu, 1964) was explained by Mukherjee as a possible result of the intermittent replication of the cow X chromosome at the beginning of the 'S' period.

He found that in pulse labelling experiments, both X chromosomes started replication at a comparable time, but after a short time one of them ceased synthesis entirely. It was only during the last part of the 'S' period that this chromosome finished its replication during a very short period, this being the heavily labelled X as seen at the terminal stage of the synthesis period.

One further domestic animal, the dog, has been studied by means of autoradiography by Fraccaro, Gustavsson, Hulten, Lindsten, Mannini and Tiepolo, (1965). As was found in man, cow, horse, goat and pig, one X chromosome replicated later in the 'S' than its homologue. In this species, as in the cow, the X and the Y are easily distinguished, being the only non-acrocentric chromosomes in the complement. The late-replicating X was clearly demonstrated in bone marrow cells labelled in vivo and blood cells grown in vitro. Kidney interphase cells were similarly labelled in vitro and in several nuclei, areas at the periphery were particularly heavily labelled, being interpreted as labelled sex chromatin bodies. Fraccaro noticed that there appeared to be no correlation between the size of chromosomes and the time of replication, a finding which agreed with those of Mukherjee and Sinha, (1964) in the mule.

Gustavsson, Fraccaro, Tiepolo and Lindsten (1968) found one heifer with a structurally abnormal X chromosome among a group of female cattle heterozygous for an autosomal translocation, and postulated that it originated from an interchange between an X and one unidentified autosome. The length of the abnormal X eliminated the possibility of a pericentric inversion and the possibility of the X being a isochromosome of the long arm was not considered, although from the measurements of the chromosome, this would appear to have been unlikely. Continuous labelling was carried out during the terminal stages of the 'S' period and in the 50 cells which had a single heavily labelled chromosome, this was identified as the normal X. Although these findings appeared to contradict the findings

in human females with structurally abnormal X's (Rowley etal 1964), the abnormalities were not comparable, in that in this case the abnormalities appeared to arise from a translocation of unknown origin whereas in the human cases, the abnormal chromosomes originated entirely from the X. In the mouse, (Evans, Ford, Lyon, and Gray, 1965) a similar X-autosome translocation was found to be late replicating in only about half the cells whereas in the other 50% a late replicating chromosome assumed to be the normal X was found.

The finding of a heavily labelled chromosome in the goat which was presumed to be the X (Evans, 1965) was confirmed by Short, Hamerton, Grieves and Pollard, (1968) in a study of an intersex goat with a XX sex chromosome complement. In this species the X chromosome cannot be positively identified and it was assumed that the karyotype was 60,XX as no Y chromosome was found, thus apparently confirming the diagnosis of the karyotype.

Although the amount of work carried out on the replication pattern of domestic animals has been very small, it has been found that the general picture of labelling of the sex chromosomes is similar to that in both the Chinese Hamster and Man. The case reported by Gustavson etal, (1968) is an example of the potential value of autoradiography in domestic animals and it also raises some interesting questions concerning randomness of the late replication of abnormal X chromosomes in cattle, a field which it would be hoped will be very fruitful.

Materials and Method

Tritiated thymidine of specific activity 2,000 mc/mM (Radiochemical Centre, Amersham) was used throughout this study. At an early stage of the experiments, thymidine of specific activity 3,000 mc/mM was used, but the impression was gained that the frequency of breakages and other radiation-induced aberrations was higher than that obtained by using 2,000 mc/mM 9.5 ml Sterile Hanks solution (Burrough's Wellcome) was added to make the volume of isotope to 10 mls. As the thymidine purchased contained 0.25 mc, 0.4 ml of the dilute solution added to each culture gave a final concentration of 1 uc of isotope per ml of culture medium.

Peripheral blood cultures were prepared as described previously and to each culture was added 0.4 ml of prewarmed radioactive thymidine. It was considered necessary for the added fluid to be at the same temperature as the culture, in order to avoid any possibility of cold shock to the chromosomes. The thymidine was added as quickly as possible and the bottles returned to the incubator immediately. As the isotope was usually added within 8 hours of harvesting the cultures, it was considered unnecessary to carry out this procedure aseptically. The actual time of addition of the thymidine depended on the type of labelling required. Generally, if heavily labelled cells were required, the thymidine was added at 67½ hours, whereas light labelling was obtained by addition of tritium at 69 hours. In both cases, a fairly wide variation in labelling patterns was obtained.

The cultures were harvested and stained with 2% Aceto-orcein in the manner described previously, except that those slides which were to be processed immediately were only taken through differentiation and dehydration and were not cleared in Xylene. Slides which were not to be used immediately, were in addition cleared and mounted with D.P.X. as for permanent preparations. Before processing, the latter type of slides were placed in Xylene overnight before easing off the coverslip with a scalpel

blade, taking care not to scrape the slide surface. When the coverslip was off, the remaining D.P.X. was removed by washes in Xylene and Ethanol and finally washed in fresh Ethanol before being allowed to dry in a dust-free atmosphere. Once dry the slides were dipped in a 0.5% solution of Formvar in ethylene dichloride for 10 seconds, allowed to drip for a further 10 seconds and then dried in a dust-free atmosphere. After this stage the slides were ready for application of the emulsion. It was essential that the formvar was allowed to dry thoroughly before application of the stripping film. If this was not done, it was found that the film was extremely difficult to remove, pulling off a large percentage of cells when it did come free. This was used in preference to liquid emulsion because of its long storage time (about 1 year at $4^{\circ}C$) as well as relative ease of application. All manipulations of the stripping film ~~was~~ were carried out in a light-proof dark room under a red safelight, (Kodak Wratten filter No. 1) illuminated by a 25W. bulb. Although great difficulty in handling the film was experienced in the initial stages, once experience was gained the procedure was found to be relatively simple.

The photographic emulsion is mounted on a glass plate and backed by a gelatin base for ease of handling. Using a sharp scalpel blade the outer 5 mm. of the film on all four sides of the plate was removed. The periphery of the film is more firmly attached to the plate and by removing this portion, the rest of the film is stripped off more easily. The film was then sectioned into 8 pieces of approximately 50 mm. 40 mm. using the fresh scalpel blade. Care had to be taken that the incisions completely cut the film. That this occurred could be felt by the blade cutting against the glass plate. Once the scalpel blade had been used to cut one film, it was discarded to avoid any subsequent activation of the grains in further films due to its blunt surface.

If pieces were not completely sectioned, difficulty occurred in separating them from

the plate and in some cases, tearing of the film occurred. Repeated cuts to complete the separation was not advisable as this resulted, after development in bands of exposed grain along the edges. A band of black grains was commonly found after development along the cut edge of the film even after only one incision.

The technique used was that of Schmid (1963) with modification, including the use of formvar (Hsu, Schmid and Stubblefield, 1964). The glass plate with the cut-out sections of film was placed in 95% Ethanol for 2 - 3 minutes, in order to negate any possible effects of atmospheric humidity.

It was then removed from the Ethanol and one piece of film was loosened from the plate by using a fresh scalpel blade to lift up the 4 edges of the film squares. It was then a relatively simple matter to lift off the whole square using a pair of broad-tipped forceps by holding one edge of the piece and gradually easing it off completely. Care was taken to avoid touching any more than the edge of the film. Once the piece was completely free of the glass it was dropped emulsion-side downwards in a tray of clean tap water at about 30°C. The temperature at which the water was held was important. If it was higher than about 32° - 33°C, the film sank to the bottom of the tray and was lost. If the temperature was lower than 26°C, the film did not spread out properly and difficulty was encountered in smoothing the film on the slide.

The film thrown into water at 30°C swirled for a few moments then spread out completely, although a few crinkles and bubbles were present. After 2 or 3 minutes it was completely smooth and pliable, and was subsequently easily manipulated. Using the fingers, the film was completely smoothed out and the slide to be processed was picked up by the diamond marked end and lowered into the water. Held at 45° to the surface of the water, the slide was positioned under the film so that the

short edge opposite that held in the fingers was parallel to the short edge of the film, such that there was a film overlap of about 5 - 10 mm. on three sides of the slide edge. Using the far short edge of the slide, the film was lifted from the water and the film allowed to drape over it. By holding the slide at an angle, the water drained away from the space between the film and the slide. The overlaps over three edges were flattened out on the under surface and the film in contact with the chromosomes was carefully smoothed using the fingers so that no bubbles were present on the surface. Bubbles or creases between the film and slide were liable to loosen the film during the subsequent development and fixation with resulting movement of the film.

A cardboard box threaded with string from side to side with gaps of about 5 cms. between the strands was used to dry the film. The slides, chromosome side up were placed carefully across the strings. A hair drier (Ronson Ltd) with flexible coil hose was used for this drying. Using full heat, the air flow was directed at the slides and left for about 10 minutes. At the end of this period the slides were placed upside down in the hot air to dry the overlap on the undersurface. When both sides were completely dry, they were placed in individual slide envelopes made of thick dark paper. The envelopes were wrapped in light-proof paper and placed in a tin box together with some silica gel in a gauze packet. The box was closed and the lid sealed by adhesive tape. The container was then stored at $+4^{\circ}C$ until the slides were ready for processing.

An exposure time of 5 days was found to be suitable for the study and this time was adhered to throughout the study, in order that results would be as consistent as possible. At the end of the exposure period, the slides were taken from their envelopes under a red safelight in the dark room, care being taken not to damage the film surface. The back of the slides were painted with a thin layer of DePex (British Drug Houses), in order to minimise movements of the film during the

subsequent processing. The DePeX was dried thoroughly in a flow of hot air and when thoroughly dry, the slides were carefully placed in a staining rack, leaving an empty space between each slide. This was done in case a film became loose during the processing and if a gap was present between slides, the chances of the loose film interfering with its neighbour were minimised.

The stripping film was developed for five minutes in Kodak D-19b developer made up according to the makers' instructions, five seconds gentle agitation being carried out every minute. All developing and fixing was carried out at a temperature of 18°C. After developing, the slides in the staining rack were drained of excess developer, gently lowered into a dish of distilled water and agitated gently for 60 seconds. At the end of this time they were drained again and placed into Kodak Unifix fixative, again made up according to manufacturer's instructions. Fixation time was twice the time taken to clear the film, namely four minutes. The slides were then placed in very gently running water at 18°C for approximately 30 secs. and then dried thoroughly in the hot air flow, after which they were ready for examination.

Care had to be taken all through the process to treat the slide and films as carefully as possible. The slides were lowered into and removed from solutions very gently. The running water had to be extremely slow and at the same temperature as the other solutions, as it was found that if the water was too cold or hot, film-slip was more likely to occur. Depending on the degree of labelling expected the chromosomes were either photographed before application of the stripping film, and re-photographed with a superimposed labelling pattern, **or** the film was applied, the labelled chromosomes photographed and re-photographed with the film removed.

In chromosomes preparations which were made from cultures in which the tritiated

thymidine was added at 67½ hours, the chromosomes were photographed before application of the film. The preparation was mounted with coverslip and DePeX before photography, as described above. This was necessary as in heavily labelled cells it was impossible to determine the quality of the underlying chromosomes. The chromosomes were photographed and the co-ordinates noted, in an identical manner to that used for standard blood cultures. 35 mm. panchromatic Microneg film (Ilford) or Microphile (Kodak) was used with development in Ilford ID 36 developer and fixation in acid fixative. At this stage, the negatives were not printed, unless required for another purpose. The coverslip was removed as described earlier and the slides coated with Formvar. The stripping film was applied and processed as described previously. It was found that by applying the Formvar and allowing it to dry before application of the stripping film, the staining quality of the chromosomes was not adversely affected by the processing and it was not necessary to restain them. However, if Formvar was not used, or if it was not dried thoroughly before film application the Aceto-Orcein was taken out of the chromosomes, resulting in relatively unstained chromosomes which had to be counterstained. If this was required, the staining method of Schmid, (1963) was followed. This was based on Giemsa stain and was made up as follows.

Distilled water	100 ml
M/10 Citric acid	3 ml
M/5 Ma_2HPO_4	3 ml
Methyl Alcohol	3 ml
Stock Solution of Giemsa (Gurr)	5 ml

The staining time depended on the degree of decolourisation of the chromosomes, and varied from 1 - 4 mins. at room temperature, before being washed in distilled

water twice. Once the chromosomes were suitably stained and dried, the DePeX and the overlapping film on the undersurface of the slide was scraped off with a new scalpel blade. The slide was then mounted using DePeX and 50X24 mm. coverslips and allowed to dry before viewing. The metaphase spreads previously photographed were located and re-photographed in an identical manner to that described previously.

In the case of chromosome spreads which were expected to have only light labelling i.e. the tritiated thymidine had been added late in the 'S' period, the stripping film was applied and processed without prior photography. In such cultures, the incidence of unlabelled cells was fairly high and time was wasted in photographing large numbers of cells out of which only a small number were expected to be labelled. Once the film was dry, it was examined for staining quality of the chromosomes and if decolourisation had occurred, counterstaining was applied. When the film was finally dry, the DePeX and overlapped film was removed and the chromosomes were ready for photography.

It was not necessary to mount the slides, as the labelled chromosomes could be examined directly by application of microscopic oil to the film without damaging it. The slide was scanned for good quality metaphase spreads with suitable labelling. These were photographed as before with 35 mm. film and the co-ordinated recorded. When all the spread had been photographed the stripping film was removed and the unlabelled chromosomes re-located and photographed.

The method used to remove the stripping film was that of Hsu, Schmid and Stubblefield, (1964). The slide bearing the film was immersed in warm water for about 10 seconds, at the end of which time the excess film on the edges of the slide was scraped off together with about 1 mm. of film at the marked end. It was then replaced in the water for a further 10 secs., and then using a pair of broad-tipped forceps the film

was gently pulled off. The slides were then dehydrated in ascending strengths of Alcohol before being cleared in Xylene, mounted, examined and the spreads re-photographed.

After printing both labelled and unlabelled metaphase spreads at X 3000, karyotypes of the unlabelled cells were prepared as described previously. Prior to permanent attachment of the chromosomes to the cardboard, the labelled chromosomes were cut out and paired using the previously arranged karyotype as a guide. With two exceptions, no re-arrangement of the karyotype was carried out, even if the labelling pattern of the homologous chromosomes was dissimilar. One exception to this rule was the members of pairs 14 - 15. In this sub-group, one pair of chromosomes had a very distinctive labelling pattern, compared with the other two and as only a slight difference in length existed between the larger and the smaller pair, in some cases this being non-existent, it was considered valid to pair the chromosomes on the basis of labelling pattern.

In female cells, the indentification of the X chromosomes led to occasional difficulties. In most cells, the karyotype did not need to be altered, as one late replicating chromosome was present in the position assigned to the X. Occasionally a late chromosome was found in the chromosome pair 10 or 12, i.e. the pairs on either side of the X. Unless the labelling pattern was such that the chromosome was obviously very far out of synchrony with its partner, no alteration in position was carried out. However, in certain cases the replication was such that there was no option but to re-arrange two pairs in order to make this chromosome the X. However, this was necessary in only very few cells and under the circumstances was considered valid. With exception of pairs 14 - 15 and the occasional X, no re-arrangement was carried out. Both labelled and unlabelled karyotypes were tacked on to cardboard, an extra print attached to the card and the two karyotypes stored

until evaluation of the labelling.

It was not considered feasible to evaluate the labelling patterns of the chromosomes by exact grain counting, due to the very heavy labelling of those cells which were in early part of the 'S' period when the tritiated thymidine was added.

Evaluation was thus carried out by the allocation to each segment of a score of 0 - 8, depending on the amount of label present. Absence of any grains was scored as 0, 1 - 10 grains as 1, 11 - 20 as 2 and so on up to grade 8 which contained more than 70 grains. In those cases in which the actual numbers of grains present in any segment could be assessed, this count was re-calculated as a grade number. Otherwise, an attempt was made either to estimate the grain count by counting or a subjective assessment was made of the grain density and the segment was assigned a score. Each segment of every cell was evaluated by one of the two methods and the scoring was kept as consistent as possible from cell to cell, by rescoring random cells and comparing the two scores obtained. Using this method, it was found that repeatability, using this method, was remarkably accurate.

In order that the replication patterns of chromosome segments, as well as entire chromosomes, could be evaluated, each chromosome was divided into a number of segments such that the length of each segment was approximately 2% of the total chromosomal length of the haploid cell.

160 cells were selected for analysis, the criteria for selection being that the chromosomes were free of overlapping and were not unduly contracted. Each of the 106 segments in the diploid female cell and 105 in the diploid male cell were scored as described above. After analysis, the cells were divided into groups for detailed evaluation of replicating patterns. The three groups selected were based on total grain score for the cells of 0 - 100, 101 - 200 and over 200, these

representing lightly labelled cells which were in the late stages of the 'S' period when the tritium was added, medium labelled cells at the middle of the 'S' period and the third group, heavily labelled cells. The number of cells in each group was 47, 56, 57 respectively. Each group of cells was then treated separately although in an exactly similar fashion.

The autosomes and the early replicating X chromosome were analysed together, the late X in the female and the Y in the male being evaluated later. The total number of grains (represented by grain scores) over all the cells in the group was calculated for each segment of the chromosomes. In order that the X was given the same weight as the autosomes, the values for the autosomes were meaned so that the cell might be considered as a haploid set including the single X. The total values for the segments were then recalculated to give each segment an equal length, the original segments, although being approximately the same length, in a few cases differed quite markedly. Thus the value for each segment was multiplied by the factor:- $\frac{1000}{L}$ where L was the segmental length expressed in parts per thousand, the total length of the cell being one thousand. The recalculated value of each segment was then used to establish the ratio of the grain score in the formula:- $\frac{g \times 1000}{G}$, where g was the grain score for the segment and G, the total grain score of the group.

A final calculation for the haploid set gave each of the three groups of cells equal weight. The grain scores previously calculated to take account of segmental length were multiplied by $\frac{50}{n}$ where n was the total number of cells in the group. This meant that direct comparison was possible between segments of the three groups, thus allowing an assessment to be made as to the time of replication. The arbitary number of 50 cells was chosen as the actual numbers of cells selected in each group were ranged about this number.

The late replicating X

Due to the unique asynchrony found in the X chromosome, it was decided to analyse its replicating pattern separately. Although to calculate the late X in any other manner than to repeat the entire procedure described previously is liable to lead to slight inaccuracies, it was considered that the inaccuracy resulting from the method described below would be so slight that its use was justified. The method used involved comparison with the early replicating X, subsequent corrective factors being applied.

The total grain scores for each segment were totalled as before, this value being multiplied by the factor $\frac{E}{L}$ where E was the number of early replicating X's and L the number of late replicating X chromosomes. This resulted in both types of X's having the same number of samples and thus direct comparison was possible. For each of the three segments of the X a ratio $\frac{GL}{GE}$ was calculated where GL was the grain score of the late X and GE that of the early X. The grain scores for the early X, after correction for segmental length were multiplied by their respective factor to give the corresponding score for the late X.

These values, however, had to be reduced in order to be in direct comparison with the early X for the subsequent steps. As the early X and haploid autosomes had been recalculated to give a fraction of the total count, it was not considered valid to recalculate the late X by using this same total. Thus, before this recalculation for the late X, the grain scores were multiplied by $\frac{T}{T+t}$ where T was the total score of the haploid set, and t, the difference between the grain scores of the late and early X's. After applying this factor, the segmental scores for the late X(S) and the early (s), were applied to the ratio of the early X by applying the factor $\frac{S}{s}$, thus giving the corresponding ratios of the late X.

As described previously, the total grain score for each segment was multiplied by $\frac{50}{n}$ to give each group of cells equal weight i.e. 50 cells.

The Y chromosome

Evaluation of the replication of the Y was carried out in a similar fashion to that of the autosomes. The total grain count of each segment of the Y, was multiplied by $\frac{A}{Z}$, where A was the number of haploid sets counted and Z was the number of Y chromosomes counted in each group. The resulting value was then multiplied by $\frac{1000}{L}$ where L was the segmental length. By these two calculations, the Y was given equal weight with the autosomes. In order to arrive at a ratio comparable to that calculated for the other chromosomes, the total grain count for each segment was divided by the total grain counts for all the cells in the group plus the grain counts over the Y. This value gave a ratio of almost comparable weight to that calculated for the autosomes. Finally, the total grain score for the Y was multiplied by $\frac{50}{n}$, as previously, to give the total Y score over 50 cells.

From the results obtained for each of the three groups of cells, histograms were constructed for the ratios of grain scores over each segment as compared with total counts and also the total scores over each segments.

Results

Due to the large amount of raw data accumulated during the preliminary work on the autoradiographic study, only the final results will be presented. As well as the two types of results, namely total grain scores and the proportions of this score contained in each segment, histograms have been constructed to demonstrate, for each group of cells, the total scores and also, for certain groups of chromosomes, the segmental proportion of the total score.

Lightly labelled cells (Group A) Grain score 0 - 100

56 cells were present in this group, 26 of which were female and 30 male. Table 16 combines the total grain counts and also the proportion in each segment. The latter results are displayed in histogram form in figure 17.

Medium labelled cells (Group B) Grain score 101 - 200

57 cells were selected in this group, 35 female and 22 male. As in group A, total grain counts and segmental proportions are presented together in table 17. The segmental proportions of the total score are shown in fig. 18.

Heavily labelled cells (Group C) Grain score - over 200

20 female cells and 27 male cells were selected for this group. Again the results are presented in both tabular form (table 18) and the segmental proportions in histogram form (fig. 19).

In addition to the results presented concerning the groups of cell types, histograms have been constructed to determine to what extent chromosomes of similar morphology can be separated by labelling patterns. Four such sets are presented in histogram form, utilising both types of data, the sets being numbers 3 - 4 (fig. 20), 14 - 15 (fig. 21), 17 - 18 (fig. 22) and in addition the early X, late X and the Y (fig. 23).

Interpretation of the Labelling Patterns

Group A

Chromosome 1

At all stages of replication, chromosome 1 displayed a similar form of labelling, in that the two proximal segments of the long arm were less heavily labelled than the remainder of the arm. This feature, together with the light labelling of the distal segment of the short arm in medium and lightly labelled cells, gave the chromosome a characteristic appearance (fig. 24). In medium-labelled cells, a further noticeable feature was the heavy labelling of the proximal segment of the short arm when compared with the remainder of the chromosome. Although this segment contained a higher proportion of grains than the remainder in all types of cells, it was especially noticeable in medium labelled cells, at which stage the segment contained almost twice the amount of label found in the rest. It appears from the results that with the exception of this segment, the chromosome replicates mainly in the early part of the 'S' period. The out-of-phase segment does not replicate until the middle of synthesis and as such a large peak appears in the histogram representing the medium labelled cells, but this peak has disappeared from the histogram of the lightly labelled cells.

Group B

Chromosomes 2 - 4

A feature of the group was that, in most cases, the proximal segment was much more heavily labelled than the rest of the chromosomes, (figure 25). The sole exception to this rule was, generally speaking, chromosome number 2.

Chromosome 2

In heavily labelled cells, chromosome 2 was more lightly labelled than the remaining

members of the group. At this stage no distinctive labelling pattern was observed, thus distinguishing it from chromosome 3 - 4. This indicates that in the early 'S' period the entire chromosome underwent a certain amount of replication at a rate which was reasonably constant throughout the chromosome. However, when the results of the medium labelled cells were analysed it appeared that by that time, both the proximal and middle segments had completed further replication, whereas the distal segment had approximately the same amount of labelling present, indicating that, in this segment, replication was delayed. At this stage, therefore, chromosome 2 had the distinguishing feature of the distal segment being the heaviest labelled part of the chromosome, a unique picture in group B chromosomes. By the time the cells reached the late 'S' period stage, the distal segment had undergone the main part of its replication and the whole chromosome was very lightly labelled, this appearance again distinguishing it from the other two members of the group.

Thus chromosome 2 at all stages of labelling can be relatively easily distinguished from the remaining members of group B.

Chromosome 3 - 4

At all stages of replication, both chromosomes 3 and 4 have the typical appearance of the proximal segments containing a higher amount of label than the rest of the chromosome. In heavily labelled cells the grain scores of the middle and distal segments of both chromosomes 3 and 4 were approximately the same, whereas the proximal segment of number 4 was more heavily labelled than number 3. An almost identical picture was present in the medium labelled cells, whereas in lightly labelled cells, the proximal segment of 3 was later replicating than that of number 4. Similarly, the middle segment of 3 contained more label than the corresponding segment of number 4.

It appears therefore that throughout the 'S' period, both chromosomes replicated continuously throughout their length with two exceptions. The first of these was that the two distal segments of both 3 and 4 began replication earlier than their corresponding proximal segments, thus resulting in the characteristic appearance of the proximal third being more heavily labelled than the rest of the chromosome.

The second exception to the rule of continuous replication throughout chromosomes 3 and 4 was the proximal and middle segments of number 3. This is seen in detail in fig. 20. It can be seen that whereas the proportion of grains in all segments decreased between the medium and lightly labelled cells, these two segments of number 3 increased their proportion of the total grains this being especially noticeable in the proximal segment, thus indicating that in contrast to the rest of the segments which replicated during the middle of the 'S' phase, these segments were late replicating.

It is concluded from the analysis of group B that chromosome 2 can be distinguished from 3 and 4 by the absence of a more heavily labelled proximal segment. It also appears likely that chromosomes 3 and 4 can be separated on labelling patterns, although the difference is less marked as both chromosomes have their proximal segments more heavily labelled than the other segments.

Group C

Chromosomes 5 - 8

Morphologically, group C consists of 2 distinct sets of chromosomes. Numbers 5 and 8 have a subacrocentric appearance, whereas 6 and 7 are more submetacentric, having nearly identical arm ratios of about 1.80. Although it is considered that all four members of the group can be identified, it has been found that additional aid in identification has been forthcoming from the autoradiographical study (fig. 26.)

Chromosome 5

This chromosome had a replication pattern which distinguished it from all the other members of group C in that the proximal segment of the long arm was much lighter labelled than any other segment of the chromosome. As can be seen from fig. 19 this segment was the earliest replicating part of the whole chromosome complement, only chromosome 15 replicating at a similar time. At all stages of labelling this feature was apparent, although not being so marked in lightly labelled cells due to the fact that most of the other chromosomes completed their replication between the middle and end of the 'S' period and thus in lightly labelled cells only a few grains were present. In heavily labelled cells, a higher concentration of grains was present on the short arm than on the distal segment of the long arm, whereas in medium labelled cells the position was reversed. As the grain scores for the distal long arm stayed virtually constant between the two groups, this means that between early and middle 'S' phase, replication had occurred on the short arm whereas the distal long arm underwent virtually no DNA synthesis. However by the time of lightly labelled cells, the grain score on the distal segment had dropped to almost the same score as the short arm. Thus, the three segments in chromosome 5 underwent replication at different times, the proximal part of the long arm being the earliest, followed by the short arm and finally the distal long arm, which although later than the rest, completed its replication early enough not to be considered late replicating when compared with certain other segments in the complement, (fig. 17a).

Chromosomes 6 and 7

These two pairs which are very similar in morphology, although distinguishable on size, were found to be similar also in labelling pattern in heavily and medium labelled cells. In both types of cells there was virtually no detectable difference in labelling pattern, all segments containing approximately the same grain scores,

although 6 contained slightly more than 7 but the difference was not enough to warrant separation of the two pairs. However, in lightly labelled cells, this difference was more marked in that the short arm and proximal part of the long arm of 6 were more heavily labelled than the corresponding segments of chromosome 7. This is seen more markedly when the proportions of the total grains contained in each segment are compared. The values for the segments are 2.28 and 1.98 in 6 and only 1.39 and 1.24 in number 7. Thus throughout the synthesis phase, chromosomes 6 and 7 replicate continuously and at an equal rate until towards the end when chromosome 7 undergoes an earlier termination than does number 6.

Chromosome 8

At no labelling stage did chromosome number 8 have a distinctive labelling pattern, in that, generally speaking, all three segments replicated at a constant rate and thus the grain scores were virtually identical at any one stage. The one exception was the short arm at the early stages of replication which had a markedly higher grain count than did the rest of the chromosome. At the other stages the replication pattern was very similar to those of 6 and 7, but markedly different to that of 5, due to the unique picture found in number 5. Thus no difficulty is encountered in separating 5 and 8. Replication in 8 appears to continue constantly through the 'S' phase, with the exception that the short arm initiates replication later than the remainder of the chromosome, but having undergone its preliminary replication, continues in a similar fashion to the rest of the chromosome during the remainder of the 'S' period.

Group D.

Chromosomes number 9 - 12 including the X

Group D chromosomes, with the exception of the X, all have the same morphology,

i.e. an arm ratio of about 1,40, although their lengths allow differentiation to be made. A distinct length difference exists between chromosome number 9 and the remainder of the group, number 9 being significantly longer. A similar difference is present between the remainder of the group and chromosome number 12, the latter being the smallest in the group. Chromosome 10 and 11 have a slight difference in length, but as all the chromosomes were originally paired on a length basis, the problem of whether the pairs selected were in fact homologues always exists. However, on the basis of the autoradiographic findings it appears that pairing using length as the criterion is valid (figure 27).

Chromosomes 9 and 10

The replication pattern of number 9 had at no stage a distinctive pattern, all segments replicating at approximately the same time. However, the amount of labelling present can be used to differentiate it from the autosomes most likely to be confused with it i.e. number 10, as number 9 tended to be more heavily labelled throughout all labelling stages. Chromosome number 9 also tended to have its short arm more lightly labelled than the remainder of the chromosome throughout all stages. In contrast to this pattern, the proximal segment of the long arm of number 10 was the latest replicating part of the chromosome. It appears therefore, that one segment in each of chromosome 9 and 10 replicated out of phase with the remainder of the chromosome in the early 'S' period and this differential was kept constant throughout the remainder of synthesis, all segments replicating at the same rate. A similar procedure occurred with the entire chromosomes 9 and 10, in that 10 underwent more early replication than did number 9, but once this burst of activity was over, the two chromosomes then continued at the same rate.

Chromosomes 11 and 12

Chromosomes 11 and 12, although neither displayed a distinctive pattern, can be separated by their rate of synthesis during the early synthesis period. In

heavily labelled cells, number 11 had a higher grain score than number 12, indicating that replication had begun earlier in the former chromosome. As with chromosomes 9 and 10, once this early synthesis had occurred, both chromosomes then proceeded to replicate at approximately the same rate. By this feature number 11 and 12 can be reasonably easily separated.

The replication patterns of the various segments of number 11 and 12 had slight differences, although they are not distinctive enough to allow them to be identified by this feature. During the early and middle replication stages, the centromeric segment of the long arm of 11 was more heavily labelled than the remainder of the chromosome, although by the stage of lightly labelled cells, this difference had disappeared, thus indicating that this segment had undergone less sythesis early in the 'S' phase, but later on a burst of replication had occurred out of phase with the other two segments. However, all through the 'S' phase, the three segments of 11 underwent constant replication. Similarly, the long arm of 12 started replication earlier than the short arm, both segments then continuing replication until after the middle period of DNA synthesis, when the short arm underwent an out-of-phase period of replication, so that by the lightly labelled stage, both arms were equally lightly labelled.

Thus, although no very distinctive replicating patterns were seen amongst the autosomes, the labelling pattern have been found to be of great use in separating chromosomes of similar appearance. Generally speaking, chromosome 9 is the heaviest labelled autosome in the group, at all stages, with number 12 the lightest.

X chromosomes

At all three stages of replication, marked asynchrony was present between the two X chromosomes in the female, this difference being most noticeable in medium and lightly labelled cells.

Early replicating X

It was found that at all stages, the short arm was lighter labelled than the long arm, indicating that the short arm replicated earlier than the long arm, although it did not complete its synthesis at this stage, but continued throughout the 'S' phase. The long arm in contrast replicated to a certain extent during all the 'S' period, but completed its replication late, the distal segment being especially late replicating. With the exception of its late replicating homologue, the long arm and especially the distal segment was the latest replicating part of group D.

Late Replicating X

In female cells, one X chromosome was noted to be markedly out of phase with its homologue at all stages of replication, this being much more evident at the later stages. A similar pattern of replication to its homologue was observed in that the short arm underwent much more replication at the early 'S' period than did the long arm. This differential existed at all stages studied, indicating that a certain amount of synthesis did occur throughout the 'S' period, although most of the chromosome underwent a large amount of late replication. The extent to which the chromosome replicated late is very clearly seen in fig. 17a. From the idiogram results X was found to contain approximately 5% of the total chromosomal length of the cell. From the lower section of the histogram (fig. 27) it can be seen that as the labelling became lighter throughout the cell, the proportion of the total grains present on the late X increased continuously, so that in cells with scores of 0 - 100, almost 15% of the total grains were found on the late X, compared with 11.79% and 9.22% in the medium and lightly labelled groups.

It is clear from the results obtained that in the female no difficulty is encountered

in identifying the X chromosomes, especially the late X whose long arm, especially at later stages, is much more late replicating than any other chromosome in the set. The early replicating X however, was also among the latest replicating in group D. In lightly labelled cells, the distal segment of the long arm was, with the exception of the long arm of the late X, the latest replicating segment in the group. A further feature of the late replicating X was its variation in morphology (figure 27). This is a finding which requires further investigations, having been similarly noted in the human (Bishop, Lees and Blank, 1965).

Group E

Chromosomes 13 - 15

Morphological identification of the largest chromosomes in the group, number 13, is made simple by the presence of a secondary constriction. However, chromosomes 14 and 15 are very similar morphologically, only a slight difference in length separating them. As described below, it was observed that at all stages of replication, two chromosomes were late replicating and the other two were among the earliest replicating parts of the chromosome complement (fig. 28). Because of this, separation of the two pairs was carried out on the basis of replication rather than on length.

Chromosome 13

Chromosome 13 was found at all stages to be earlier replicating than 14, but later than 15, and by this intermediate position could be further identified. From figs. 17 to 19 it can be seen that the short arm had a higher grain score than did the long arm, this feature being observed at all stages of replication, indicating that the long arm began replication slightly earlier than the short arm. Replication

in both arms continued in the earlier part of the 'S' phase, but from table 17, it can be seen that the percentage of grains present on chromosome 13 increased in the later stages. Thus, chromosome 13's final replication was delayed until the end of the 'S' period, its synthesis rate apparently slowing down towards the end and completing its replication late. However, it was clear from the study that the secondary constricted area was not markedly late replicating, many segments in the set completing their synthesis after this area (Figure 17a).

Chromosomes 14 - 15

In the whole of the chromosome complement, the difference in labelling patterns between 14 and 15 was the most distinctive found between morphologically similar chromosomes. This difference was most marked in heavily labelled cells due to the fact that chromosome 15 was, with the exception of the proximal part of the long arm of 5, the earliest replicating segment in the karyotype. The distinct difference in grain scores occurred throughout all stages of replication, because, as can be seen from fig. 17a, both arms of 14 are late replicating. Fig. 21 which displays both the total and percentage grain scores for the chromosomes, shows clearly the late replicating quality of chromosome 14, in that the percentage of grains present increases in lightly labelled cells. The times at which chromosomes 14 and 15 replicated, can be interpreted from fig. 21. Although chromosome 14 was late replicating, DNA synthesis appeared to occur continuously throughout the stages studied until towards the end of the 'S' period when a final burst of synthesis occurs. The activity of number 15 was almost a mirror image, in that very early replication occurred, the remainder of the synthesis being completed gradually through the rest of the stages.

Thus chromosomes 14 and 15 can be very easily separated on labelling patterns. However, as chromosome 14 and 15 were originally paired using labelling patterns, this very distinct difference was not unexpected. In order to determine the

relationship between size and labelling, 138 cells which showed a distinct difference in labelling between pairs 14 and 15 were examined, (table 19). In all 138, two heavily and two lightly labelled chromosomes were present. The two larger chromosomes were late labelling in 103 of the cells, one larger and one smaller chromosome were heavily labelled in 29 and in the remaining six cells, the two late replicating ones were either the two smaller chromosomes or else no difference was present in size.

Thus it appears that in 75% of the cells, by either numbering the two larger chromosomes number 14 or by calling the two late replicating ones number 14, the same arrangement will result. However, in 21% of the cells examined the late replicating pairs consisted of one small and one larger chromosome. From these results, therefore, it is not valid to positively identify chromosome pair number 14 on the basis of length alone.

However, as 87% of the late replicating ones were the larger chromosomes, if any definite numbering of chromosomes has to be carried out, the most accurate way is for the two larger chromosomes to be numbered pair 14.

Group F

Chromosomes 16 - 18

Group F consists of one pair of acrocentrics (number 16) which is markedly larger than the other two pairs which make up the group. Pairs number 17 - 18 are very alike in length, only a slight difference existing between them. However, the results of the idiogram indicate that the difference is sufficient for them to be separated. On this basis therefore, no attempt was made to pair the chromosomes using labelling patterns and the original karyotypes based on morphology were left unaltered, (figure 29).

Chromosome 16

During all three stages of labelling, the proximal, or centromeric segment of 16 was found to be later replicating than the distal part. This difference was especially marked in lightly labelled cells when the proximal segment was observed to be distinctly late replicating (fig. 17a). Prior to the earliest stage examined, the distal segment had presumably undergone a greater amount of replication than the proximal part. From this stage onwards, both segments continued replication at an approximately identical rate until towards the end of the synthesis period when the distal segment underwent a further replication earlier than the proximal segments' final late replication.

During the first two stages of labelling, chromosome 16, as well as being identifiable by size, was the heaviest labelled chromosome in group F. At the final stage, however, it was found that number 17 was later replicating than was number 16.

Chromosomes 17 - 18

As with chromosome 16, at all labelling stages, the centromeric segments of chromosomes 17 and 18 were found to have a higher grain score than had the distal segments. In heavily labelled cells there was no detectable difference in grain scores between numbers 17 and 18, both having the distinctive labelling pattern of the group, in that the proximal segments were the heaviest labelled.

At the next two stages, however, identification of the pairs was possible using replication patterns, chromosome 17 being more heavily labelled than number 18. This difference was much more marked in lightly labelled cells, as chromosome 17, especially the proximal segment, was much later replicating than number 18. It appears therefore that in the early synthesis period, both pairs underwent

approximately the same amount of replication, the distal segments completing more than the proximal segment. By the time of the medium-labelled cell stage, chromosome 18 had undergone replication at a faster rate than 17 and this continued to be the case until the end of the 'S' period, when the late replicating proximal segment of 17 and, to a lesser extent the distal segment, underwent its final DNA synthesis at a much greater rate than the remaining replication of number 18.

Y Chromosome

At all stages of replication, the Y chromosome was found to have the heaviest labelling per unit length of all chromosome segments in the complement. Fig. 23 shows the comparison with both the X chromosomes, the late replicating X being the latest replicating chromosome in the female. From this histogram the labelling pattern at different stages can be seen. The total grain scores show that in the earlier part of the 'S' period, a certain amount of replication had occurred in the long arm of the Y, but between the early and middle stages this rate slowed down to almost nil. In comparison the short arm underwent some synthesis at this stage, although still remaining more heavily labelled than the long arm. From the medium labelled stage until the very end of synthesis, virtually no replication occurred in the Y, resulting in the very heavy labelling pattern in lightly labelled cells, the grain score in the Y being greatly in excess of any other chromosome.

By this labelling pattern, absolutely no difficulty is encountered in the identification of the Y in male cells by the use of autoradiography (figure 28).

Discussion

The present study, which is the first comprehensive analysis of the replication behaviour of pig chromosomes, has shown that autoradiography is a most useful aid to identification of individual chromosomes in the normal karyotype. It also seems likely that in the future, identification of the missing or supernumerary chromosomes in animals with aneuploidy, will be simplified by the asynchrony found to be present between chromosomes of similar morphology.

Prior to the present autoradiographic analysis only two reports had been published concerning labelling patterns of pig chromosomes. Evans, (1965) reported the presence of a late-labelling X chromosome in female pigs, but presented no further data as to the labelling patterns of the entire cells or of the individual autosomes. Similarly, Cornefert - Jensen. Hare and Abt, (1968) found a single late-labelling chromosome to be present in females but absent in the male, identifying it as an X. In male cells, Cornefert - Jensen and his co-workers, (1968) reported that the Y chromosome was late replicating, but in no case was an attempt made to carry out grain score analysis or any objective assessment of labelling. From the studies of Evans, (1965) and Cornefert - Jensen etal (1968), with the exception of the late-replicating properties of the Y and one X in the female, no comparison is possible with the present study, and as such the latter findings can only be discussed as an isolated entity.

The first and possibly most important finding was that of the late replication of certain sex chromosomes. In female cells, one X was much later replicating than either its homologue or any autosome, thus confirming the findings of Evans, (1964) and Cornefert - Jensen, (1968) as discussed above. This finding is in agreement with similar findings in the human (Morishima, Grumbach and Taylor, 1962 and German, 1962) the cow (Mukherjee and Sinha, 1963) the goat (Evans, 1965 and Short, Hamerton, Grieves and Pollard, 1968) and the sheep, (Bruere, personal

communication).

One finding concerning the late replicating X of the pig which is of extreme interest, is that the short arm was earlier replicating than the long arm. Although this difference was made more noticeable by the correction for arm length, the long arm being divided into two segments, whereas the short arm was undivided, the fact remains that the labelling per unit length indicated later replication in the long arm than in the short arm.

From work carried out on pig embryos, Axelson, (1968) postulated that the sex chromatin body is formed from only part of the X chromosome. This theory was derived from rather tenuous evidence of observations of thin chromatin strands stretching from the sex chromatin body to the nucleolus, a feature never seen in the present study, although the tissues examined were not comparable. At present, this theory must be viewed with care and must await further confirmation. Nevertheless, if it does eventually become proven, it may be one possible explanation for the earlier replication of the short arm of the X. It has been shown repeatedly that in the human female with an isochromosome of the long arm of the X, a larger than normal sex chromatin body is observed, together with a late replicating chromosome with the appearance of the isochromosome, (Muldal, Gilbert, Lajtha, Lindsten, Rowley and Fraccaro, 1963 and Gianelli, 1963). This evidence of the late replicating X chromosome forming the sex chromatin body, together with reports of patients with multiple late replicating X chromosomes forming the same number of sex chromatin bodies, (Gianelli, 1963, and Grumbach, Morishima and Taylor, 1963) indicates strongly that the late replicating part of the X chromosome forms the sex chromatin body. Grumbach etal, (1963) further found, by autoradiography of skin explants from a penta - X patient, that in some cells four areas of high grain density were found, which in a few cases were found to overlay the sex chromatin body.

If this is also the case in the pig, which appears not unlikely, the part of the X chromosome which Axelson, (1968) theorised was not involved in sex chromatin formation, would probably not be late replicating. Thus the short arm of the X, which was found to be earlier replicating than the remainder of the chromosome, might possibly be this segment. In the human female, the long arm has been found to be later replicating then the short arm. Schmid, (1963) noted that in many cases, the distal segment of the long arm of the late replicating X was more heavily labelled than was the short arm or the centromeric region. Similarly, Gianelli, (1963) in a study of a Triplo-X female, found that in a number of cells, part of the long arm of the late X tended to be more heavily labelled than the short arm, but neither Schmid, (1963) nor Gianelli, (1963) carried out grain counts to substantiate their claims. However, grain counts were carried out by Gilbert, Muldal, Lajtha and Rowley, (1962) and from this they constructed a histogram similar to those presented in the present study. Although these workers did not comment on the fact, the grain counts of the long arm of the late X tended to be higher than those of the short arm thus apparently confirming the work of Schmid and Gianelli. Unfortunately, neither Evans, (1965) nor Cornefert - Jensen, (1968) attempted grain counting of the pig X and thus the present study is the sole report on this feature in the pig. In the cow, Mukherjee and Sinha (1963) similarly noted that one portion of the late replicating X was out of phase with the rest of the chromosome, in that the long arm completed replication before the short arm. Although no confirmation is at present available for the finding that one segment of the late X of the pig is out of phase with the rest of the chromosome, evidence from other species suggests that the pig is not unique in this behaviour.

It was also found in the present study that the early X chromosome in the female and the single X in the male were among the latest replicating chromosomes in

Group D, with the exception of the late replicating X. Although the labelling pattern of the 'cold' X was not such that it was distinctive on first inspection of the cell, in the way that chromosomes 5, 15, the late X and the Y were, it may be useful for chromosome identification. This feature of the relatively late replication of the single X in the male and the early X in the female has been found to be also the case in both humans and cattle. Schmid, (1963) found that the long arm of the human 'cold' X displayed a diffuse type of late replication. Similarly Hsu and Lockhart, (1965) found in the Group C chromosomes of a 49,XXXXY child, in addition to three late replicating X's, a fourth chromosome possessed a moderate amount of labelling over its long arm. Neither of these publications however, contained grain counts and the labelling intensity relative to the autosomes cannot be determined.

This feature of the 'cold' X has not been reported often in the literature, possibly due to the difficulty in accurately identifying the human X. Morishima, Grumbach and Taylor, (1963) found that the early replicating X in the female did not display a conspicuous difference in labelling pattern from the autosomes. However, they used a pulse-labelling technique compared with the continuous labelling technique used in the present study, that of Schmid, (1963) and also of Hsu and Lockhart, (1965). As such, the results are not comparable and this cannot be considered to invalidate the findings. The original paper on mammalian autoradiography by Taylor, (1960) contained a description of the replicating behaviour of the sex chromosomes of the Chinese Hamster. In this, Taylor reported that in the X chromosome of the male the long arm was late replicating, this also being a feature of the 'early' replicating X in the female. A third species, namely the bovine, has been found to display a similar labelling pattern of the 'cold' X. In this species, the X chromosomes are morphologically distinguishable from the autosomes, being submetacentric as opposed to the acrocentric structure

of the autosomes. Thus, in comparison to the human, there can be no doubt about the identification. Mukherjee and Sinha, (1963) using continuous labelling, found that the early replicating X completed its duplication much earlier than the late X, although it was still replicating when approximately three-quarters of the autosomes had completed their synthesis.

Therefore, in the pig, the X chromosomes, as well as having replication patterns which aid identification, have been found to have many features in common with other species. The significance, if any, of this species similarity must await clarification. It was found during the study that the X chromosome appeared to have a polymorphic structure. It can be postulated that due to the unique despiralisation of the late replicating X it can adopt varying forms depending on the stage at which it is examined. It is hoped in the future, to make a detailed study of this feature.

In the present study, it was found that the Y chromosome was the heaviest labelled chromosome at all the three stages studied, the difference in labelling patterns between the Y and the other chromosomes being most noticeable in the lightly labelled series. From this study therefore, there can be no doubt that the Y is late replicating and by this feature can be easily recognised. A similar finding was reported by Cornefert-Jensen etal, (1968) who simply stated that the Y was late-replicating but gave no indication as to the degree of difference between the Y and the autosomes.

In addition to the valuable information obtained concerning the sex chromosomes in the pig, the analysis of the autosomes has shown that certain pairs of chromosomes which are morphologically similar, can be differentiated on their labelling patterns. As can be seen from figures 24 - 29 homologous chromosomes replicate synchronously, thus allowing identification to be made. The most

distinctive example of this is the sub-group 14 - 15 of Group E, in which one pair of chromosomes was much later replicating than the other two members of the group. On the basis of the idiogram results, the larger chromosomes were designated number 14 and the two smaller ones number 15. This arrangement on the basis of size has been found to be erroneous after analysis of the chromosome size of the late replicating pair, 85% of the heavily labelled chromosomes being found to be those of the larger size. If it is therefore necessary to specifically number chromosome pairs, it seems reasonably valid, in the absence of autoradiographic studies, to pair the chromosomes on a length basis, accepting that less than one in five of the arrangements will be incorrect. However, in those cases in which the need for accurate numbering is encountered, it would be expected that autoradiographic studies would be carried out as the difference in labelling patterns is so distinct.

Less distinctive labelling differences were found between the members of pairs 3 - 4 and 17 - 18 than was found between 14 - 15. Pairs 3 - 4 and 17 - 18 were found, on idiogram measurements, to be separable only with a slight degree of certainty. Nevertheless, the unlabelled chromosomes were arranged purely on size and the labelled karyotypes arranged likewise. Between pairs 3 and 4, no clear labelling difference was evident and because of this no effort was made to carry out arrangement of the pairs using replication patterns as a guide. From the analysis of the labelling patterns of pairs 3 - 4, it was found that in heavily labelled cells virtually no difference was present between the pairs, but in medium labelled cells, number 4 was found to be more heavily labelled than 3. However, in lightly labelled cells, this position was reversed, chromosome 3 being quite distinctly later replicating than number 4. Thus by selecting cells in which the labelling was relatively light, differences in labelling pattern can be detected. A similar situation was found to exist in pairs 17 - 18, in

that the slightly larger pair, number 17 was later replicating than the smaller pair, number 18.

A very similar replicating pattern was found in all but one of the acrocentrics of Group B and E, the one exception being chromosome 2. In pairs 3 - 4 and 16 - 18, the centromeric regions were the heaviest labelled segments in the chromosomes, indicating that replication tended to start at the distal end of the arm and proceed towards the centromere. The same general pattern of more heavily labelled centromeric segments was also found in Group D chromosomes, but in only 60% of all non-acrocentric chromosomes in the pig was the pattern repeated. Thus, this apparent zipper-like replication of the acrocentrics may be more than pure coincidence. One possible factor which might play a part in this striking feature of the acrocentrics is the replication pattern of the short arm. Although these are not seen in every chromosome, enough have been observed to make it appear likely that they are present on all such chromosomes. If this is the case, a certain amount of labelling is bound to be present on the short arm, thus increasing the grain count of the centromeric segment. However, it seems rather unlikely that this radioactivity would account for what is, in some cases, a considerable difference in labelling between the proximal segment and the remainder of the chromosome. At present, therefore, it must be considered that the pattern of replication in the acrocentrics is purely coincidental.

From the present study, it is clear that asynchrony of replication between homologous pairs of chromosomes, exists in pig chromosomes. It is further evident that such differences will be most useful in chromosome identification in the future, both for diagnosis of supernumerary chromosomes and for, in some cases, the detection of structural aberrations such as deletions and translocations. This, the first such study in the pig, has also raised some problems, notably the polymorphism of the X chromosome. It is hoped that future studies can be based on the present work and such problems as the X chromosome may be further explored

in more detail.

Tritium Induced Abnormalities.

Introduction

The use of tritiated thymidine in autoradiographical studies of chromosomes by Taylor, Woods and Hughes, (1957) led to the discovery that the soft radiation emitted by the tritium which was incorporated into the deoxyribonucleic acid (DNA) caused chromosomal aberrations. This effect was first noted in Bellavalia root tips by Taylor, (1958) who reported that in a high proportion of chromosomes, both single and twin exchanges occurred. He further found that the frequency of aberrations was higher in chromosome 1 than in the smaller chromosomes and concluded, that the frequency of abnormalities was nearly proportional to chromosomal length.

Confirmation of the damaging effect of tritium was presented by Wimber, (1959) who used the root tips of Tradescantia Paludosa to study post-metaphase breakages after tritiated thymidine treatment. From studies carried out by varying the length of time in contact with the tritium and also the time during which the plants were left with tritium incorporated in their molecules, Wimber concluded that the cells which had absorbed the largest amount of tritiated thymidine contained the highest frequency of anaphase fragments. Using different plant species, McQuade and Friedkin (1960) and Natarajan, (1961) similarly demonstrated that tritiated thymidine caused chromosomal breaks and that the frequency increased with the amount of tritium incorporated into the DNA.

The first report of similar findings in mammals was by Bender, Gooch and Prescott, (1962) who studied the effect of tritiated thymidine on human chromosomes obtained from lymphocytes. The workers pulse labelled the cells for 30 minutes and continued the cultures for either 24 hours or 60 hours after removal of the tritium. They found that the frequency of aberrations was higher in the cells which were

allowed to continue to grow for the longer period. Bender and his workers concluded as did Wimber, that most of the tritium-induced aberrations occurred in heavily labelled nuclei, thus indicating that the frequency of breakages was dependent on the dosage of tritium received. However, it did not appear that the breakages were more frequent over specific heavily labelled areas and they concluded that the distribution of breakages was independent of label distribution. It was further found that the breakage distribution was random over the chromosome complement, although no detailed study was carried out to prove this.

Gilbert, Muldal, Lajtha and Rowley, (1962) in an autoradiographical study of human lymphocytes, observed that the chromosomal areas which had the highest frequency of breakages corresponded to the late replicating areas. However, they were unable to conclusively demonstrate this because of the low grain counts in their study but stated that a high yield of breaks was present on the heavily labelled X chromosomes.

Dewey, Humphrey and Jones, (1965) studied hamster cells grown in vitro, to which tritiated thymidine was added. After 1 hour, the tritiated thymidine was removed and the cultures then incubated for a further 10 hours. They found that chromosomal damage, consisting of single breaks and chromatid exchanges, was seen in both labelled and unlabelled metaphases, but that the frequency of aberrations increased in proportion to the number of disintegrations in the cell. From studies of the labelling patterns of the chromosomes, these workers were unable to correlate the location of the aberrations and the distribution of the tritiated thymidine in the chromosomes.

Hsu and Zenzes, (1965) confirmed the work of previous authors in that they found the frequency of breakages to increase with both increased dosage and increased

duration of tritium treatment. In a detailed study of the correlation between breaks and labelling patterns they found that certain late replicating chromosomal areas contained more breakages than would have been expected on the basis of the length. They concluded that the occurrence of breaks was not a random process but was related to thymidine incorporation.

Further studies on human chromosomes were carried out by Vig, Kontras and Paddock, (1968) who found a variety of chromosome aberrations in cells from peripheral blood cultures to which the tritiated thymidine had been added. As virtually no abnormalities were seen in the control material, it was concluded that these abnormalities were tritium-induced. Vig and his co-workers found no apparent correlation between the position of breakages and the degree of labelling over the breakages, and further that the frequency of aberrations and the length of chromosomes or group of chromosomes were in reasonable agreement.

Materials and Method

Blood was collected from six young pigs at the Glasgow Slaughter House. The carcases were examined for evidence of disease, none being found in any of the pigs. Blood from three animals was used for each experiment, four cultures being set up from each animal. To two of these was added 0.4 ml. of tritiated thymidine to give a final concentration of 1 microcurie/ml. of culture and to the two control cultures, 0.4 ml. of sterile Hank's solution. Two experiments were run using this procedure, to the cultures of the three animals comprising the first experiment, the tritiated thymidine was added 68 hours after initiation of incubation and to the cultures of the second experiment it was added at 69 hours. In both experiments, the Hank's solution was added to the control cultures at the corresponding times. Thus, the whole experiment consisted of three cultures to which tritium had been added 6 hours prior to fixation and three to which it had been added 5 hours pre-fixation. For each of the six sets of cultures, the control cultures were processed in an identical fashion except for the absence of the radioactive material. 100 cells were examined from each animal, 50 from the cultures to which tritium had been added and 50 from the controls. Each cells was analysed at x1250 for chromosome number and for the presence of any breakage or abnormality. Six types of abnormality were found and the classification and criteria used were as follows:-

(1) Single Breaks. An abnormality was scored if one part of a chromatid arm showed non-alignment with the rest of the chromatid, and there was no evidence of visible chromatid material joining the separated parts (fig. 30a).

(2) Double Breaks. (Isochromatid breaks). If both chromatids were broken with no evidence of continuity with the rest of the chromosome and malalignment was present, the breakage was classed as an isochromatid break being scored as two breaks (fig. 30b).

(3) Single Fragment. A fragment was scored if a single chromatid dot was present, either related to, or apparently independent of any chromosome. This abnormality was scored as a single break. (fig. 30c).

(4) Double Fragments. If two chromatid dots were present in close apposition such that they appeared to be the fractured ends of a chromosome arm, the aberration was scored as two breaks (fig. 30d).

(5) Complexes. These were complicated abnormalities in which two or more chromosomes were involved, having apparently arisen through breakages occurring in adjacent chromosomes with subsequent repair involving, and thus joining the affected members (figure 30e). An estimate of the number of breakages which had probably occurred was made and the complex scored accordingly.

(6) Gaps. Non-staining or poorly staining areas of a chromatid arm when present, were judged to be non-staining gaps if no evidence of displacement of the part was evident (fig. 30f).

Each cell in which an abnormality was noted was photographed and karyotyped. From these, every abnormality in which the chromosome involved could be identified was scored accordingly and the position on the chromosome noted. Each chromosome arm was divided into three segments and the abnormality assigned to one of the segments. The total number of breaks which occurred in each chromosome segment was calculated and the final frequencies superimposed on the idiogram (fig. 31), which had been previously prepared. Finally, the frequency of breakages on each chromosome was compared with the expected frequency related to length, (table 20).

Results

The distribution of the chromosome counts for both the cultures to which tritium was added and the control cultures are presented in table 21. The results of the control cultures have been pooled, as they all had similar modal frequencies and spreads about the mode. It can be seen from the results that there is very little difference between the counts for the two culture groups to which tritium was added. However, in comparison with the controls, a higher frequency of aneuploidy was found, due to loss of chromosomes, although this was still within the range of spread found in normal cultures, (table 1). It was found by karyotyping a number of these aneuploid cells, that the loss of chromosomes appeared to be at random as no one chromosome was lost selectively.

The frequency of breakages found in the cultures (a) and (b) and the control cultures are presented in table 22. As before, the results from the control cultures are pooled. From these results it can be seen that no breakages were present in the control cultures, but there was present a low level of non-staining gaps. Although the number of cells examined in the two experiments (a) and (b) were relatively small, 150 cells in each, the results show that the frequency of breakages increased quite markedly from the relatively low frequency of 0.14/cell to 0.51/cell. Applying the student's 't' test to these results, P was less than 0.01 when the control was compared with both experiment (a) and (b), this being significant. Comparing experiments (a) and (b), P was less than 0.05 (probably significant).

In both experiments, the majority of the breakages were of the single chromatid-break type, in experiment (a) making up 77% of the total breaks and in experiment (b), 60%. In both types of cultures the incidence of fragments was low, possibly due to either the loss of the tiny pieces of chromatin at harvesting or

overlaying by other chromosomes, thus rendering them invisible.

As was noted in the case of breakages, there was a significant increase in the frequency of non-staining gaps seen in experiment (a) compared with the control cultures (P less than 0.01, this being highly significant). A further increase was noted between (b) and (a), but this was found to be not significant (P greater than 0.5).

Combining the results of the two series of cultures which were exposed to the tritium, a total of 98 breaks was found. Of these, 17 were fragments, the origin of which could not be determined. By karyotyping the cells containing breakages, the chromosomes on which the remaining 81 were present were identified and their position on these chromosomes noted. Each chromosome arm was divided into three segments, and the position of each break assigned to one of the segments. The final frequencies for each segment was traced onto the idiogram and this is shown in figure 31. As can be seen, the majority of breakages (59%) were found on chromosomes 1 - 4. Table 20 shows the observed percentage frequency of breaks on each chromosome and also the expected frequency similarly expressed as a percentage, this based on length. These findings show that, although the majority of breakages do appear on the largest chromosomes, the frequency does not parallel the chromosome length, and certain chromosome segments do contain a higher proportion of breakages than was expected. Applying the X^2 test to the observed and expected frequencies, P was less than 0.01, this being highly significant.

An attempt was also made to correlate breakages with the amount of radioactivity incorporated in the segment involved (table 23). In experiment (a) the labelling patterns of 16 out of the 22 breakages found were examined. 8 of these were labelled and the remaining 8 were devoid of radioactivity. In experiment (b) the results were again equivocal. In only 52 of the 76 breakages found, could the associated labelling pattern be determined. 35 breakages were associated

with heavy labelling, 14 with light labelling and 3 were apparently free of radioactivity. However, as the vast majority of segments in these cells were themselves labelled, it was impossible to correlate the frequency of breakages with the amount of labelling. Furthermore, the positions of the breakages appeared to bear no relationship to either specific late-replicating segments (figure 17a) or secondary constrictions (figure 5).

Discussion

Many papers have been published concerning studies on the relationship between tritiated thymidine and induced chromosomal abnormalities. However, most of this work has been carried out on tissues other than lymphocytes, such as plants, (Taylor, 1958; Wimber, 1959; McQuade and Friedkin, 1960; Natarajan, 1961) and Hamster tissue (Dewey, Humphrey and Jones, 1965; Hsu and Zenzes, 1965). Thus the premise must be borne in mind that results obtained in different species and tissues are not directly comparable. Nevertheless, it is possible to draw comparative conclusions from the present study and the literature.

The results of the present experiments show quite clearly that the addition of tritiated thymidine to cultures of growing pig lymphocytes results in significant damage to the chromosomes. The fact that in the control cultures no breakages were seen, further indicates that all the breakages were induced by the tritiated thymidine, as all other conditions of culturing were identical. It was also seen that the frequency of breakages was markedly increased between experiments (a) and (b) being 0, 14 and 0, 51 per cell respectively. As the cells in the latter experiment were exposed to the isotope for 1 hour longer than in (a) this clearly indicates that chromosomal damage increases with the length of time of incorporation of the isotope.

The finding of tritium-induced damage has been reported many times in various biological systems. In plants, Taylor (1958) first suggested that tritium might be the cause of sister chromatid exchanges, but later reported that the hypothesis was not tenable (1958a). Soon after this, however, many workers were able to demonstrate that tritium could in fact, induce chromosomal damage (Wimber, 1959; McQuade and Friedkin, 1960; Natarajan, 1961). In human lymphocyte cultures

tritium has also been shown to cause damage to chromosomes (Bender, Gooch and Prescott, 1962; Vig, Kontras and Paddock, 1968).

It has also been shown previously that the frequency of breakages increases with the time of exposure. In the present study, there was more than a three-fold increase in breakages observed for a one hour increase in exposure time (from 5 hours to 6 hours). Hsu and Zenzes (1965) obtained similar results in Chinese Hamster cells when the time of treatment was increased from 3 to 4 hours, finding an increase in breakage frequency of from 0, 13 per cell to 0, 33 per cell. These studies however are not directly comparable, for as well as working with different cells, Hsu and Zenzes used tritiated thymidine of greater specific activity, a factor which Natarajan, (1961) found to increase breakage frequencies. However, as Hsu and Zenzes used the isotope of high specific activity throughout the study, the factorial increase in the breakage frequency can be considered to be a comparable one to the present study.

Bender, Gooch and Prescott, (1962) similarly found that an increased frequency of breakages occurred when tritiated thymidine was incorporated for longer periods, although in their case, a standard time of pulse labelling was carried out, followed by a variation in time before fixation. The only contradictions to the correlation of increased breakage and increased incorporation time were reported by Wimber, (1959) and Natarajan, (1961). Both workers found that, although an increase in breakage frequency was found as the time of treatment increased, after a certain point the frequency then dropped. This anomaly was explained by the fact that gradual dilution of the isotope had occurred, resulting in a lowered uptake of thymidine by the cells which divided later in the culture period.

The latter findings of Wimber and Natarajan illustrate very clearly the difficulties in attempting to correlate results obtained in two different ways. In both these papers, it is clear that the drop in breakage frequency after a prolonged period was not a true decrease in frequency, being most likely due either to dilution of the isotope or to the death of cells with multiple aberrations. Thus, although Bender and his co-workers (1962) found only a small increase in breakages between the cells grown for a further 24 and 60 hours before fixing, there must be some doubt as to whether the frequencies were a true reflection of the status of the system. Because of this, any attempt to compare the comparative radiation-sensitivity of cells of different species is bound to fail and until identical experiments are carried out in different species, it is impossible to determine how sensitive pig lymphocytes are to tritiated thymidine in comparison with human cells.

In the present study, non-staining gaps were found to increase in frequency from 0, 03 per cell in the control cultures to 0, 16 in experiment (a) (highly significant, P being less than 0.01) and 0.25 in experiment B). Thus, as was found with breakages, non-staining gaps appear to increase with increased contact with tritiated thymidine, although this difference between a and b was shown not to be significant (P being greater than 0.5). These aberrations appear to be poorly staining or non-staining area of chromatin, which show no evidence of breakages. Revell, (1959) in his study of Vicia Faba found these aberrations to be present in post-irradiated root tip cells, increasing in frequency to a maximum which occurred just as true breaks started to appear. He suggested that some of the gaps may have been areas under repair, but stated that they could not all be explained as such, and that no explanation was at the time forthcoming. However, if, as Revell postulated, gaps are a heterogeneous mixture of aberrations, his data indicated that the frequency did bear some relationship to radiation dose.

Bender, Gooch and Prescott, (1962) similarly found the frequency of gaps to bear some relationship to the type of radiation applied. A higher level was found in the cultures to which thymidine had been added than was found in uridine-added or control cultures, Bender further stating that although gaps were seen after X irradiation, they were never found in such high frequencies. Although the frequency of gaps was found to be highest in the tritiated thymidine cultures, the level stayed constant in the 24 and 60 hour cultures, although the frequency of true breaks increased.

The reason for the discrepancy between Bender's and the present work is rather obscure. One possible explanation was the small number of cells involved in the studies, making the results less significant. A second possibility is that, if the gaps are indeed abnormalities under repair, they would have had time to undergo this repair during the 60 hours of culture, whereas in the much shorter time of the present study, the chances of this occurring were much less. It must be borne in mind, however, that it might not be valid to compare these findings due to the differences in techniques.

Further confirmation of the association of gaps with radiation has been produced by Vig, Kontras and Paddock, (1968) who found that in human lymphocytes exposed to tritiated thymidine for the last 6 hours of culture, the gaps were seen frequently, whereas in the control cultures, only very small numbers were observed. Vig and his co-workers found that the gaps were positioned at random and theorised that they were subchromatid breaks, points of future true breaks and fragmentations. Taylor, (1963) suggested that the gaps were small areas of incomplete DNA replication and, if supplied with exogenous non-radioactive thymidine before complete breakage occurred, could heal themselves. This is a possible explanation for the lack of increase in the frequency of gaps in the work of Bender etal,

in that some of the gaps had time to heal themselves before harvest.

In the present study, the distribution of non-staining gaps was not found to be a random distribution. The correlation between the expected and observed frequencies of gaps, (Table 24) did not show a close fit (P being less than 0.01; highly significant) and in the case of the first 4 chromosomes there appeared to be hot spots, i.e. areas which were found to have a disproportionate frequency of gap present. However, the sample taken was rather small and these results will have to await further confirmation.

The findings from the present study are confirmed to a high degree by the previous work done in this field. Thus, it seems very likely that non-staining gaps are indeed associated with radioactivity and it appears possible that, as in the case of breakages, the frequency increases in step with increases in exposure times. Further, it would appear that the distribution of gaps is a fairly random process not associated with either areas containing large amounts of isotope, i.e. late replicating areas, or with specific weak spots on chromosomes.

As discussed previously, it is clear from the present study that the frequency of breakages increases with the time of contact with labelled nucleosides. In order to investigate the mechanisms involved in the breakages, all the cells which contained breakages were karyotyped and those in which the chromosome involved could be identified were further analysed. Their distribution in the idiogram is seen in figure 31. From this and ~~and~~ from table 20 which shows the observed and the expected frequency, it can be seen that the distribution of breakages is not a purely random process. Almost 60% (48 out of 81) identifiable breakages were found on the first 4 chromosome pairs, chromosome 2 being very frequently the site of abnormality. As can be seen from table 20, the frequency with which breakages are found on chromosome 2 differs markedly from the expected.

Table 23 shows the findings of the attempt to associate position of breakage with the amount of isotope incorporated. Although 83.8% of the breaks were on heavily labelled segments, apparently significant, it was found that the majority of segments which did not contain breaks were also heavily labelled, and it was found impossible to correlate labelling and breakages.

The conclusions from these findings therefore, are that the distribution of breakages appears to bear no connection with labelling patterns. When the distribution is related to chromosome length, it is found that the relationship is not purely a random one, certain chromosomes, especially number 2, possessing high spots of breakages in that certain areas appear to contain a disproportionate frequency of breaks.

Section V.

Cytogenetic examination of abnormal animals.

KLINEFELTER'S SYNDROME

Of the three most common chromosomal aberrations found in human, namely Klinefelter's, Turner's and Down's syndrome, sex chromatin positive, Klinefelter's syndrome is the only one to have been found to have a chromosomal counterpart in domestic animal, thereby enabling comparisons to be made on an interspecies basis.

Klinefelter, Reifenstein and Albright, (1942) described a syndrome which was first observed during male adolescence and was characterised by bilateral gynecomastia, small testes, lack of spermatogenesis, normal to moderately reduced function of the Leydig cells and an increased urinary excretion of follicle stimulating hormone (F.S.H.). They concluded that the condition was a degenerative lesion of unknown aetiology, starting early in life. Heller and Nelson, (1945) examined a series of male patients with similar microscopic testicular appearance and found that some of the cases did not have gynaemastia, but had a similar raised urinary F.S.H. level.

It was not until 1956 that a number of workers, using the newly developed Buccal smear technique, (Moore and Barr, 1955 ; Marberger, Boccabella and Nelson, 1955), examined males with Klinefelter's syndrome with a view to determining the genetic sex of these patients. Plunkett and Barr, (1956) found that two patients with congenital testicular hypoplasia were sex chromatin positive and they postulated that these patients had two X chromosomes, having either the sex chromosome complement XX or XXY and that a congenital error in sex development resulted in these patients' abnormality.

Similar findings were reported by Bradbury, Bunge and Boccabella, (1956) who examined 5 males showing the feature of the syndrome and found them to be sex chromatin positive, drawing the conclusion that they were genetic females.

In 1957, Ferguson-Smith, Lennox, Mack and Stewart examined 831 patients in a male infertility clinic, and of 91 with oligospermia, 10 were found to be sex chromatin positive. From that survey it was further concluded that about 40% of all Klinefelter's syndrome in humans were sex-chromatin positive. Ferguson-Smith studied testicular biopsies of both sex chromatin positive and negative cases and found that it was possible to distinguish two types on histological grounds, although it was not possible to differentiate them clinically. The findings of these two types ~~was~~ were confirmed by Nelson, (1957), who re-examined four of the original cases reported by Klinefelter, (1942) and found that two were sex chromatin positive and two were sex chromatin negative.

Evidence that non-sexual abnormalities were present in XXY patients was presented by Ferguson-Smith, (1958) who carried out a survey of 325 mental defective males and found 4 sex chromatin positive patients, while Prader, Schneider, Zublin, Frances and Ruedi, (1958) found the frequency among the feeble minded to be approximately double that of Ferguson-Smith's survey i.e. 2.4%.

Prior to 1959, the only patients with Klinefelter's syndrome which had been examined histologically were post-pubertal and had shown lack of germinal epithelium, clumps of Leydig cells and hyalinised tubules, (Klinefelter etal, 1942, Ferguson-Smith, 1958). In 1959, Ferguson-Smith carried out a survey of 663 mentally handicapped prepubertal school children and found 8 to have female nuclear sex. From these figures Ferguson-Smith estimated that the minimum incidence of Klinefelter syndromes in Glasgow was 1 in 3000 and that it was possibly considerably higher. Examination of the testes in these pre-pubertal children revealed a reduction in, and in one case complete absence of, germ cells. An occasional fertile tubule with normal spermatogenesis was seen in six of the cases, but the proportion of these tubules to sterile tubules containing no spermatogonia was much lower than in controls of

the same age. The testes of the oldest of the children, aged 12, showed signs of maturation with differentiation of Leydig, Sertoli cells, but a complete lack of germinal cells. The hyalinisation and formation of ghost tubules typical of adult cases was seen in this case. Ferguson-Smith concluded that although a lack of germ cells was an important associated defect, it could not be considered the main aetiological factor in the syndrome.

Moore, (1959) carried out a survey of 3715 new born babies and found that of 1911 males, 5 were sex chromatin positive, an incidence of approximately 2.5 per thousand of the population. This figure was much higher that both the estimates of Ferguson-Smith, 1 in 3000 (1959) and that of Prader etal, (1958) who found 1 in 1000. Moore reiterated the belief that these babies were sex reversed females, but this hypothesis was proved to be wrong soon afterwards, (Jacobs and Strong, 1959). These workers used the technique of bone marrow culturing to obtain chromosomes and found that a case of sex-chromatin positive Klinefelter's syndrome had 47 chromosomes. They found that as well as having a Y chromosome, the patient had 16 chromosomes in the middle-sized group of chromosomes and postulated that the patient was probably 47,XXY.

Soon after Jacobs and Strong's report other workers also found that Klinefelter's syndrome was associated with an XXY karyotype, (Ford, Jones, Miller, Mittwoch, Penrose, Ridler and Shapiro, 1960; Harnden, 1960). In the same year, a variation in the chromosomal findings was identified in Klinefelter's syndrome patients who were also suffering from severe mental retardation, (Barr and Carr, 1960; Ferguson-Smith, Johnston and Handmaker, 1960). Both groups found their patients to have the karyotype 48,XXXY and to have double sex chromatin bodies in the majority of cells. From these findings it was suggested that the presence of the extra X chromosomes might be associated with the more severe mental retardation, (Ferguson-Smith etal, 1960).

Multiple physical abnormalities in a child with 49,XXXXY were found by Fraccaro, Kaijser and Lindsten, (1960) and Fraccaro and Lindsten, (1960), thus suggesting that the postulate of Ferguson-Smith and his co-workers, (1960) was possibly correct.

A third variation of karyotype in Klinefelter's syndrome was found by Muldal and Ockey, (1960) who examined a patient with symptoms similar to the case studied by Ferguson-Smith, (1960) and found that the subject was 48,XXYY. This finding was confirmed by Carr, Barr and Plunkett, (1961) and subsequent patients found to have XXYY sex chromosome construction have been clinically indistinguishable from 47,XXY patients.

Further evidence that the extra chromosomes in Klinefelter's syndrome were indeed X chromosomes, was presented using autoradiography. Rowley, Muldal, Gilbert, Lajtha, Lindsten, Fraccaro and Kaijser, (1963) and Atkins, Book, Gustavson, Hansson and Hjelm (1963), reported that studies of XXXXY male children showed the presence of three chromosomes which were late replicating in a similar manner to one of the X's in normal female cells. In addition, Atkins and his co-workers found that in a number of interphase cells, three areas of heavy labelling were present over the three sex chromatin bodies found in lightly labelled cells from the patient, indicating that the chromosomes which formed the sex chromatin bodies were the same that appeared late replicating at metaphase.

Hsu and Lockhart, (1965) studied a similar case of XXXXY, their findings agreeing with those of Rowley, (1963) and Atkins, (1963) finding in addition that four chromosomes were unlabelled at the beginning of the 'S' period, these being identified as the Y chromosome and three X chromosomes which corresponded to the three late replicating X's. As would be expected from these findings, the simple XXY Klinefelter's syndrome demonstrated one late replicating chromosome, (Atkins and Gustavson, 1964) which was

tentatively identified as an X.

The origin of the supernumerary X chromosomes in four patients was studied by Froland, Johnsen, Andresen, Dein, Sanger and Race, (1963) using the X linked red cell antigen Xg^a discovered by Mann, Cajan, Gelb, Fisher, Hamper, Tippett, Sanger and Race, (1962). Froland and his co-workers, (1963) found that in three of their four cases, both X chromosomes were maternally derived and in the fourth patient, one X was maternally and one paternally derived. Ferguson-Smith, Mack, Ellis, Dickson, Sanger and Race, (1964) similarly found two groups of XXY patients, as classified by the parental derivation of their X chromosomes. From these findings it is clear that the abnormal sex chromosome complement can derive from non-disjunction of either maternal or paternal origin.

In Klinefelter's syndrome the cause of the gonadal abnormality can, at present, only be hypothesised. Ferguson-Smith, (1966) postulated that as in the germ cells of females both X's are isopyknotic, (Ohno, Kaplan and Kinosita, 1961) this imbalance may interfere with the differentiation and development of germ cells in subjects with two X's in addition to a Y chromosome. Ferguson-Smith, (1959) studied prepubertal cases of Klinefelter's syndrome and found that a deficiency of germ cells did exist. Mikamo, Aguercif, Hazeghi and Martin-duPan, (1968) carried out a more detailed study of the testes of neonatal XXY children and found that the spermatogonial population was only 20% of the control population at 3 and 4 months and the frequency was only 0.1% of the normal at 9 months. It was further theorised by Ferguson-Smith, (1966) that the high output of urinary F.S.H. was due to the failure of interstitial cell hormone output and the absence of germ cells. Failure of the secretion of the interstitial cells led to tubular degeneration and as there was a deficiency of germ cells, no feedback mechanism by "Inhibin" was present to stop the F.S.H. secretion from the pituitary. This hypothesis explains most of the facts and at present would appear to be the best explanation for the infertility found in

90% of XXY patients, (Ferguson-Smith, 1966).

In Klinefelter's syndrome, various abnormalities have been reported but the only constant finding has been micro-orchidism, which can be explained by the germ cell deficiency, contraction of the tubules and clumping of the Leydig cells which make up the bulk of the intertubular tissue. More abnormalities are observed in patients with further increases in the number of extra X's (Ferguson-Smith, Alexander, Bowen, Goodman, Kaufmann and Heller, 1964). As a result of this, the imbalance of genetic material becomes greater, with the resultant abnormalities being more severe.

Klinefelter's Syndrome in Domestic Animals

The first case of a domestic animal with a karyotype similar to that found in the chromatin positive Klinefelter's syndrome in man, was reported by Thuline and Norby, (1961). Using the sex-linked coat colours found in cats, as a marker, they found two phenotypically male cats to have chromatin positive buccal smears. In both these animals a diploid number of 39 was found, instead of the modal number of 38. Only a few cells were counted and neither the karyotype nor the sex chromosome complement was reported. However, the presence of sex chromatin together with an extra chromosome was very strong evidence for the existance of XXY cells. In one cat, no gonads nor internal reproductive tract ~~was~~ were found, whereas the other showed abnormal meiosis with no spermatids nor spermatozoa, but spermatogonia were present. Almost simultaneously Frota-Pessoa, (1962) postulated that tortoiseshell male cats could be XXY on the same grounds that Thuline and Norby had used to detect their cases, namely the coat colour. Further evidence that the two tortoiseshell males studied by Thuline and Norby, (1961) might indeed have been 39,XXY was put forward by Chu, Thuline and Norby, (1964). They quoted Jones as finding a male tortoise-shell cat with the expected 39,XXY karyotype and also reported their own findings

in studies on another tortoiseshell cat. In this case cells obtained from tissue cultures of ear and peritoneal biopsies revealed an unusual chimerism of 38, XX/57,XXY. The cat was phenotypically male with slightly small testes which on squash preparation revealed the absence of mitotic figures. An occasional type A spermatogonia was observed but the general picture was one of seminiferous tubules lined by only Sertoli cells. A fourth aneuploid tortoiseshell male was reported by McFeely, Hare and Biggers, (1967). In this apparently normal cat, the testes were composed of seminiferous tubules with some spermatogonia, some having started to divide, but only a few spermatids were found and no sperms.

A brief description of an intersex bovine with an extra chromosome was given by Scott and Gregory, (1965). The animal was found to have the karyotype 61,XXY but no anatomical details were presented.

Two cases of an XXY karyotype ~~has~~ have been found in sheep, (Bruere, Marshall and Ward, 1969). These animals were phenotypically male, the only apparent abnormalities being hypoplasia of testes, azoospermia and aspermatogenesis.

It has recently been reported that intersexes in the pig may have a 39,XXY karyotype as well as the previously found 38,XX complement, (Johnson, Cantwell and Zeller, 1958, Hard and Eisen, 1965, Vogt and Gerneke, 1967). Breeuwsma, (1968) examined an apparently phenotypic male pig with a small penis and a unilateral scrotal hernia. Single sex chromatin bodies were present in over 90%. The majority of cells had 39 chromosomes and ~~was~~ were interpreted as being of an XXY sex chromosome complement. At autopsy, the internal genitalia consisted of small testes with a uterus-like organ ending in a short urethra and penis, the seminal vesicles being poorly developed. The histology of the testes showed Sertoli cells in the seminiferous tubules, but no spermatogenesis was seen. A similar chromosomal finding was reported by Glyhovschi, Bistriceanu, Rosu, and Brata, (1968), in three intersexes. These

were found in a study of 12 cases of intersexuality in which vaginae, uteri and oviducts were found together with seminal vesicles. However, in their brief description of the gonads they failed to make clear the structure of the gonads, as to whether they were ovotestes or testes. Thus, in domestic animals the only species, apart from man, in which a clear picture of the sexual development of XXY animals has emerged is the cat.

The usual findings in this species are phenotypic male tortoiseshell cats with grossly reduced spermatogonial counts and subsequent infertility. A similar picture has been seen in the two sheep studied, but in the ox and pig, not sufficient cases have been reported for a picture to be formed, although it appears likely that intersexuality can be seen.

Materials and Method

The animal under examination, code number AE32, was a ten month old large white pig, a case at the Royal (Dick) School of Veterinary Studies, Edinburgh. It was being used in a study of lymphosarcoma, being one of a litter of ten, from which one had died of lymphosarcoma, the remainder of the litter all being apparently healthy. AE32 was suspected of suffering from the disease on the grounds of its poor weight gain, compared with its litter mates, and also a high and fluctuating white cell count which ranged from 20,000/cu. mm. to 80,000/cu.mm. The animal had been castrated at the age of three weeks and there was no record of any abnormality of the genitalia at that time. The pig was probably a normal phenotypic male, but there is always the possibility than an abnormality might have been present without it being noticed.

A heparinised 20 ml. blood sample was supplied by Mr. H.S. McTaggart of the Edinburgh Veterinary School. This was transported at $+4^{\circ}C$ to Glasgow where the cultures were set up as follows, the technique differing slightly from the described previously. One ml. of reconstituted phytohaemagglutinin was added to the blood and this was kept at $+4^{\circ}C$ for one hour before centrifuging for 5 minutes at 750 r.p.m. The supernatent fluid and buffy coat were drawn off and the red cell layer was centrifuged at 1500 r.p.m. for 15 minutes to yield cell-free plasma. 0.8 ml. of the white-cell-rich plasma, 1.2 ml. of cell-free plasma and a further 0.2 ml. of phytohaemagglutinin were added to 8 ml. of medium 199 (Glaxo Ltd.), thus giving a final white cell concentration of 0.9×10^{6} cells per ml. and a plasma concentration of 20%. The cultures were then incubated and processed as previously described. Before further blood samples for autoradiography or biopsies for fibroblast culturing could be obtained, the animal died, of what was, at autopsy, diagnosed as lymphosarcoma.

At post-mortem which was carried out by Dr. K.W. Head, cervical spinal cord was

removed and fixed in 5% Formal Saline. After standard histological procedures, the spinal cord sections were stained with 1% Cresyl Violet, (Matheson, Coleman and Bell), and permanently mounted using DPX, (British Drug Houses, Ltd). The slides were scanned under low power and suitable cells chosen according to the criteria reported in the sex chromatin studies. The selected cells were then evaluated for the presence of the sex chromatin body or bodies.

Results

One hundred and seventy two metaphase spreads were examined and the chromosome content of each one counted (Table 25). In addition, the presence of a Y chromosome was scored in all 172 cells. As the Y is the smallest chromosome in the complement, no difficulty was encountered in identification. In every cell examined, a Y was present.

Seventeen of the 120 cells containing 39 chromosomes were photographed and karyotyped, Fifteen of these showed a consistent picture in that, having removed the Y, 19 pairs of chromosomes were left, as is found in the female cell. Thus the karyotypes appeared to have the constitution 39,XXY, (Figure 32). As can be seen, all the homologous pairs of chromosomes are present and in addition two chromosomes with the morphology of the X. Of the cells containing 40 chromosomes, only two were of sufficient quality for karyotyping, but both had different supernumerary chromosomes. Three cells with 38 chromosomes were karyotyped and all of these had an apparently male constitution, namely 38,XY (Figure 33).

One hundred and fifty neurones from the spinal cord, stained with Cresyl Violet, were scored for the presence of sex chromatin. The distribution of the chromatin counts in the neurones is shown in Table 26. From this it can be seen that about 70% of the cells were sex chromatin positive and a further 6% contained two such bodies, (Figures 34 and 35).

Autopsy

The autopsy was carried out by Dr. K.W. Head, Department of Pathology, Royal (Dick) School of Veterinary Studies, Edinburgh 9. The findings were that the pig had lymphosarcoma with widespread metastases to liver, spleen, kidneys and lymph nodes. Histology revealed that gross lymphocytic and abnormal cell-form infiltration had

occurred. Unfortunately, histology was not carried out on the genital tract, but it was reported that the tract did not appear abnormal on macroscopic examination.

Discussion

The finding of cells with 39 chromosomes together with neurones containing one sex chromatin body in a male animal is good evidence for the existence of 39,XXY cells. Similarly, the presence of over 17% of the lymphocytes with 40 chromosomes and 6% of neurones with a double body is indicative of a cell line, 40,XXXY. These findings, together with a low frequency 38,XY line, means that the animal under study was a mosaic 38,XY/39,XXY/40,XXXY, in both its lymphocytes and its nerve tissue. However, a number of complications arise when the question is posed of how this aneuploidy could have occurred.

Firstly, the presence of lymphosarcoma means that there is a possibility that the aneuploid cells could be neoplastic. Two factors make this hypothesis rather unlikely.

(a) The presence of sex chromatin bodies in the neurones of the spinal cord indicate that cells with the extra X chromosomes were the original karyotypes of the animal. As mitosis of neuroblasts ceases very early in post-natal life, in the human within the first year, (Arey, 1965), this suggests that the lymphosarcoma would have to have been present virtually pre-natally to have altered the sex chromatin content of the neurones. Furthermore, as there was no indication of any neoplastic change in the nerve tissue, the sex chromosome complement would appear to have been unaffected by the tumour.

(b) Leucosis in the pig is rare as shown by recent surveys at abattoirs. Renier, Friedmann, Chevral, Gacquiere and Guelfi, (1966) found an incidence of about 6 per 100,000 whereas Beutling, (1968) in a similarly sized survey of about 700,000 pigs diagnosed the condition in only four, an incidence of about 0.6 per 100,000. Because of the rarity of the disease

no reports have been published concerning the chromosome complement in the condition. However, in other species of domestic animals, chromosomal analysis has been carried out on cases of lymphosarcoma. Basrur and Gilman, (1966) found that in five dogs with lymphosarcoma, nearly all cells from peripheral blood culture had normal karyotypes although aneuploidy was found in many of the cells taken directly from lymph nodes without resort to culture. Similarly, in cattle, Hare, McFeely, Abt, and Feierman, (1964) found that in the four cases in which peripheral blood cultures using phytohaemagglutin were carried out on animals with aneuploidy of cells from lymph nodes, the karyotypes were found to be normal. From these findings, it would appear that in dogs and cattle, cells from peripheral blood cultures, to which phytohaemagglutinin has been added, do not reflect the aneuploidy of the neoplastic lymph nodes. More recent work by Hare, Yang and McFeely, (1967) showed that in eight cattle with lymphosarcoma in which aneuploid cell lines were found in blood cultures with phytohaemagglutinin added, an apparently similar cell line was present in lymph nodes, but the blood cultures of the remaining 26 animals with aneuploid cell lines in lymph nodes had normal diploid mode. It would appear therefore that culturing cells in medium to which phytohaemagglutinin has been added is more favourable to non-neoplastic cells than to neoplastic ones. Hare, Yang, and McFeely found that only about 55% of the lymphosarcomatous cattle which were cultured without phytohaemagglutinin had diploid modes, whereas 80% of the same animals when their blood was cultured with phytohaemagglutinin displayed diploid modes.

If parallels can be drawn from other species it would appear that in the present case, even if aneuploidy had been seen in the neoplastic nodes, it would be unlikely that these abnormal cells would have been observed with the lymphocyte culturing as phytohaemagglutinin was used. This, together with the evidence above, suggests that the aneuploidy found in the present case was the true chromosomal constitution of the animal.

Although it is impossible to draw any conclusions concerning the co-existence of lymphosarcoma and sex chromosome aneuploidy from the present single case, this association has been reported in other species although in the case of XXY individuals, it has only been noted in the human. Basrur and Gilman, (1966) examined five cases of lymphosarcoma in dogs and found, in cells taken directly from the lymph nodes without recourse to culturing, that extra metacentric chromosomes similar to X chromosomes were present. However, in these cases variable aneuploidy of the acrocentric autosomes with possible rearrangement was present and because of this it is impossible to identify positively the X's without resorting to idiogram construction and autoradiography, neither of which were done.

However, in bovine lymphosarcoma more positive evidence exists for the presence of aneuploidy involving the X chromosome. Basrur, Gilman, and McSherry, (1964) found in a cow with lymphosarcoma that the majority of lymph-node cells cultured for only 24 hours contained an extra chromosome which was morphologically similar to the X, thus giving a karyotype 61,XXX. Seventy percent of the interphase cells from the same culture appeared to indicate that the extra chromosome was in fact an X. A second mode of normal female cells was also present indicating the animal was a mosaic 60,XX/61,XXX. In ten-day cultures of the same animal the frequency of 61 cell line was much lower, possibly indicating again that neoplastic cells are at a disadvantage in prolonged culture.

X chromosome aneuploidy was also noted by Hare, Yang and McFeely. In ten out of 47 cases of bovine lymphosarcoma, an X chromosome was missing and may also have been missing in 2 further cases, although it was not possible to be definite as other metacentric were present which could have been either deleted X's, translocations involving the X or possible rearrangement of the autosomes.

In the human, Klinefelter's syndrome co-existing with leukemia has been reported, although not frequently. Ferguson-Smith, (1966) in a survey of the literature of

354 cases of chromatin-positive Klinefelter's syndrome found only 2 cases of leukemia. One of these was reported by Tough, Court Brown, Baikie, Buckton, Harnden, Jacobs, King and McBride, (1961) who in a study of chronic myeloid leukemia found a male with the disease to have a lymphocyte mosaicism 46,XY/47,XXY together with an abnormal small chromosome. More recently Borges, Nicklas and Hamm, (1967) examined 25 non-mongoloid children with acute leukemia and found that four had cytogenetic abnormalities, 2 of which were 47,XXY. Borges and his co-workers postulated that aneuploid cells are more susceptable to post-zygotic environmental factors which may eventuate in acute leukemia. However, from Ferguson-Smith's literature survey, in the case of Klinefelter's syndrome this hypothesis does not appear to be borne out in the case of X chromosome aneuploidy.

Nevertheless, Borges' hypothesis concerning aneuploidy is certainly true in the case of two different aneuploidies of the human 21 chromosome. Firstly, in Down's syndrome, before the chromosomal basis of the condition was discovered, it was found by Stewart, Webb and Hewitt, (1958) that the incidence of leukemia in Mongols was much higher than should be expected from the frequency of mongolism in the human. Stewart and his co-workers examined the histories of 1416 children who died from malignant tumours and found that 17, or 2.6%, of all leukemias, were in mongoloid children. This incidence was about 20 times the expected frequency based on the incidence of mongolism at birth of about 1 in 700 (Penrose, 1966).

The year after Stewart's report, Lejeune, Gautier and Turpin, (1959) reported the association of a small additional chromosome in three cases of Down's syndrome. This finding was substantiated in the same year by Ford, Jones, Miller, Mittwoch, Penrose, Ridler and Shapiro, (1959); Jacobs, Baikie, Court Brown, and Strong, (1959), and Book, Fraccaro and Lindsten, (1959). This chromosome was an acrocentric chromosome being one of the smallest chromosomes in the complement, and was variously described either 21, 22 or 23. In 1960, Nowell and Hungerford reported finding an abnormal chromosome in two cases of chronic myeloid granulocytic leukemia, and suggested

that it was an abnormal Y chromosome. In the same year Baikie, Court Brown, Buckton, Harnden, Jacobs and Tough, (1960) found a similar abnormality in 8 out of 12 patients with the same condition, refuting Nowell's explanation that it was Y, it being also found in women suggesting that it was a deleted 21 or 22. In further publications, Nowell and Hungerford, (1960a and 1961) reported further cases with the abnormal chromosome present and designating it a deleted 21 or 22. In accordance with the suggestion of the Denver Report, (1960) this chromosome was named after the town in which the abnormality was first discovered and is now known as the Philadelphia chromosome (Ph'). These workers found the Ph' chromosome to be present in a patient before any symptoms were seen and stated that it was possible that the abnormality was a primary change and not a result of the neoplasia.

Although it was not until later that the mongol chromosome and the Ph' chromosome were shown to be same, (Schmid 1963), Nowell and Hungerford hypothesised that, as it appeared to be the same, the long arm of 21 might contain loci governing the production of white cells because trisomy in mongols appeared to produce leukemia in a high percentage of the cases and effective monosomy for the locus gave rise to chronic myeloid leukemia. However, the frequency of leukemia in mongols is far lower than the freqeuncy of the Ph' found in chronic myeloid leukemia and if such a locus does exist the frequency of trisomy - induced leukemia should be much higher, although environmental factors may play a part. At present, the suggestion can only be treated as an unlikely unproven hypothesis. From the findings in neoplasia in animals and man, it can be said that chromosome abnormalities occur in conjunction with tumours in three ways:-

(1) Probably as a result of tumour or as a maldivision or error in the post-zygotic period, thus possibly eventually contributing to the tumour development. Examples of these are lymphosarcoma in cattle and the Ph' chromosome.

(2) As a predisposing cause to neoplastic development, such as the trisomy in Down's syndrome.

(3) Coincidental co-existance of chromosomal abnormality with the neoplastic condition. Klinefelter's syndrome in man would appear to fall under this heading.

Under which heading the present case falls can only be a matter of conjecture, and more such cases will have to be studied before any suggestion can be made on this matter.

The method by which the XY/XXY/XXXY karyotype of the present case arose must have involved at least two divisional errors during the early development of the zygote. A number of possible explanations can be put forward, but with the exception of the following two, all require at least four errors of division to have occurred. The most likely explanations would appear to be:-

(1) Meiotic non-disjunction in either the maternal or paternal germ cells leading to either an XX ovum fertilised by a normal Y-bearing sperm or an XY sperm fertilising an X-bearing ovum. Froland, Johnsen, Andresen, Dein, Sanger and Race, (1963) showed that in cases of XXY males, the extra chromosome could be either maternal or paternal in origin. The XXY zygote at a mitotic division other than the first, then underwent either a mitotic non-disjunction or an anaphase lag of one XXY cell resulting in two cell lines XY and XXXY. The resulting mosaic zygote would then be XY/XXY/XXXY. Ferguson-Smith, (1966) reported two cases with similar karyotypes and Barr, Carr, Morishima and Grumbach, (1962) reported a mental defective male who was a mosaic XY/XXXY. Barr postulated that this arose in a not dissimilar way to the theory concerning the present case, in that an XXY zygote in the first mitotic division underwent non-disjunction resulting in XY/XXXY.

However, they accepted that the maldivision might have been at a later division and that it was XY/XXY/XXXY, but the XXY cell line had not been detected.

(2) The other possibility could involve two successive mitotic non-disjunctions.

(a) A normal XY zygote at the first mitotic division underwent non-disjunction with a resultant XXY and Y cell lines, the latter being lost as it is most likely inviable.

(b) The XXY zygote underwent a normal division and at a later stage underwent a second mitotic non-disjunction to result in a mosaic XY/XXY/XXXY.

The reason for speculating that the first error occurred at the first mitotic division is that if it was delayed, a higher percentage of XY cells would be expected to be found in the animal. Although it is not possible to assess frequencies of cells in a cell line from one culture, the combination of a low frequency line, together with an incidence of sex chromatin nuclei no lower than normal females would appear to indicate that the XY line was only a minor mode and therefore developed later in zygotic division. It is obviously impossible to state which of the two theories is correct and as such the facts must remain obscure. Other hypotheses could be put forward, but these require about four errors to have occurred and as such would appear to be very unlikely.

Although the presence of sex chromatin bodies in neurones in the pig was first described by Cantwell, Johnstone and Zeller, (1958) over 10 years ago, the present case is the first one to demonstrate that the modified Lyon hypothesis, (1962) is valid for the pig and, in fact, for domestic animals. In this hypothesis, Lyon stated that in individuals with supernumerary X chromosomes, the number of sex chromatin bodies is one less than the number of X chromosomes. Thus the presence of double sex chromatin bodies together with a presumed 40,XXXY cell line, appeared to allow the reasonably valid assumption to be made that the Lyon theory holds good in the pig.

It is known that double sex chromatin bodies in normal female pigs are very rare, (Table 9), and the finding of 6% in this case must be taken as significant especially when found together with 17% of cells having 2 chromosomes more than found in the normal male. Barr, (1966) states that in the human although buccal smears are essential in screening, the frequency of double bodies should not be relied upon for statistical analysis. In his opinion the findings of such double bodies in nerve tissue give a much more reliable assessment and in a retrospective analysis of a previous case, (Barr, Shaver, Carr, and Plunkett 1959), he was of the opinion that from the presence of double sex chromatin bodies in a Klinefelter's case, it could be assumed with reasonable confidence that the patient was XXXY, even though no chromosome analysis was carried out. Judging the present case by Barr's opinion, there would appear to be no doubt concerning the conclusions drawn from the cell line containing 40 chromosomes.

Unfortunately, in the pig under study the anatomical findings were sparce. The only factors which were communicated between the clinician in charge of the case and the author were (1) The pig had a very poor growth and lymphosarcoma

(2) It had been castrated early in life without any abnormalities being noted and, at autopsy, the genital tract did not appear abnormal.

Due to the co-exisiting lymphosarcoma it is impossible to attribute growth abnormalities to any factor other than the tumour. With reference to the second point, at castration, pigs are routinely examined for the presence of hernias, and at this examination the presence of a vulva or abnormal genitalia would certainly have been noted. Thus, although a rather incomplete history is present, it can be stated descended testes and an apparently normal penis were present and no ambiguity of the genitalia suggestive of intersexuality was observed. It would appear, therefore, that the present case was a normal phenotypic male.

From the findings, the presence of supernumerary X chromosomes appears to have no effect on the genitalia in the pig, being in this way similar to the human Klinefelter's syndrome. However, in the human, cases with 49,XXXXY, tend to have some abnormalities of the external genitalia, (Fraccaro, Kaijser and Lindsten, 1960; Joseph, Anders and Taylor, 1964), this being probably explained by the finding of Ferguson-Smith etal, (1964) that not all of the inactive X is in fact inactive. If this is so, as the number of X's increase, the amount of heterochromatin derived from the X's will similarly increase, this being in contradiction to the original Lyon hypothesis, (Lyon 1961). By this theory, an XXXXY individual would be expected to be effectively XY, with the extra X chromosomes inactivated. Lyon, however, modified her theory to include the increase in abnormalities which occurred with the increase in X's (Lyon, 1963).

The present case appeared to parallel the findings in the human Klinefelter's in having normal genitalia, although obviously the main findings in man, that of testicular abnormality, could not be determined. In the sheep, a similar situation occurs in XXY rams, in that the only phenotypic abnormality is testicular hypoplasia of the postpubertal animal, (Bruere, Marshall and Ward, 1969). In the human and the ram, the only consistent finding in Klinefelter's syndrome is infertility associated with lack of germ cells. As Ferguson-Smith (1966) has pointed out, as X-inactivation does not occur in germ cells, these cells have to bear the full brunt of the genetic imbalance. If this is so, germ cell deficiency may arise. Although no pre-natal XXY individuals have been examined to find at which stage this may occur, Klinefelter's babies have been studied (Mikamo, Aguercif, Hazeghi and Martin DuPan, 1968), and these showed a gross deficiency of spermatogonia. Similar findings were reported by Ferguson-Smith, (1959) who found that it was at puberty that the deficient tubules underwent hyalinisation. In Turner's syndrome in which the lack of an X chromosome must again leave the germ cell with a genetic imbalance,

early human embryos and fetuses with XO constitutions were studied, (Singh and Carr, 1966). They found that in the fetuses up to an estimated age of 43 days, the ovarian structure was similar to that of XX embryos of similar age. However, in embryos of about 70 days and older, an increase in the connective elements occurred instead of the normal sequence of primary follicle development. It was noted that the deficiency in these XO ovaries was of follicular cells, possibly leading to primordial follicle mal-development and degeneration of germ cells. After birth, similar happenings to those observed in Klinefelter's syndrome occur, in that germ cells are still present at and after birth, (Carr, Haggar and Hart, 1968), but these have virtually disappeared by puberty. It is quite possible that very similar steps occur in pre-partum Klinefelter's syndrome foetuses as occur in XO foetuses, the post-partum and pre-pubertal behaviour of the germ cells in both syndromes being very similar. As the gonads of the XY/XXY/XXXY pig were not available, it is not possible to know if the same mechanisms function in the pig as in man. However, the testes at castration did not appear abnormal and from this it can be presumed that they were of normal size. The absence of pre-pubertal changes in the macroscopic appearance of the testes is also a finding in the human Klinefelter's syndrome, (Ferguson-Smith, 1958) and thus from the findings in the present case, there is no reason to assume that there is any difference in the phenotype as compared with Klinefelter's syndrome in man, in that the pig was a phenotypic male with apparently normal testes.

However, recent findings of intersex pigs by Breeuwsma, (1968) and Gluhovschi, Bistriceanu, Rosu and Brata, (1968) appear to indicate that a different mechanism. so that in the human may exist with some XXY pigs. The possibility of mosaicism XX/XXY cannot be ruled out in any of these cases. Breeuwsma counted only 32 cells of which 30 contained 39 chromosomes. No indication was given of the karyotype of the other 2 cells, nor of how many of the 30 cells were in fact XXY and how many

contained an extra autosome. If there was indeed a 38,XX cell line present, it would also show up as sex chromatin positive and thus a frequency of 90% sex chromatin positive cells could still be obtained. Although it would appear unlikely, an undetected cell line could possibly be present in the 3 cases of Gluhovschi and his co-workers as again no indication is given as to number of cells counted or what percentage of cells did contain 39,XXY.

These two reports indicate two ever-present failures in cyto genetic studies. Firstly, the failure to report the numbers of cells counted, and secondly, the failure either to report the frequency of chromosomal distribution found or to omit to karyotype other aneuploid cells. These omisions invariably result in inconclusive findings and in the possibility of undetected mosaicism which can negate otherwise valid work because of its inconclusiveness. However, although this possibility exists, it would appear unlikely that in all four of the cases mosaicism existed and in every case was undetected. The report of Gluhovshi etal, (1968) is so incomplete with no anatomical details, chromosome counts or karyotypes presented, that it must be ignored as a valid finding. Breeuwsma, (1968) did present one karyotype, but in it a certain amount of dubiety is present. Chromosome pair number 15 was completely unlike small submetacentric chromosomes, and, in fact, was not dissimilar to a single chromosome 13 with its secondary constriction. Because of this, doubt must be present as to his interpretation of the karyotype presented. However, it does seem unlikely that he has miscounted all 30 cells which were claimed to contain 39 cells. If it is assumed that the pig is 39,XXY, it must be assumed that intersex pigs can have this karyotype. An unusual feature of this pig is that externally it resembled a male, in that it had a small penis and a scrotum. This is in complete contrast to the usual appearance of the pig intersex, which has normally a female external appearance, (Johnson, Cantwell and Zeller 1958; Makino, Sasaki, Sofuni and Ishikawa, 1962; Gerneke, 1964; Hard and Eisen, 1965 and Vogt, 1966).

However, what was described a small penis, more closely resembled an enlarged clitoris as it pointed posteriorly and was in a position approximately halfway between scrotum and anus. At post mortem, the reproductive tract was more typical of an intersex having two small testes and a uterus-like organ. Thus, this case appears to be similar to the typical pig intersex, with testes, uterus and seminal vesicles, with the exception that the external genitalia were more masculinised.

The mechanism of sex determination in the pig is highly complex and one which is still not fully understood. To attempt to explain it, it is necessary to draw on the findings in the goat. In this species, intersexuality has been known for about 30 years to be associated with the dominant gene for hornlessness. Eaton and Simmons (1939) and Asdell (1944) in their studies on the problem, concluded that it was a recessive character associated with the hornlessness gene. They furthr found that when the intersexes were classed as males, which they resembled, a sex ratio of male to female of 150: 100 was found. They therefore assumed that these goats were genetically female. Detailed work by Soller and Angel, (1964), revealed that intersexes only appeared in the offspring of mating types which could give rise to animals homozygous for the polled gene. They further found that even if the intersexes were counted as female the male/female sex ratio was high and in the mating type expected to give 50% homozygous offspring, the ratio was even higher. They postulated that either some genetic females were completely sex reversed to apparently normal males or a lethal factor was occurring in the homozygous females.

Cytogenetic evidence for the theory that intersex goats were genetic females was produced by Luers and Struck, (1959); Basrur and Coubrough, (1964) and Biggers and McFeely, (1966). In virtually all the cases reported, although the genital tract was found to vary from virtually normal female to virtually normal male together with all gradations between the two extremes, the one constant feature which was found was the presence of testicular tissue. Short, Hamerton, Grieves and Pollard, (1968)

found that one gonad of an intersex goat produced amounts of testesterone comparable to that produced in the normal male. Thus in the goat, genetic females can possess testicles, produce testosterone and have a genitalia which in some cases can mean that the animal is considered a normal male, (Short, R.V. personal communication).

A rather similar situation exists in the pig, with the exception that although testes develop, the rest of the reproductive tract is usually predominantly female. (Johnston etal, 1958; Hard and Eisen 1965; Vogt, 1966). With the exception of two animals with the sex chromosome complement XX/XY and which were most likely freemartins (McFee, Knight and Banner, 1966; Bruere, Fielden and Hutchins, 1968), all porcine intersexes so far found have been genetic females, both on the basis of sex chromatin studies and chromosome analysis. Johnston etal, (1958) suggested that the condition was caused by a recessive gene which was influenced by modified genes. However, the findings of two species in which testicular tissues develops in genetic females makes it appear possible that a similar mechanism may work in both the goat and pig.

The method by which these abnormalities develop can only be a matter of conjecture. One explanation which was considered by Biggers and McFeely, (1966) was that intersex goats were in fact mosaics in which XY cells can be found only in the testes. This theory was rejected on the grounds that this would mean that about 10% of goats were mosaics and it would be difficult to imagine a mechanism which would result in such a frequency of meiotic or mitotic errors.

Another possibility which must be considered is that put forward by Ferguson-Smith, (1966a) to explain the occasional true hermaphrodite and Klinefelter's syndrome patients with an XX chromosome complement. He postulated that as the X and Y chromosome paired end-to-end at meiosis, occasionally an interchange of material between the two chromosomes could occur with the result that the X chromosome was carrying some male determining genes. As a result the individual who inherited this X plus part of Y could develop testicular tissue when the normal X was inactivated. Recent evidence

involving Xg studies have indicated that in some cases of XX Klinefelter's syndrome this has most likely happened, (Ferguson-Smith, Personal Communication). However, although this mechanism may occur occasionally it seems unlikely that it could explain the large number of XX intersexes in both goats and pigs.

Recently, two groups of workers have attempted to explain the testicular development in XX goats. McFeely, Hare and Biggers, (1967) suggested that if the theory that the X and Y were originally evolved from homologous chromosomes was correct, (Ohno, 1965), it is feasible to assume that maleness genes are present on the X chromosomes and that these are capable of being repressed or derepressed. They postulated that in the presence of a Y chromosome the male genes on the X are derepressed allowing the animal to develop as a male. If the Y was absent the animal developed normally as a female. McFeely and his co-workers explained the intersexes by the fact that the autosomal factor linked to the polledness gene can derepress the maleness genes on the X allowing development of testes, the degree of maleness depending on the amount of derepression.

Hamerton, (1968) similarly suggested that the Y chromosome acted as a controlling centre for factors on the X chromosomes. He postulated that in the presence of the Y, the structural gene on the X switched on and thus mediated the production of a medullary stimulator or a cortical inhibitor. Whatever the factor produced, be it stimulator or inhibitor, the result was the development of testicular tissue. In the case of the female, both the controlling centre and the gene for cortical stimulator or medullary inhibitor were carried on the X and in the absence of the Y the organism developed as a female. Hamerton explained intersexuality in the goat and possibly the pig by postulating that autosomal modifiers may act in a similar manner to the Y, by switching on male factors.

On the basis of the complex sex determination in the pig, it could be postulated that the presence of XXY sex chromosomes in an intersex pig may involve an autosomal

modifier gene which acts in an opposite way to that postulated above. If such a gene exists it could repress the factors on the X chromosome which had been derepressed by the presence of the Y. Thus depending on the time at which the gene started to act, the degree of maleness would be determined. If the gene repressed the maleness factors early in development just after testes were formed, the rest of the tract would continue development along female lines as this was shown to happen by Jost, (1955). If this occurred late in gestation, a virtually normal male would result. From the findings in XX intersex pigs, it would appear that the latter does not happen. This would appear to indicate one of two possibilities. Firstly, that the gene invariably acts early in gestation, or secondly, that complete sex reversal occurs and these animals are not detected.

A second possibility involves another theory put forward by Hamerton, (1968). He suggested that autosomal modifiers could account for the presence of testicular feminisation in XY human females. It was postulated that the modifiers might make the target organs insensitive to testesterone. Jost, (1955, 1967) had suggested that two hormones are probably produced by the fetal testes, one which stimulates the Wolfian system and a second one which cases regression of the Mullerian Ducts. Thus, if the theory of Hamerton is to be accepted, it would appear necessary to postulate that the tissues are made insensitive to both hormones. The validity of these hypothesis can only be tested with the discovery of phenotypic female pigs with the sex chromosome complement of XY. Whatever the correct explanation is concerning testes development in XX pigs, it appears likely that the presence of the Y chromosome in Breeuwsma's case has caused further masculination than is usually found in the intersexes.

Whatever is the explanation of the development of intersexuality in XXY pigs, it seems feasible to have two types of XXY pigs, one developing in a similar manner to XXY humans and sheep and a second group developing testes similar to the first group

but under some unknown influence continuing development as females. The development of two distinct types of pigs with the same chromosome complement is certainly not surprising when viewed in the light of two such groups with the karyotype 38,XX developing either as intersexes or normal pigs.

With the discovery of more XXY pigs it is hoped a number of these problems concerning sex development will be clarified.

Miscellaneous Clinical Material.

10 Boars, all about 6 to 10 months of age were examined cytogentically, for sex chromosome abnormalities. Six were suspected to be infertile, as none of the sows which had been served by them had held to service. Unilateral testicular hypoplasia had been observed in two cases, and the other four pigs had apparently normal-sized testes.

The remaining four cases were referred before having been put to service, mainly because the Pig Industry Development Authority testing stations considered the testes to be rather small. In no case was the actual measurement reported.

Peripheral blood cultures were prepared from all ten cases, and the chromosomes analysed as described elsewhere. In every case, the chromosome constitution was 38, XY. Because of this, no further work was carried on the pigs.

Although this study did not reveal any chromosomal abnormalities, this line of investigation would possibly appear to be a potentially fruitful one for future studies, in view of the findings in cases of testicular hypoplasia in other species.

Section VI

APPENDIX

PHOTOGRAPHY

Camera

All Photomicroscopy was carried out using a Wild M20 binocular microscope fitted with a Wild 1.25 X camera tube with multiple variable shutter speeds. The ~~mangification~~ magnification available with system was found by the formula:

O x C x E where O is the ~~mangification~~ magnification of the objective.

C is the inherent magnification of the camera tube.

E the magnification of the eyepiece in the camera tube.

The values of the factors were O :- a) 10x b) 20x c) 50x d) 100x

C :- 1.25x

E :- 10x

Thus, the possible magnifications were 125x, 250x, 625x, 1250x, the majority of photographs being taken at 625x.

Film Magazines

The magazine used was a Plaubel miniature film Adaptor. This adaptor took 24 x 36 mm. negative held in a standard 35 mm. cassette. The film is fed round a system of sprockets on to the wind-on cassette.

Light readings were taken directly from the camera tube to a Microsix light meter (Leitz) by means of a short lead. The light reading measurements were expressed in D.I.N. units. The amount of light which passed to the film emulsion was variable by using (a) light readings (b) different shutter speeds.

A combination of meter readings and shutter speeds for different films was found by carrying out different exposures by varying the light readings. The values used for Microfile (Kodak) 35 mm. panchromatic film, the most commonly used film, was 1 sec. at D.I.N. 11-12.

Negative Film Used

Microfile (Kodak) 35 mm. panchromatic with an antihalation base was used to photograph both metaphase and cells labelled during autoradiography. Suitable lengths of Microfile film (approximately 3 ft.) were placed into 35 mm. cassettes and loaded into the Plaubel film holder in the dark-room. After the required number of photographs had been taken, the exposed film was rewound into the cassette and this was removed in the dark-room. Processing of the film was carried out in a Johnston Universal Tank (Johnsons of Hendon Ltd.). The film was removed from the cassette in the dark-room and fed into the film spiral adjusted for 35 mm. films.

Development

The developer used was Bromophen powder (Ilford), formerly ID36 (Ilford). This was made up according to manufacturer's instructions and stored as concentrated stock solution. For use, 1 part stock solution was diluted with 3 parts water to make 300 cc. of working solution at 20°C.

Fixing

Acid hypo-fixative (Johnson of Hendon Ltd.) was made up as working solution according to maker's instructions. 300 cc. of the solution at 20°C. was used for Microfile film.

Processing Times

Developing	Bromophen at 20°C.	6 mins. (10 seconds agitation every minute).
Washing	Tank filled and emptied twice.	
Fixing	Acid Hypofixative at 20°C.	10 mins. (10 secs. agitation every minute)
Washing	Running water for 30 minutes.	

The film was then placed in a dust-free drying cabinet until completely dry, before being cut in 5 frame lengths and stored in a 35 mm. film wallet until use.

Printing

Chromosome spreads were normally printed at a magnification of x 3,000. A microscopic grid of length 1 mm. divided into 100 equal parts was photographed at all possible magnifications. This was placed in the enlarger and the height adjusted until the distance between the grid spaces ~~correspond~~ corresponded to the required magnification. A mark was made on the wall so that a standard magnification could be used. In this way the height of the enlarger head was calculated in order to give a magnification of x 3,000 using negatives with magnification of both x 625 and x 1250.

The paper used was single weight Gevebrom (Gevaert Ltd.) or Kodak Bromide (Kodak Ltd.). These papers were in different gradings of hardness and the decision as to the grade suitable for different negatives was only arrived at by trial and error until enough experience was gained to be able to judge the grade required at first sight. Similarly the experience in deciding the combination of exposure time and the aperture size of the enlarger lens was only gained with time.

The negative was placed in the negative holder, and the enlarger switched on with an orange filter in placed on the light beam. The negative was focussed on a Leitz easel and the printing paper placed on the easel and held by the metal straps.

The orange filter on the enlarger was pushed aside for the decided period at the end of which the projection lamp was switched off. Once the print had been exposed, the remainder of the printing procedure was kept constant in order to reduce the number of variables to the minimum.

The developer and fixative were stored in darkened Winchesters until required for use.

Developer

D - 163 Developer Powder (Kodak) was made up according to manufacturer's instructions. For use, 1 part of the stock solution was diluted with 3 parts of water to give a working solution of developer at 20°C. This was used in an enamel developing dish into which the print was placed emulsion side up and with gentle continuous agitation was developed for 90 seconds. The time was adhered to very strictly so that the degree of development was constant. The developer was poured away when the day's printing was finished or whenever it started to discolour badly.

Washing

After removal and draining of the developed print it was washed in a developing dish of cold tap water for about 10 seconds, in order to remove developer and thus save the fixative from exhaustion. Acid Hypofixative (Johnson's of Hendon Ltd.) was made up according to instructions. This was re-used a number of times until it was considered that excess contamination had occurred, Prints were fixed face down for at least 10 minutes and agitated about once a minute during that time. It was important that the whole print surface was submerged, because if not, discolourisation tended to occur.

After fixation was completed the prints were washed for 60 minutes in a water bath with a continuous fill and re-filling action. At the end of this time the prints were removed, placed in a dish filled with water and left in this until glazing was carried out. Using a Photax double sided glazer, the prints were placed emulsion side down on the glazing surface and the excess water removed using a rubber coated glazing roller. The canvas cover was then pulled down on top of the prints. Glazing was complete when the print sprang up from the surface of the glazer and was completely free. The prints were then stored until required for use.

Washing and Sterilization of Glassware

All glassware which was used in any part of the culturing procedure was thoroughly washed, these containers in which culturing was carried out were further sterilized by autoclaving. Pasteur pipettes used in the removal of fluid during hypotonic treatment and fixation were discarded after use. Similarly, sterile disposable syringes and needles were used for withdrawal of blood from veins and also of fluids such as medium, phytohaemagglutinin and white cells.

Throughout the study, 1 ounce Universal Bottles were used for culturing of white cells. In the earlier part of the experiments, metal caps with rubber liners were used, whereas later, plastic universal bottle caps were found to be equally satisfactory. 10 ml. Centrifuge tubes (E.-Mil. Ltd.) were used during hypotonic treatment and fixation of the cells.

With the exception of the rubber liners of the caps, all materials were cleaned together and in an identical fashion. Immediately after culturing, running cold water was used to remove all traces of blood and solutions. This was done by repeated hosings using a narrow bore tube attached to the cold water tap. After all traces of fluids and cells had been removed, the glassware and universal caps were placed in a Chloros solution, made by adding about 2--3 ounces of Chloros to a gallon of cold water and allowed to steep overnight. At the end of this time, repeated washes in cold running water removed all traces of chloros before placing the containers and caps in a solution of Pyroneg (Diversey U.K. Ltd.) consisting of 2 tablespoons per gallon of warm water. After 2-3 hours soaking in this solution, vigorous brushing of the bottles and caps in Pyroneg was carried out in order to thoroughly remove all traces of grease and cells which might still be present in the classware. Pyroneg was then completely removed by about 20 rinses in running hot water followed by a

similar amount of washing with cold water. Final rinses were carried out using glass-distilled water and the glassware and metal caps placed in a hot air oven until thoroughly dry. When dry, the 10 ml. Centrifuge tubes were removed and wrapped in aluminium foil, then stored in a clean drawer.

The rubber liners for the metal caps were treated separately. After thoroughly washing to remove all traces of blood, the liners were placed in a one litre beaker with fresh cold water, this being brought to the boil and allowed to remain at this temperature for 15 minutes. At the end of this time, the beaker was covered with aluminium foil and the water allowed to cool. The liners were removed and thoroughly dried using a freshly laundered glass towel. The liners were then placed in the metal caps and screwed tightly on to the bottles. In the case of the plastic cups, after drying in the oven, they were similarly screwed on. The universal bottles were wrapped in aluminium foil and placed in an autoclave basket. Autoclaving was carried out at 15 lbs. per square inch for 30 minutes. When cool, the bottles were stored in a dust free cupboard until ready for use.

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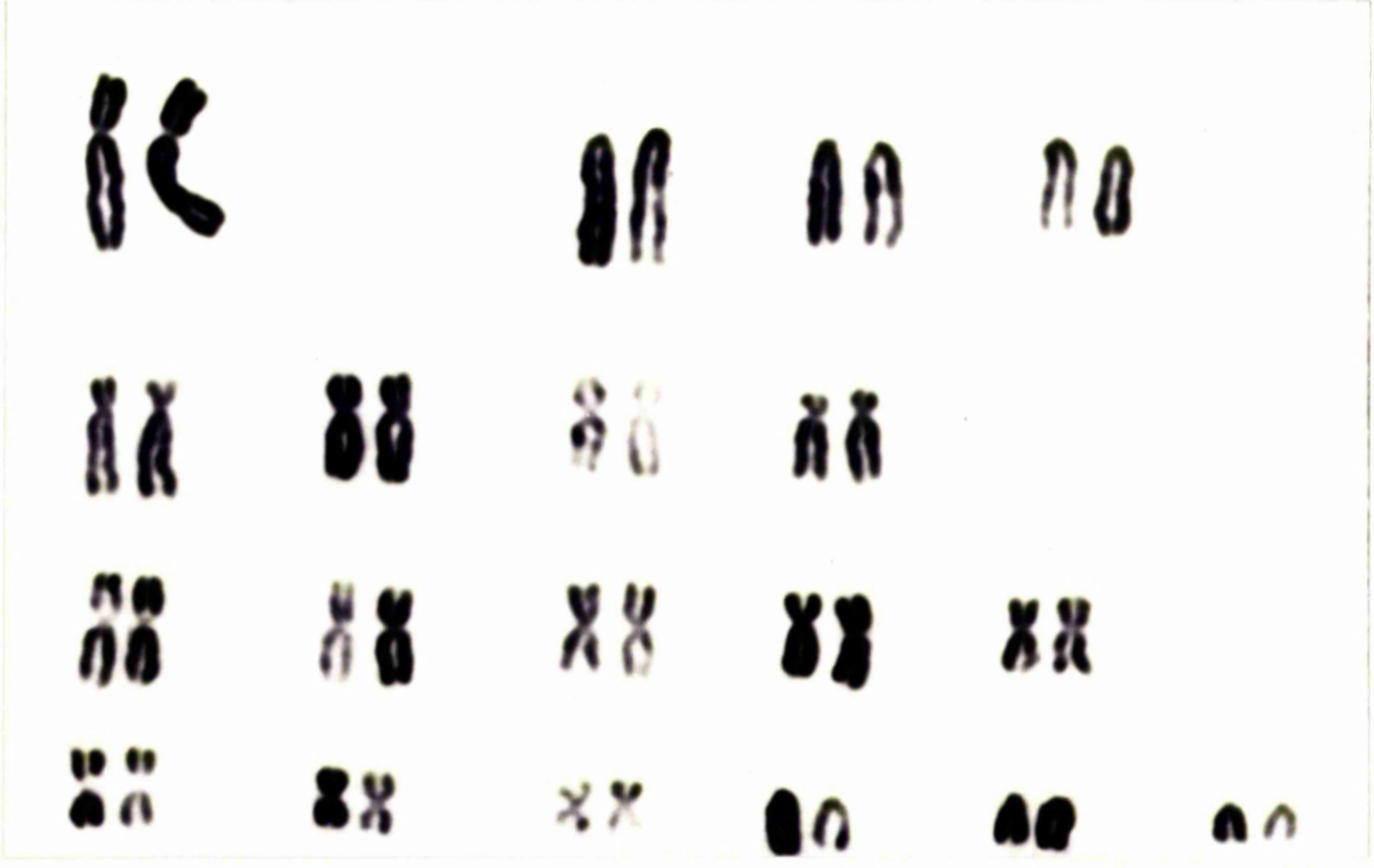
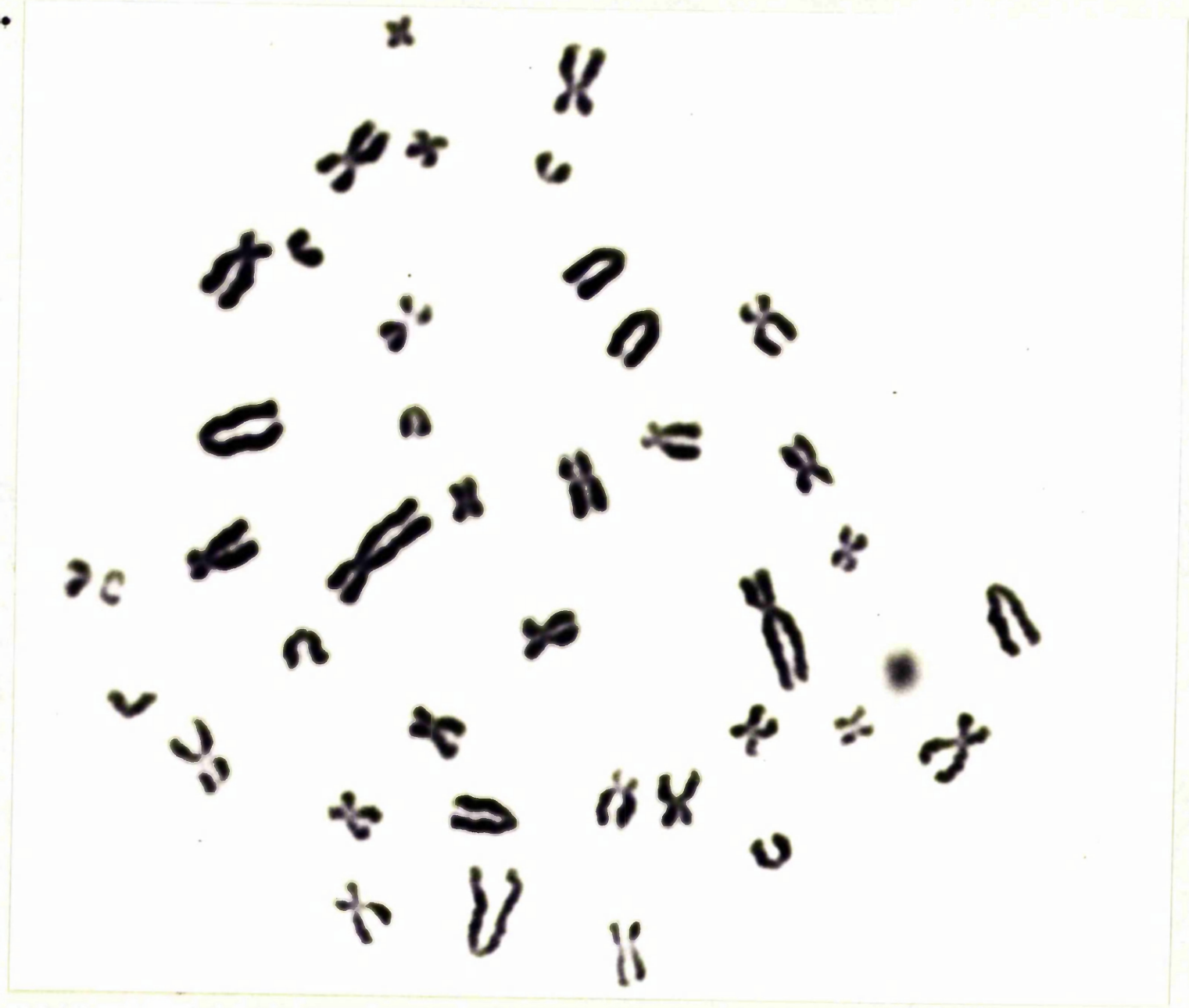
Figures.

Figure 1.



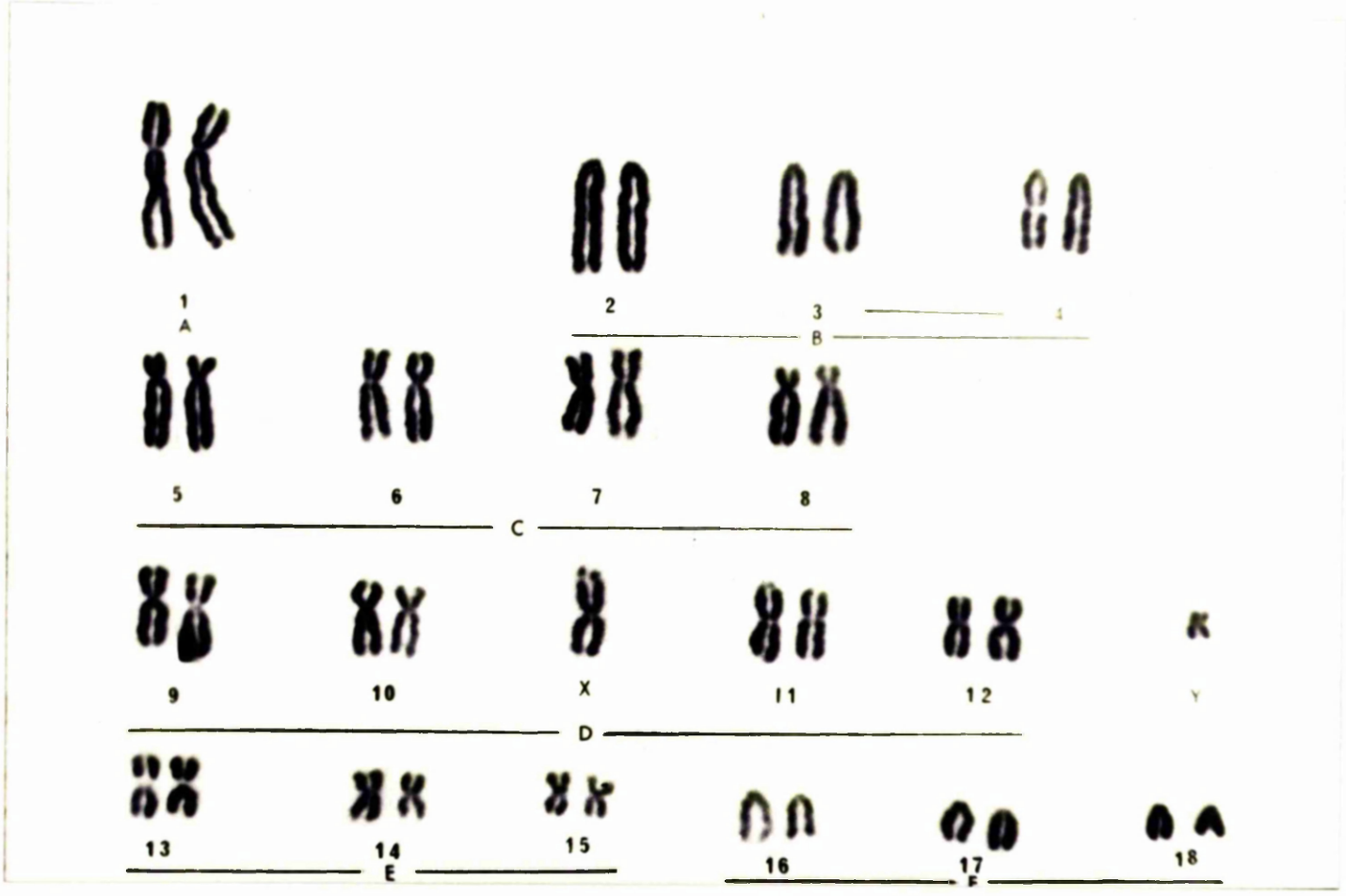
Karyotype of 38,XX cell from peripheral blood culture.

Figure 1a.



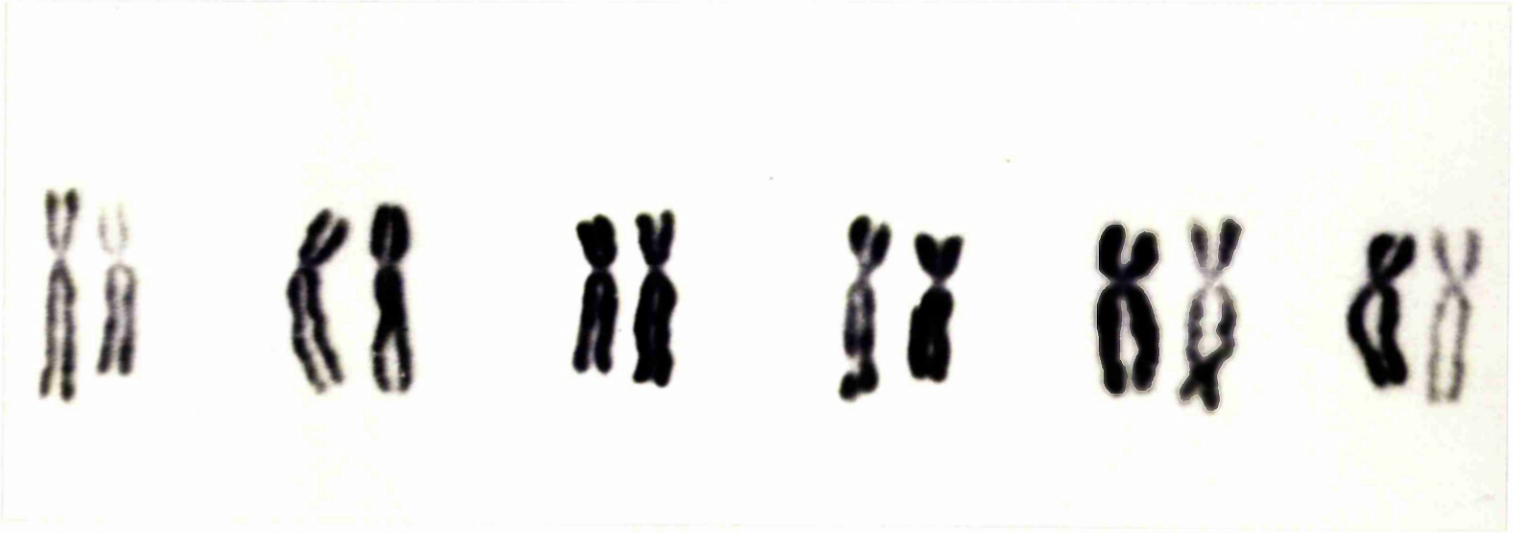
Metaphase spead from peripheral blood culture of female

Figure 2.



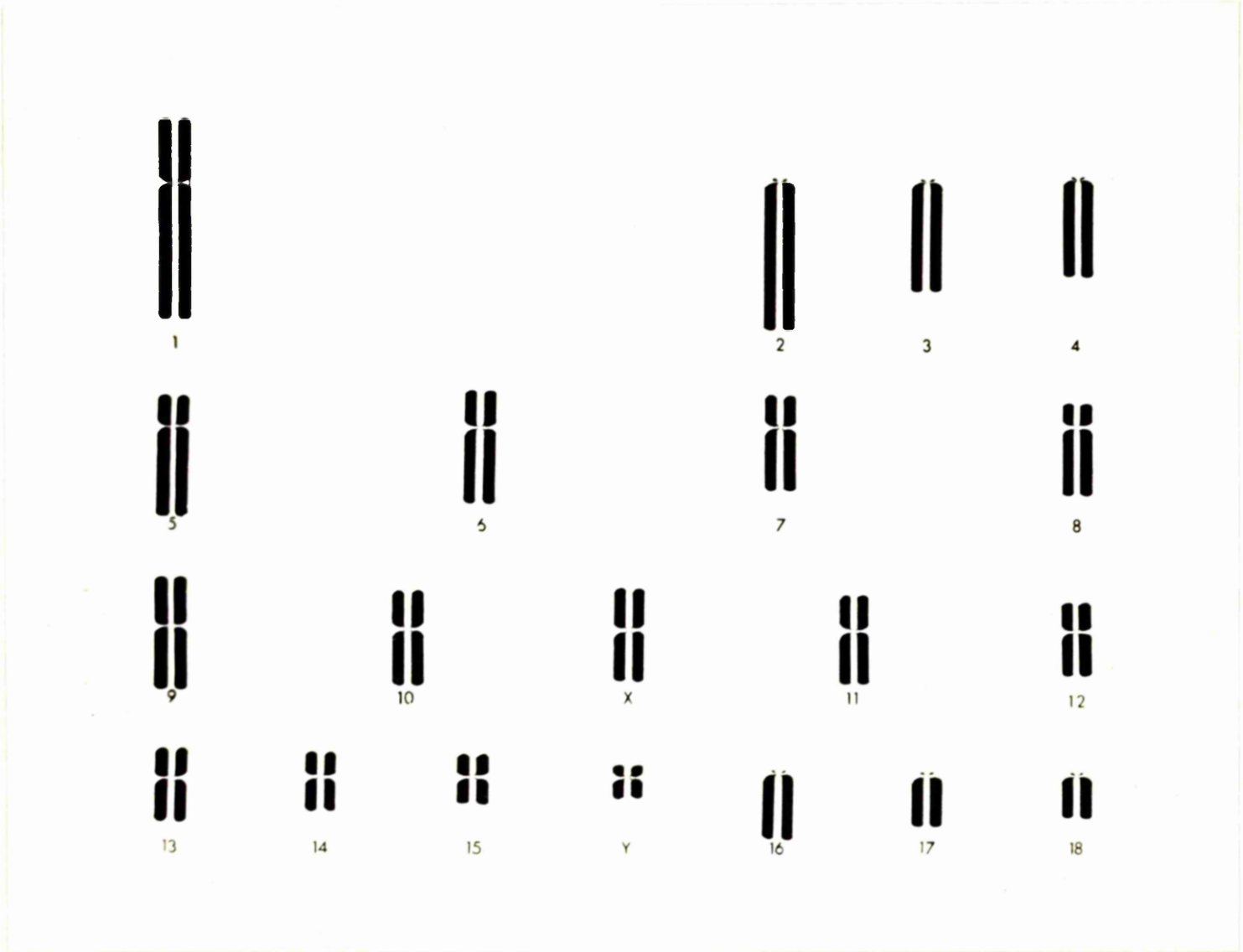
Karyotype of 38,XY cell from peripheral blood culture.

Figure 3.



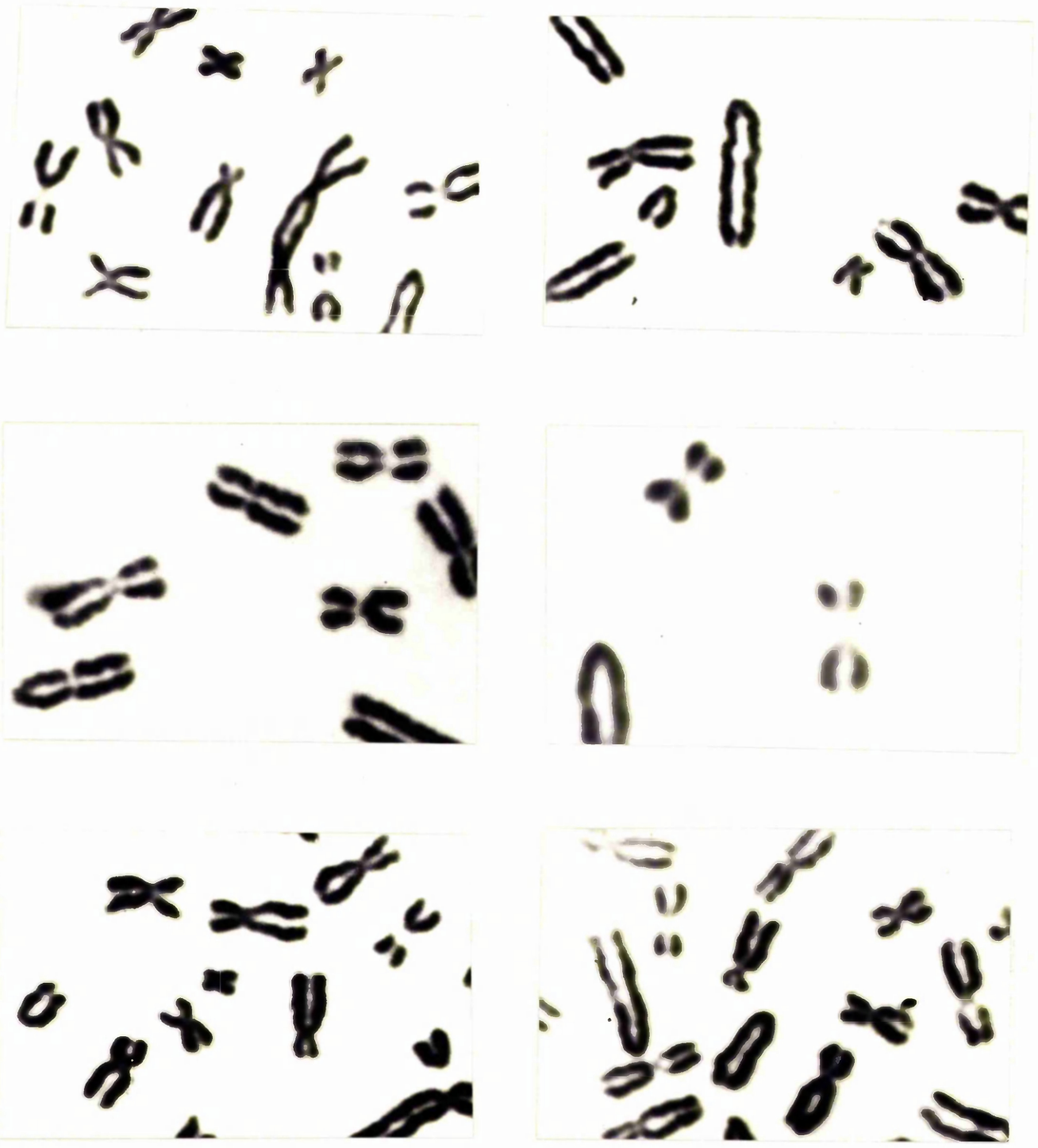
Polymorphism of pair number 1 in six different animals.

Figure 4



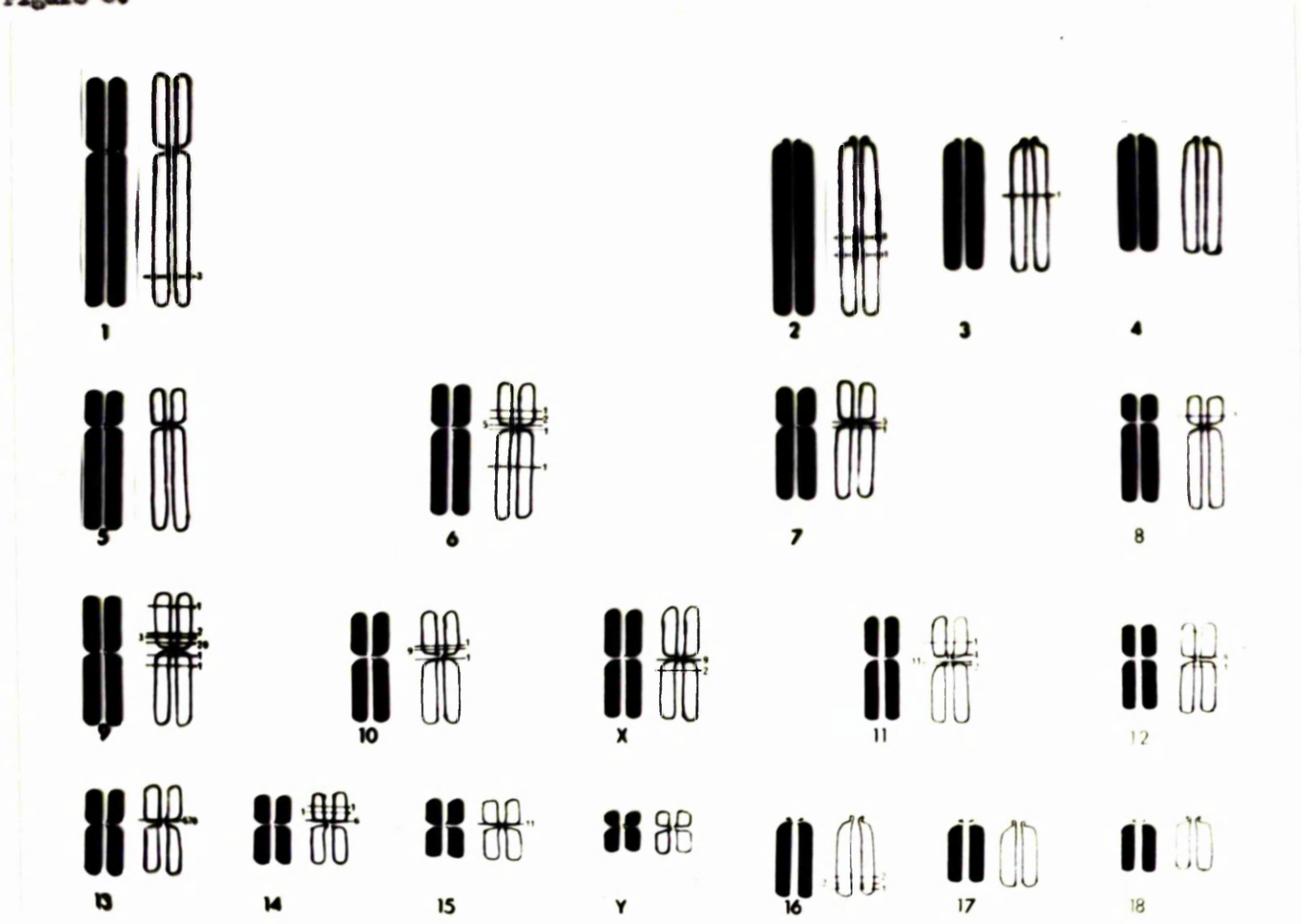
Idiogram.

Figure 5.



Secondary constrictions on metaphase chromosomes from peripheral blood cultures.

Figure 6.



Position of secondary constrictions traced onto ghost idiogram.

Figures 7-10.

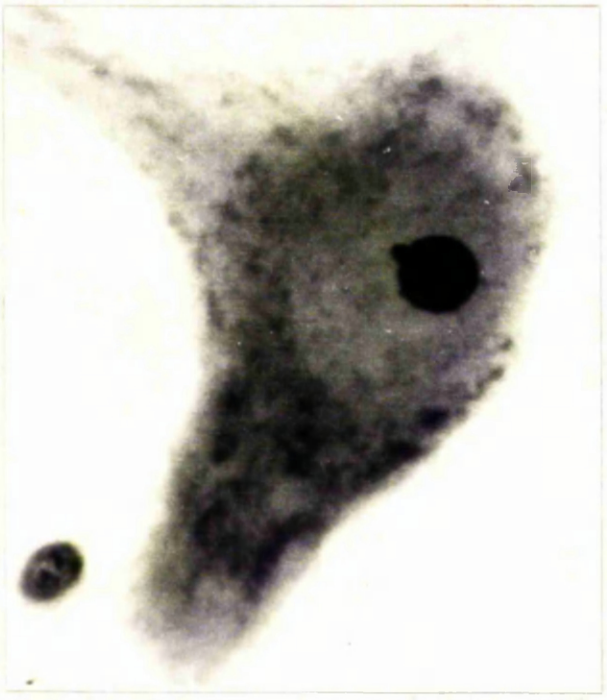


Figure 7. Sex chromatin positive neurone. Position 1.

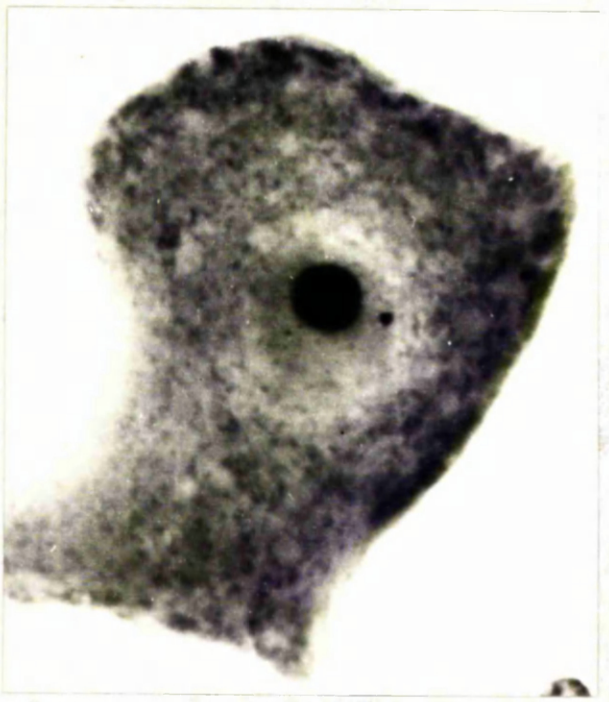


Figure 8. Sex chromatin positive neurone. Position 2.

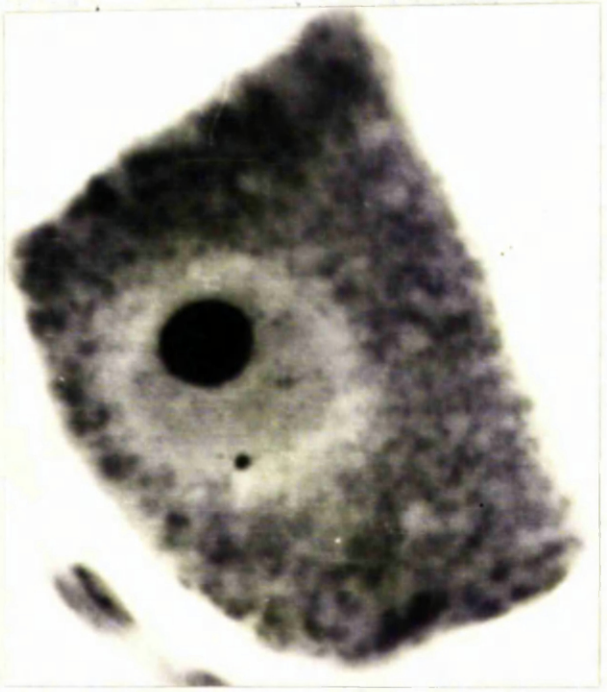


Figure 9. Sex chromatin positive neurone. Position 3.

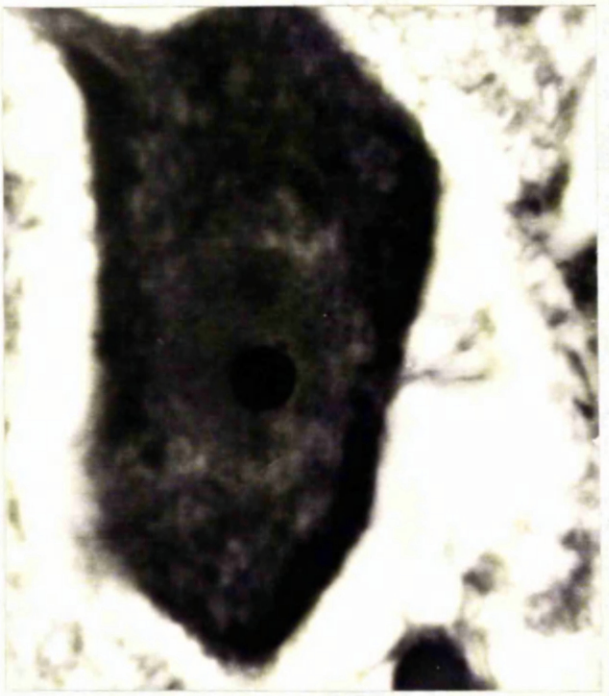


Figure 10. Sex chromatin negative neurone. Small, poorly staining body present.

Figures. 11 - 12.

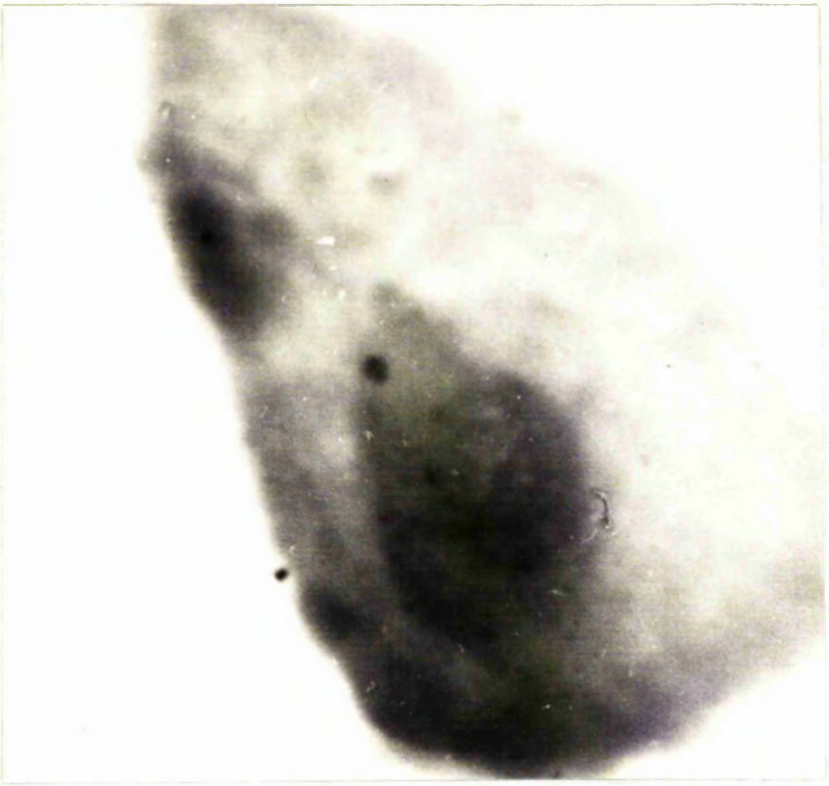


Figure 11. Sex chromatin positive cell (Feulgen staining).

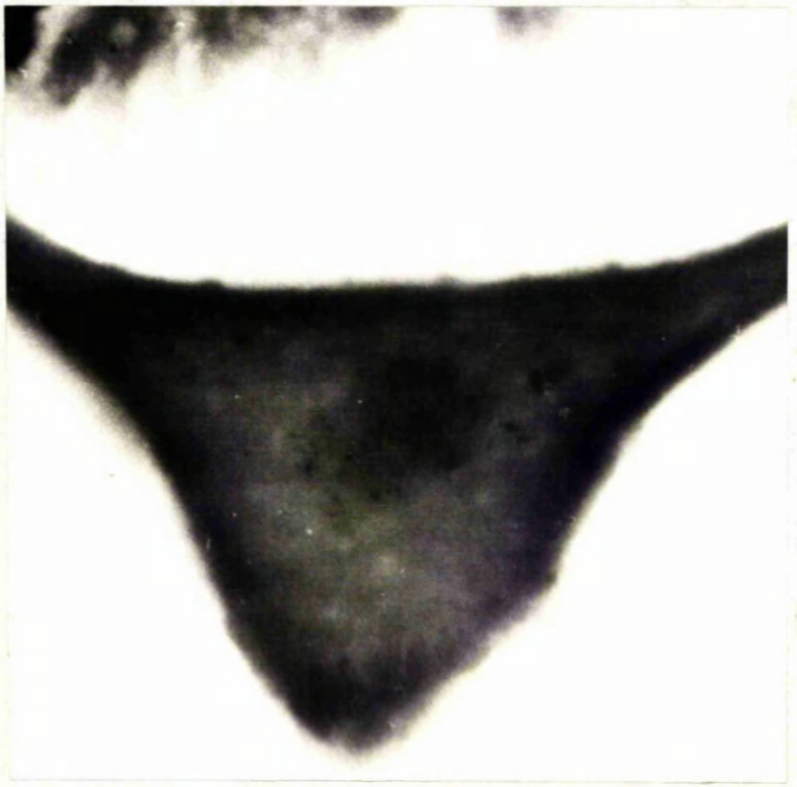


Figure 12. Sex chromatin negative cell (Feulgen staining).

Figures 13 - 14.

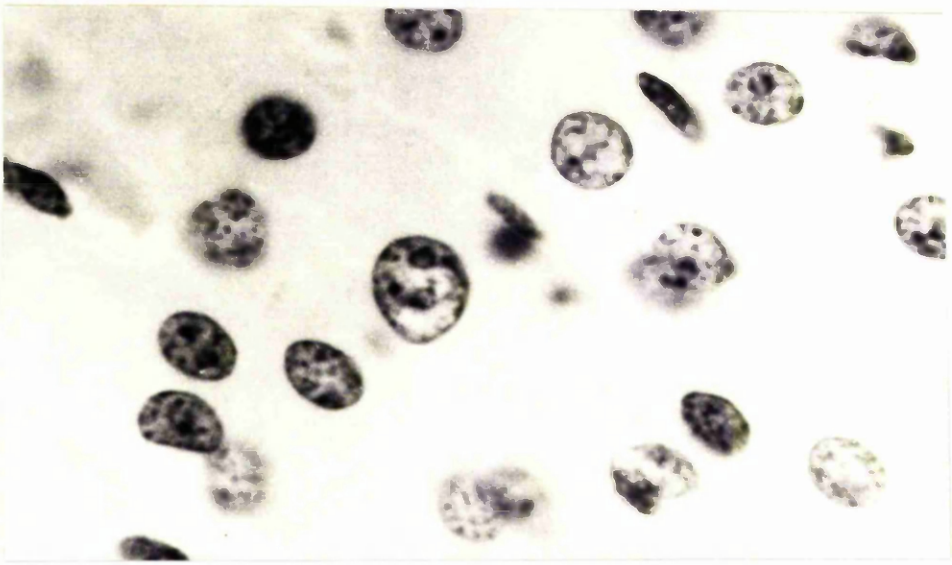


Figure 13. Liver from adult female (Cresyl Violet)

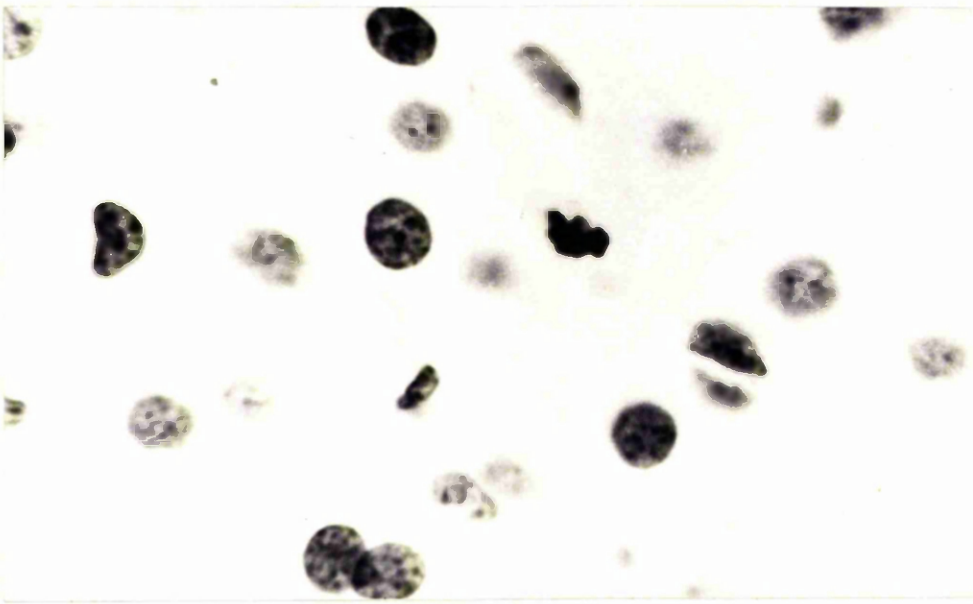


Figure 14. Liver from adult male (Cresyl Violet)

Figures 15-16.

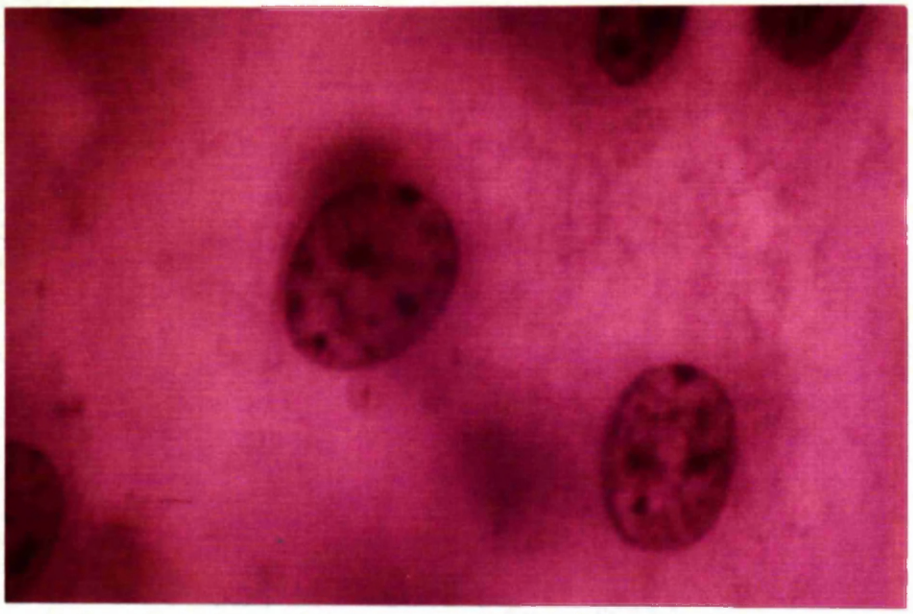


Figure 15. amnion preparation from female foetus (Cresyl Violet)

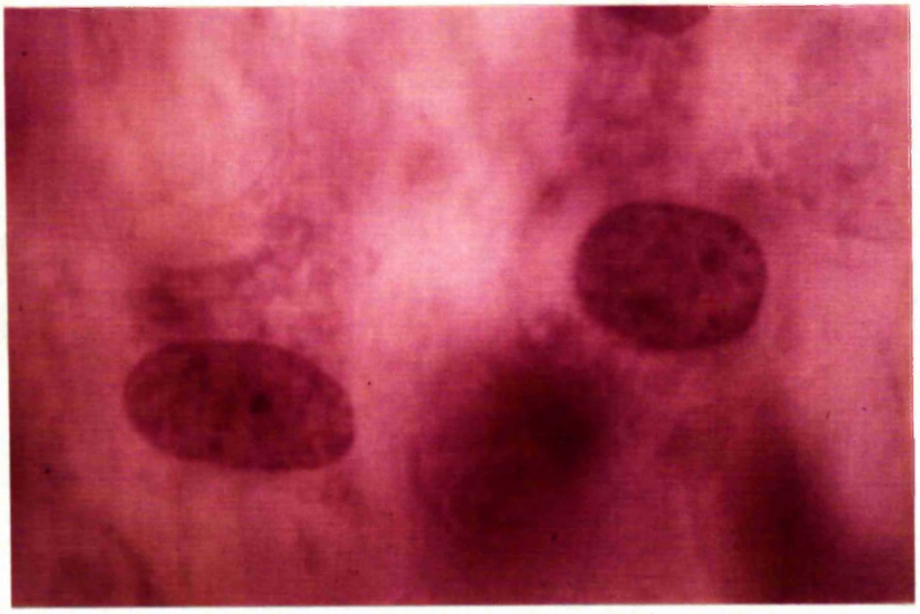
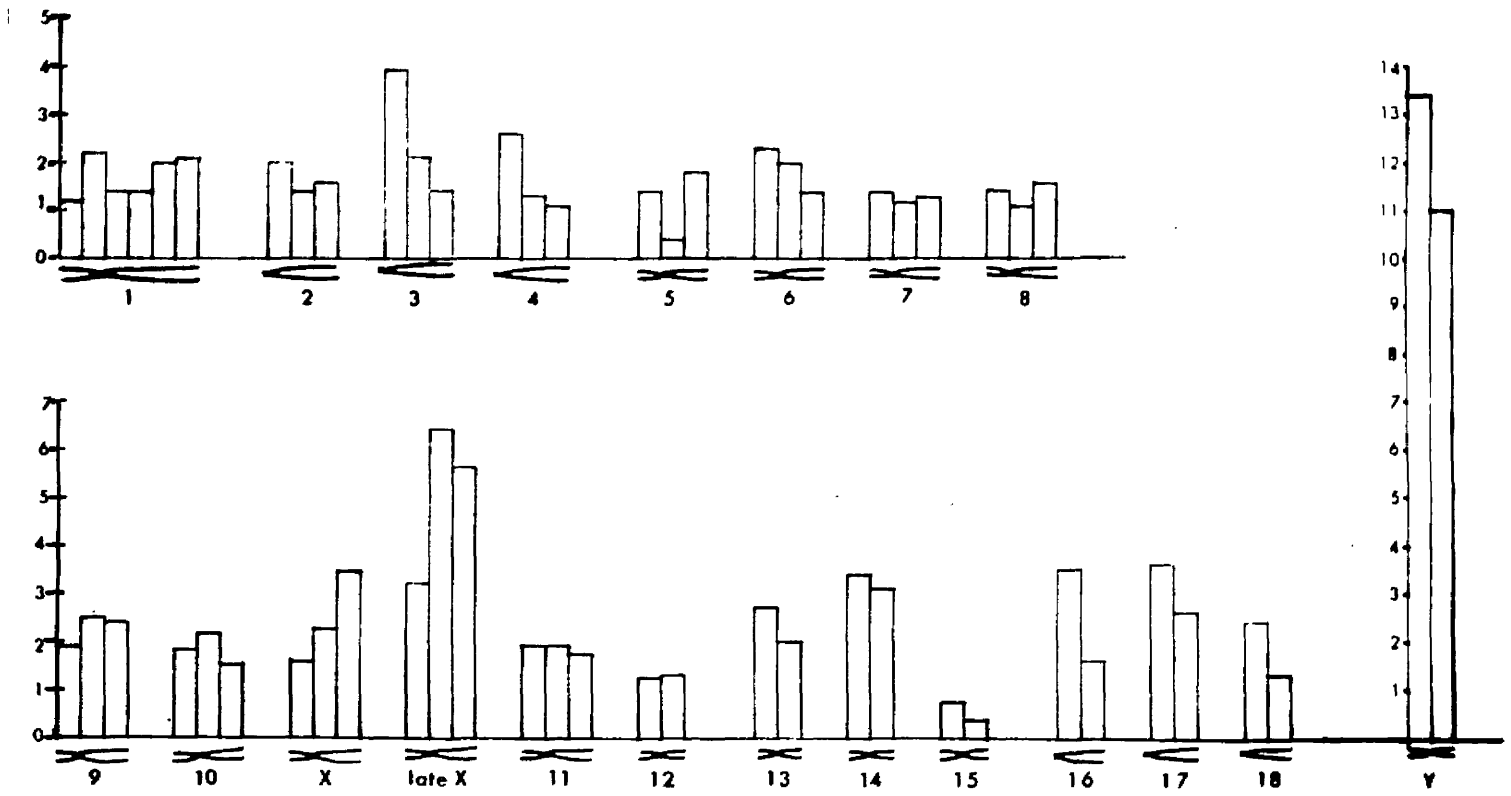


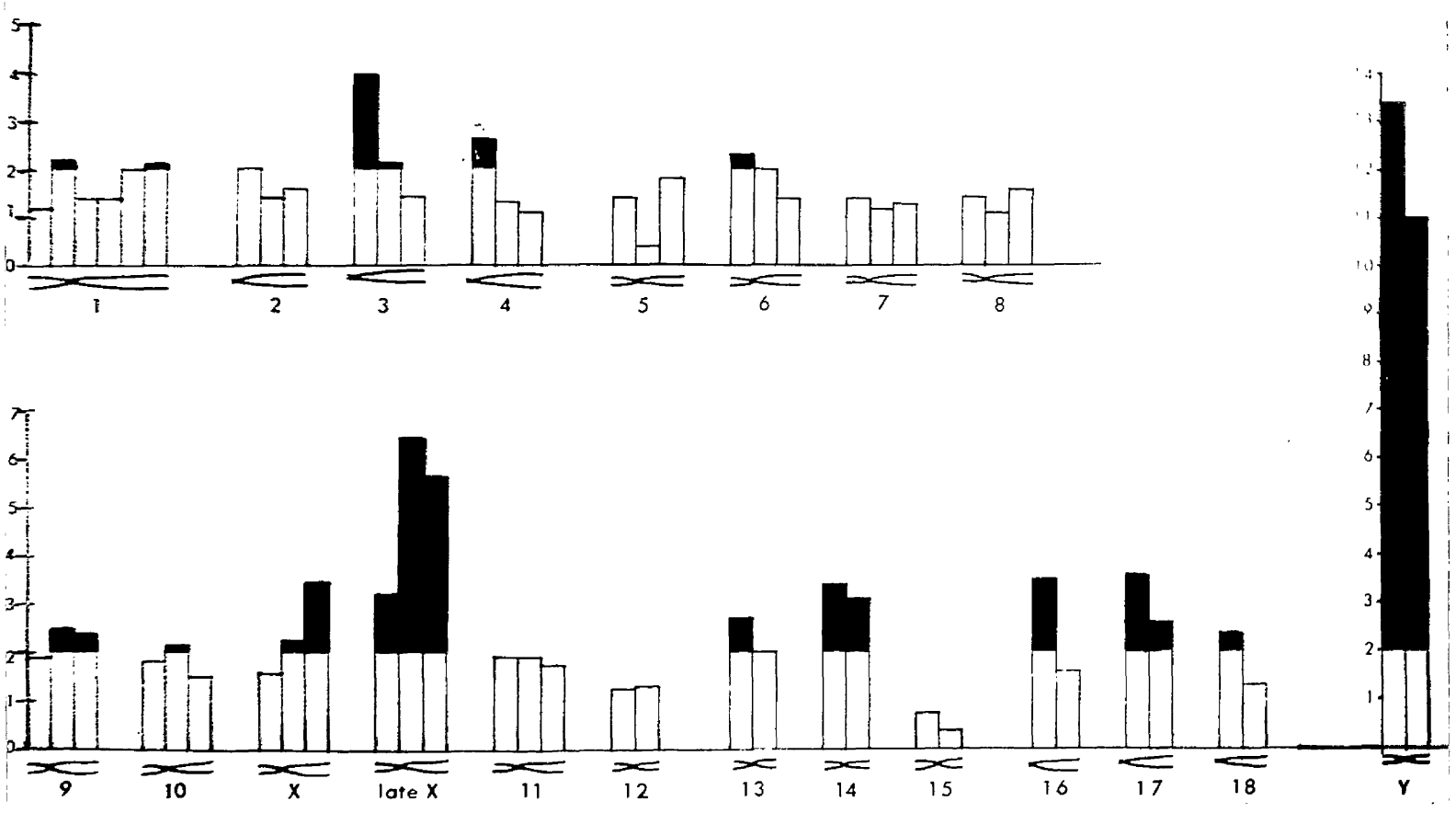
Figure 16. amnion preparation from male foetus (Cresyl Violet)

Figure 17.



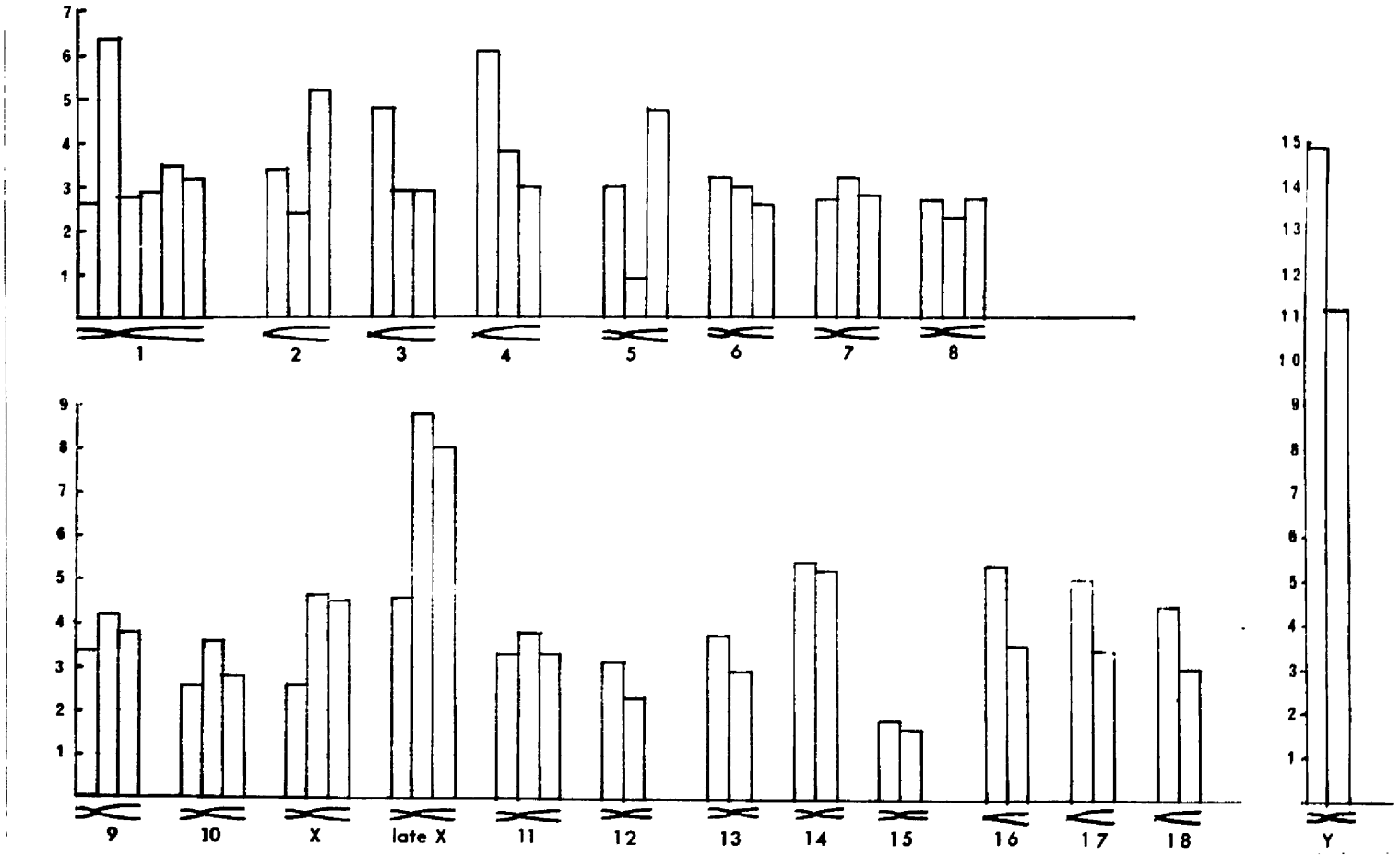
Autoradiography:- Total grain scores (X1000) for cells of group A (0-100 grains)

Figure 17a.



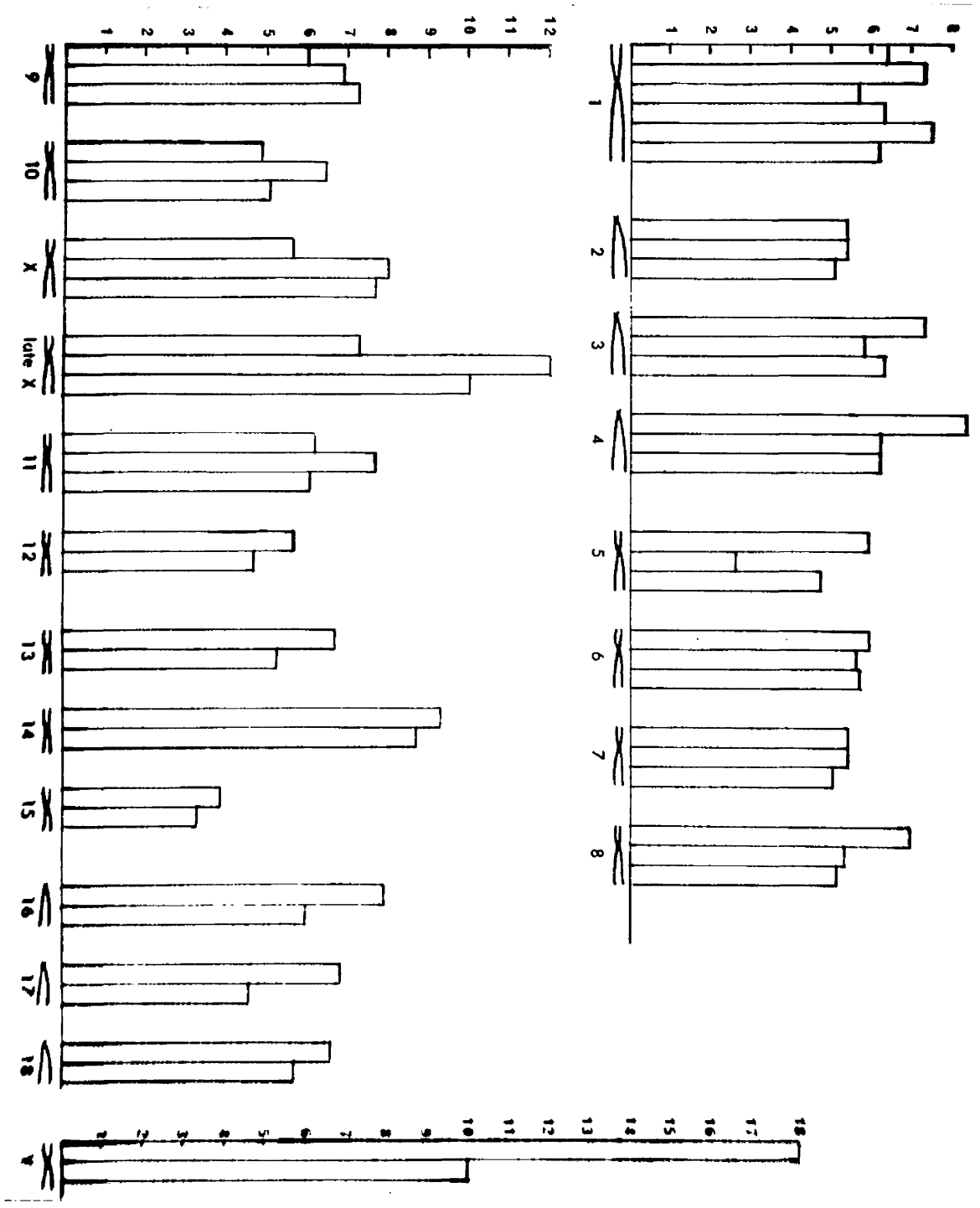
Autoradiography:- Total grain scores (X 1000) for cells of group A (0-100 grains)
Late replicating areas shaded (> 2000 grains total).

Figure 18.



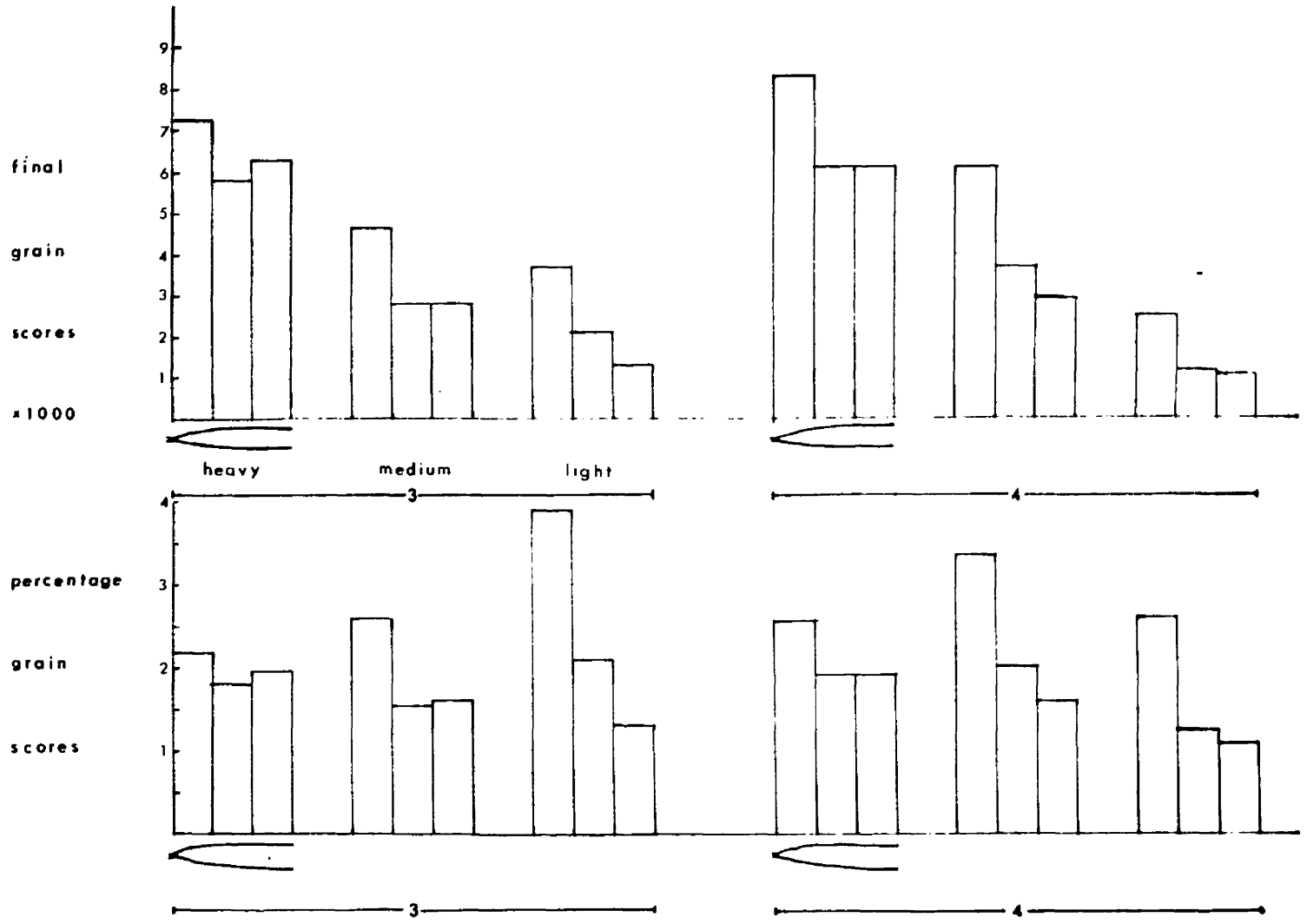
Autoradiography:- Total grain scores (X 1,000) for cells of group B (101-200 grains)

Figure 19.



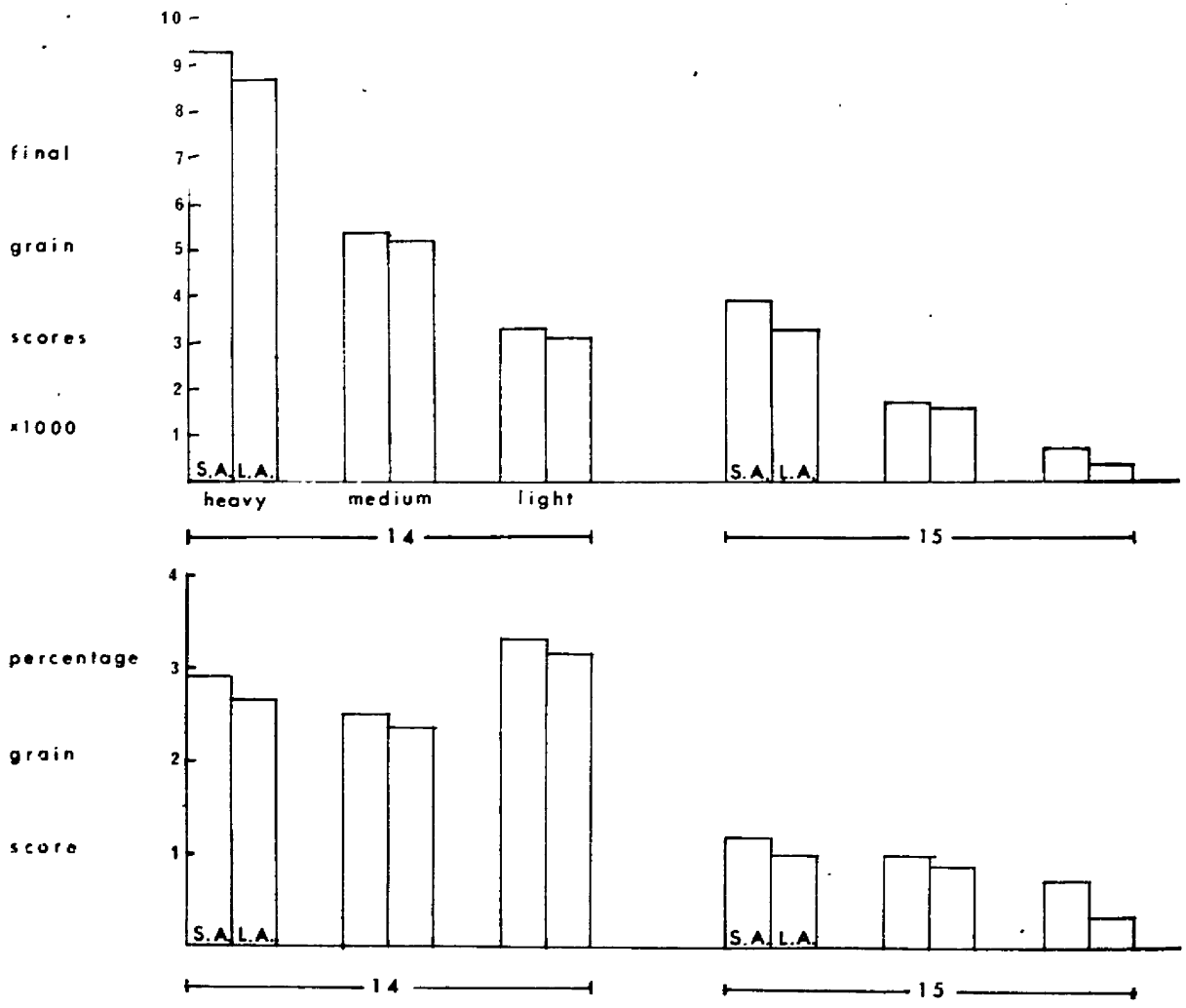
Autoradiography:- Total grain scores (X 1000) for cells of group C (> 200 grains)

Figure 20.



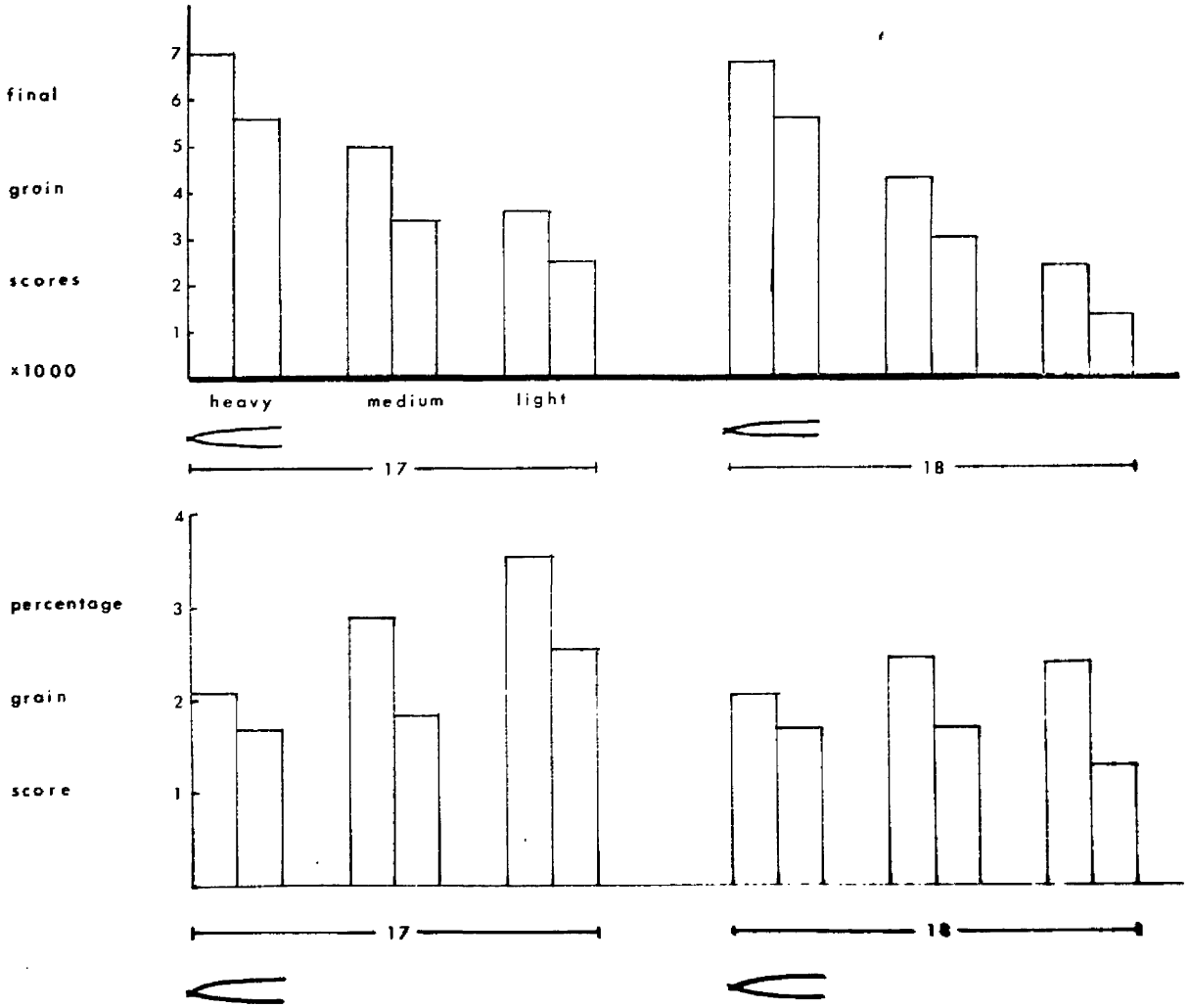
Histogram representing labelling patterns of chromosomes 3-4.

Figure 21.



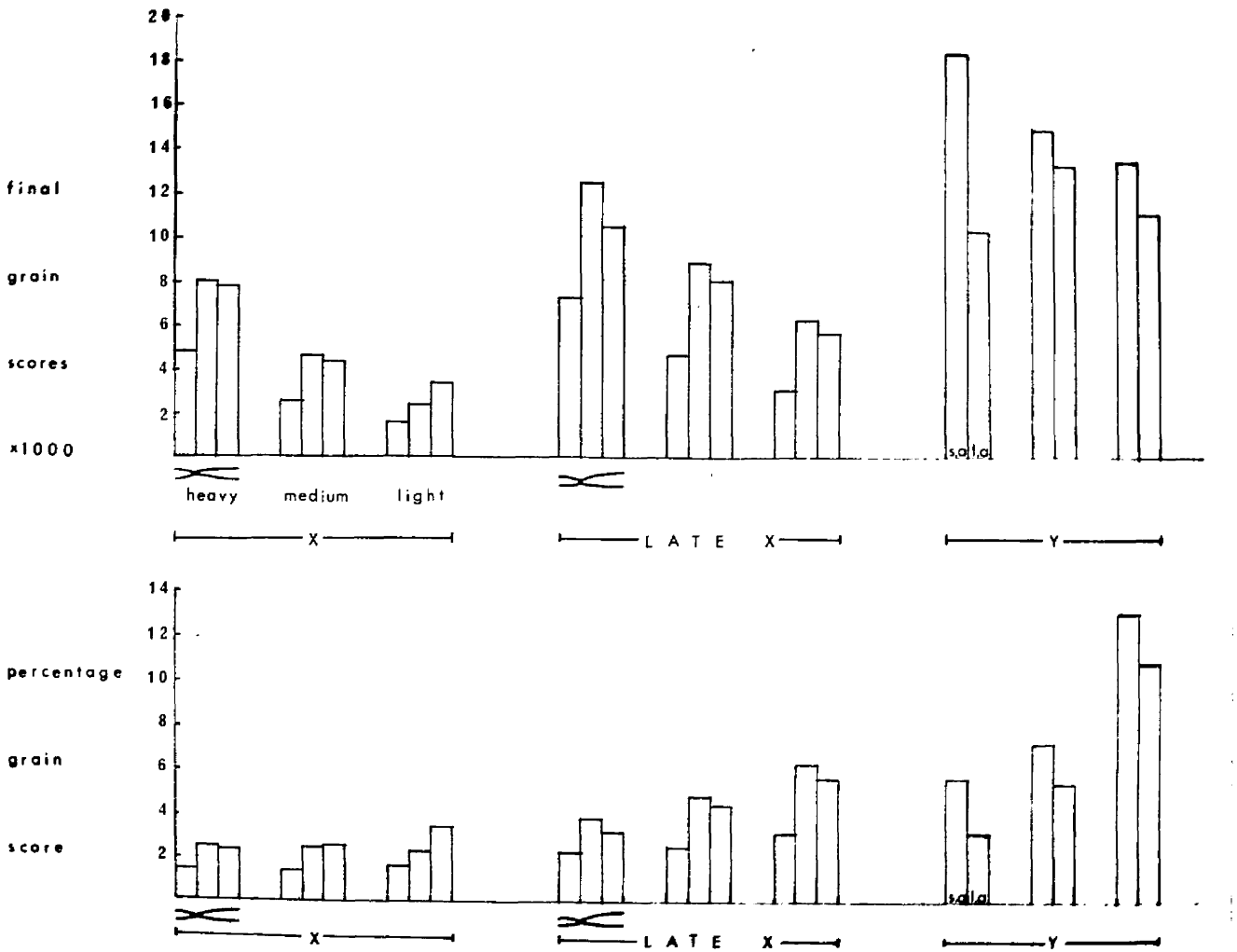
Histogram representing labelling patterns of chromosomes 14-15.

Figure 22.



Histogram representing labelling patterns of chromosomes 17-18.

Figure 23.

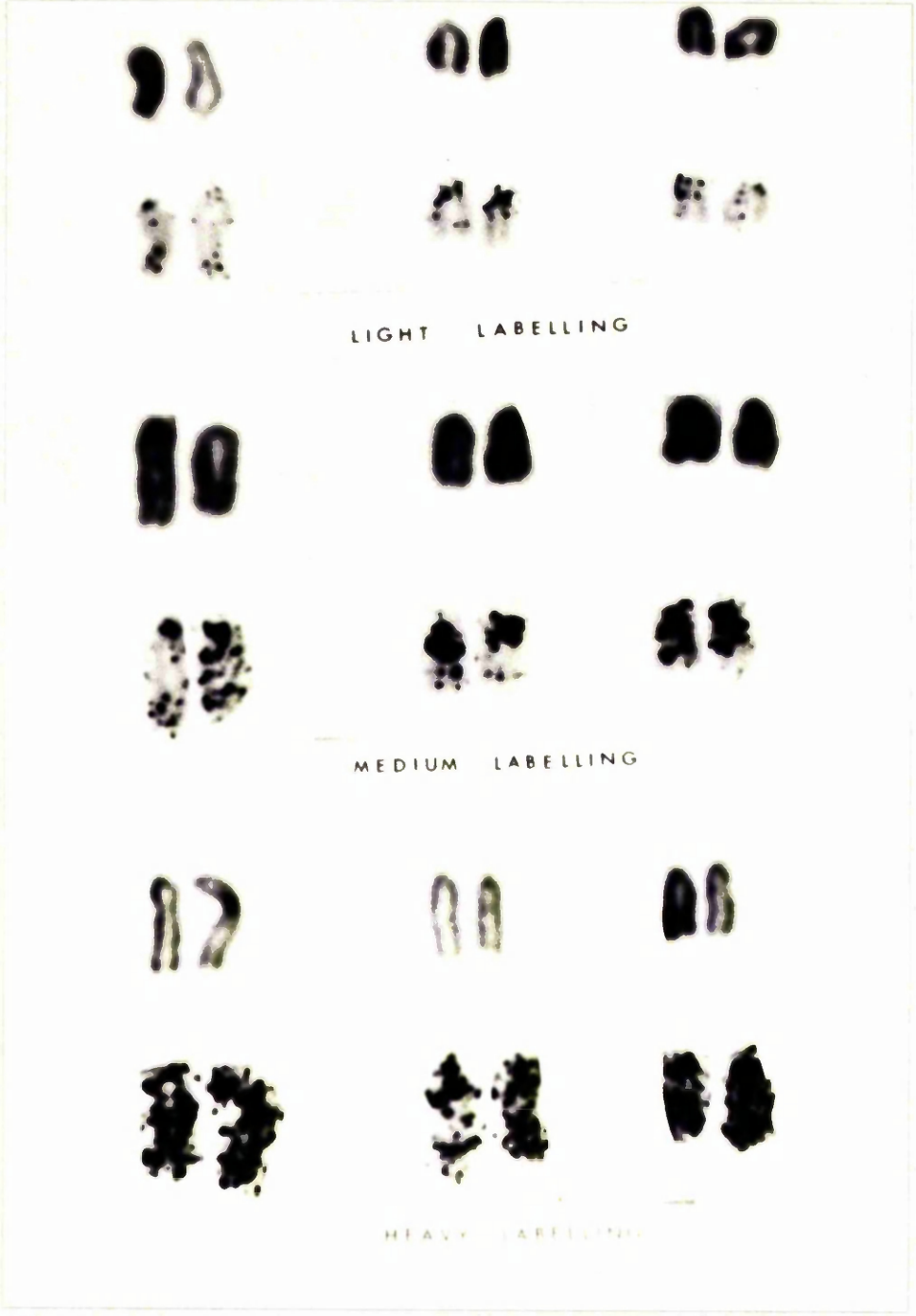


Histogram representing labelling patterns of the sex chromosomes.

Figure 24.

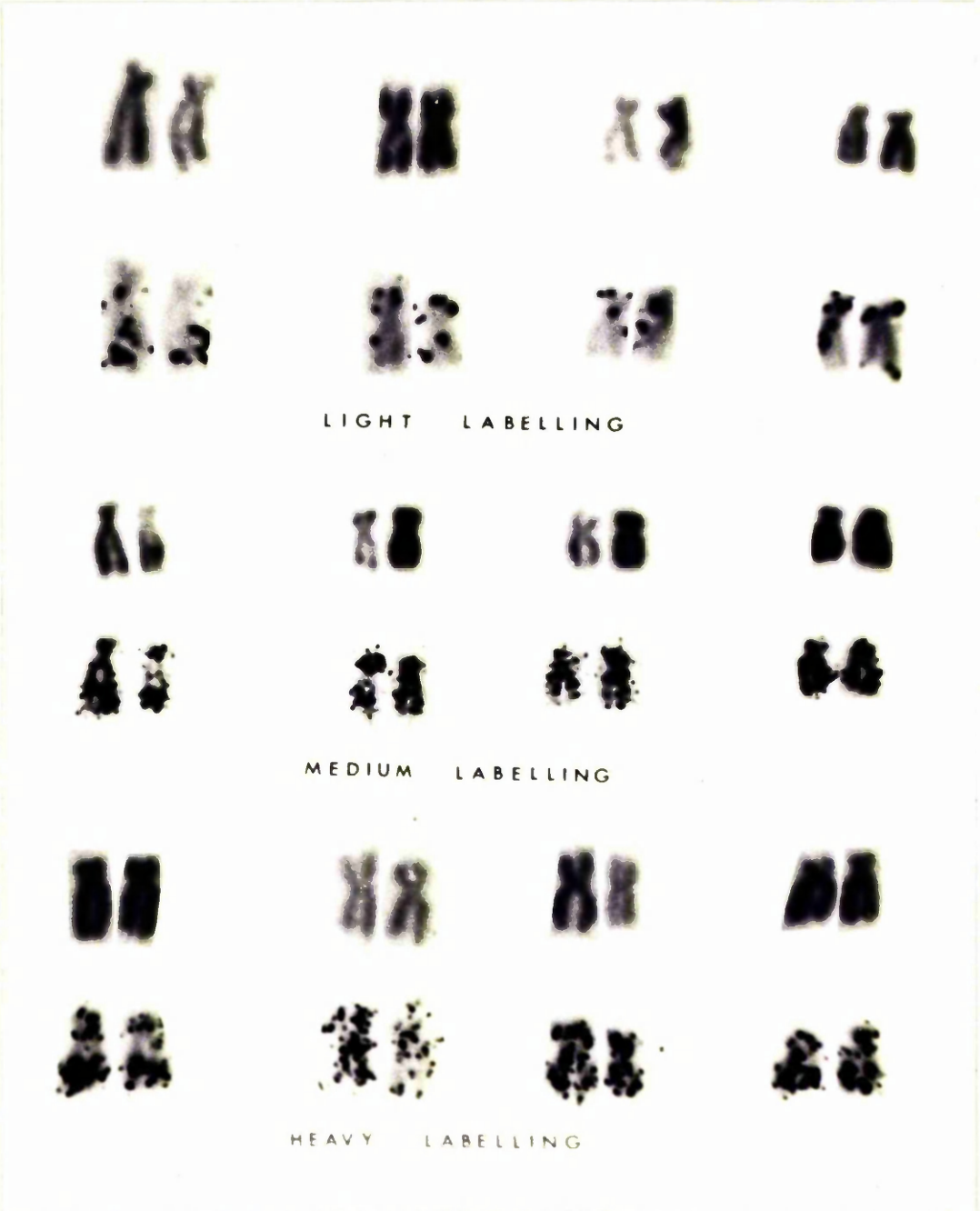
Autoradiography:- Labelling patterns of Chromosome 1.

Figure 25.



Autoradiography:- Labelling patterns of Chromosomes 2 - 4.

Figure 26.

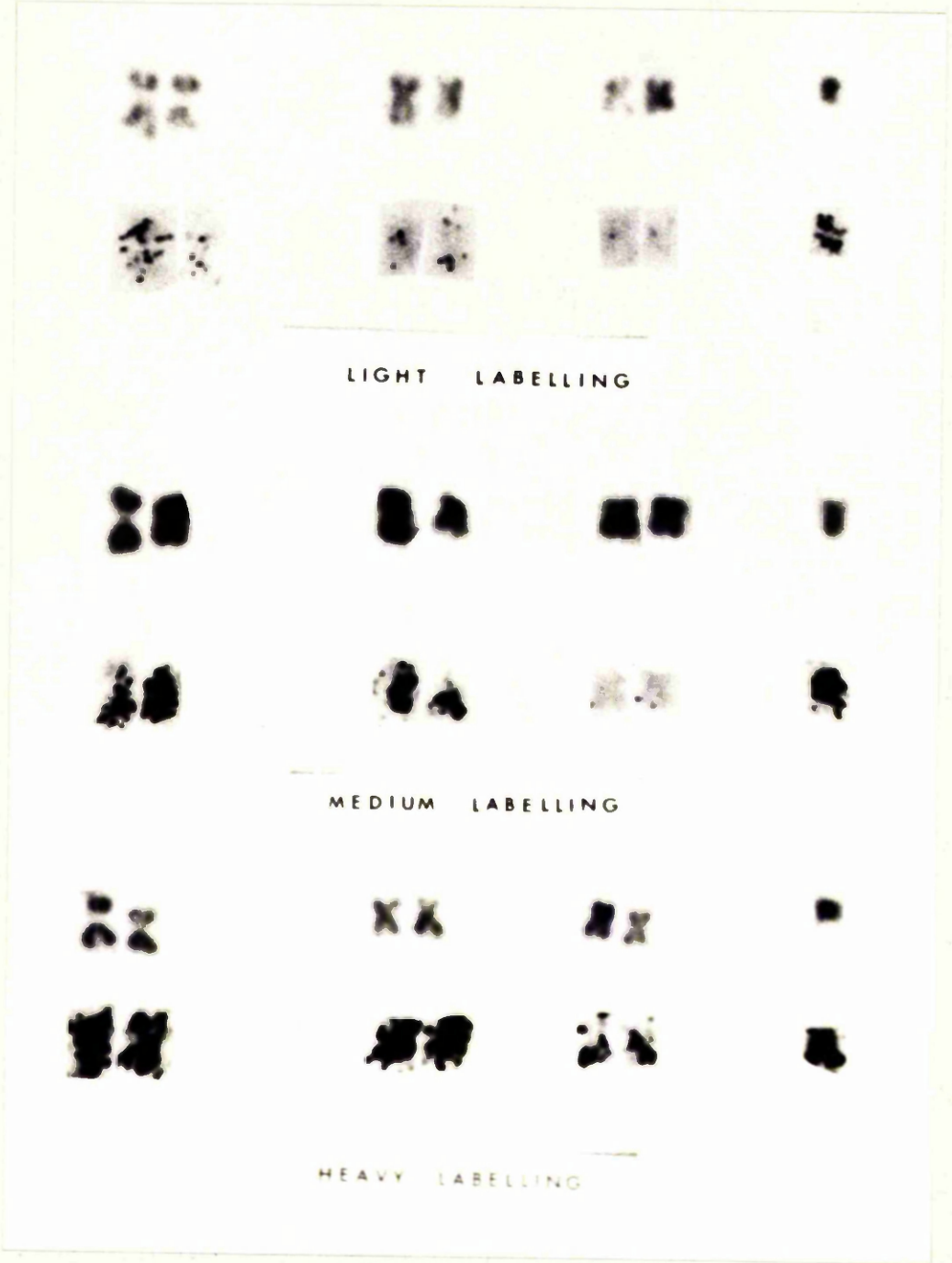


Autoradiography:- Labelling patterns of Chromosomes 5 - 8.

Figure 27.

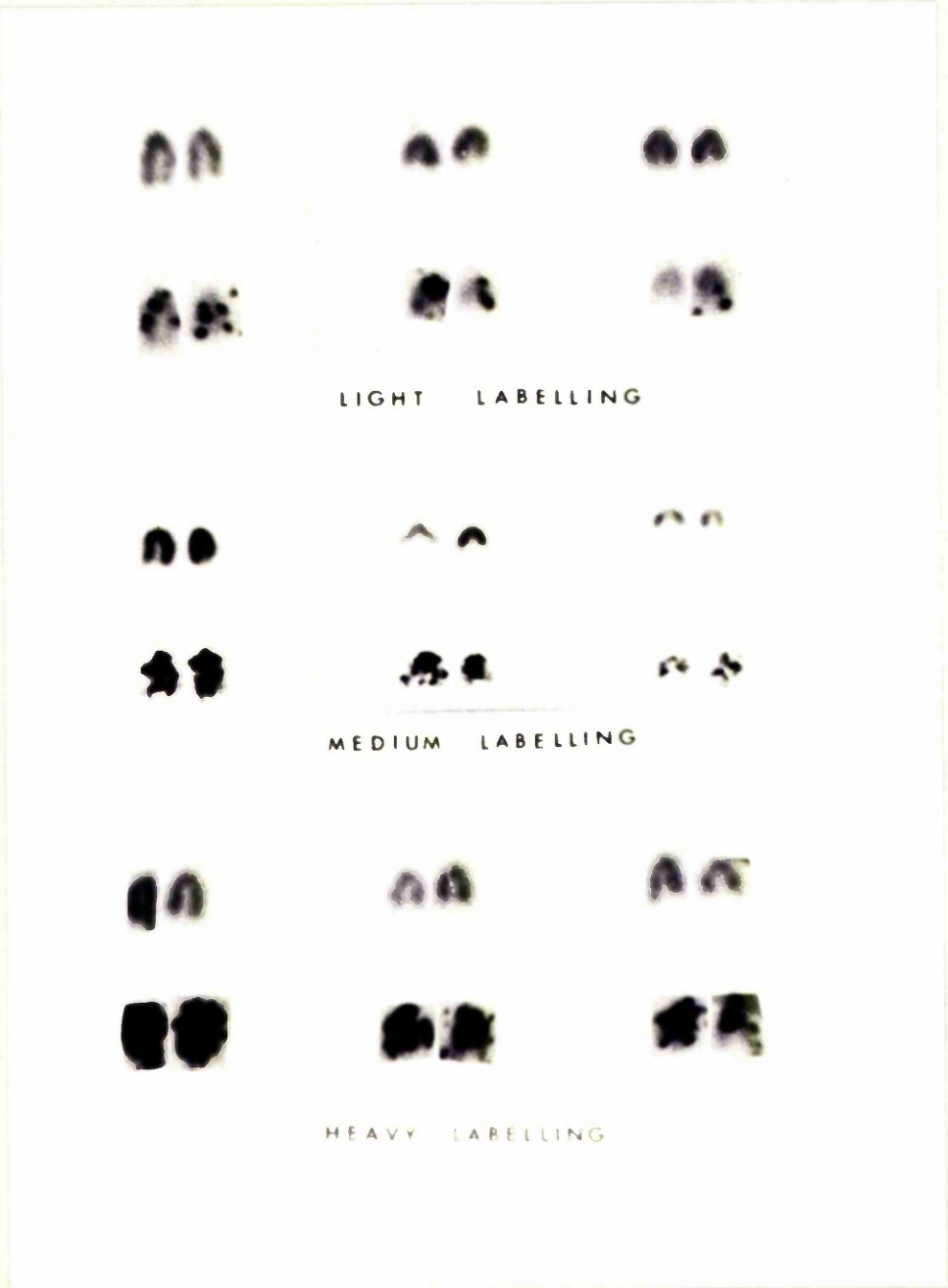
Autoradiography:- Labelling patterns of chromosomes 9-12+X (group D)

Figure 28.



Autoradiography:- Labelling patterns of chromosomes 13-15+Y (group E + Y)

Figure 29.



Autoradiography:- Labelling patterns of chromosomes 16-18.

Figures 30a-f.

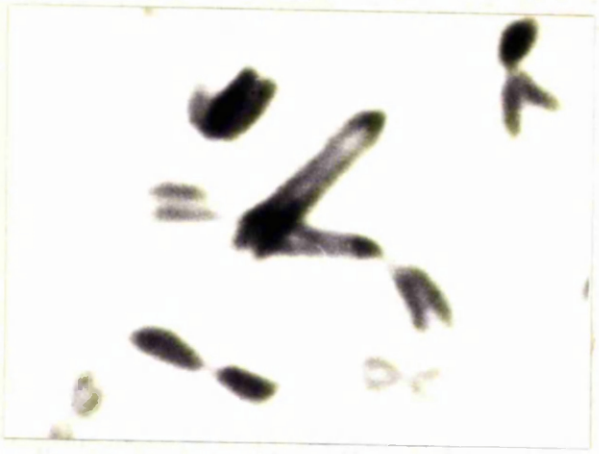


Fig. 30 a. Single break

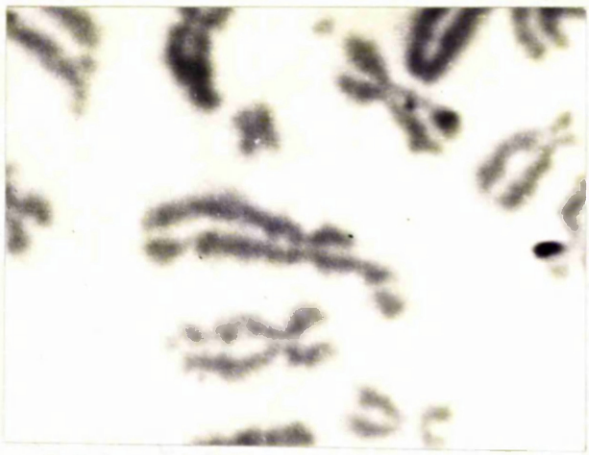


Fig 30 b Double break.

Fig. 30 c Single fragment

Fig. 30 d Double fragment

Fig 30 e Complex break

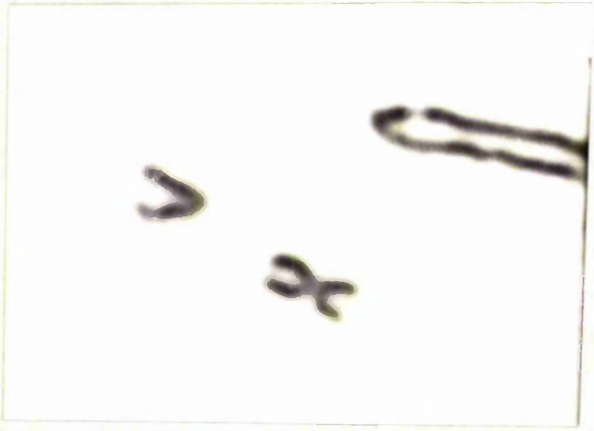
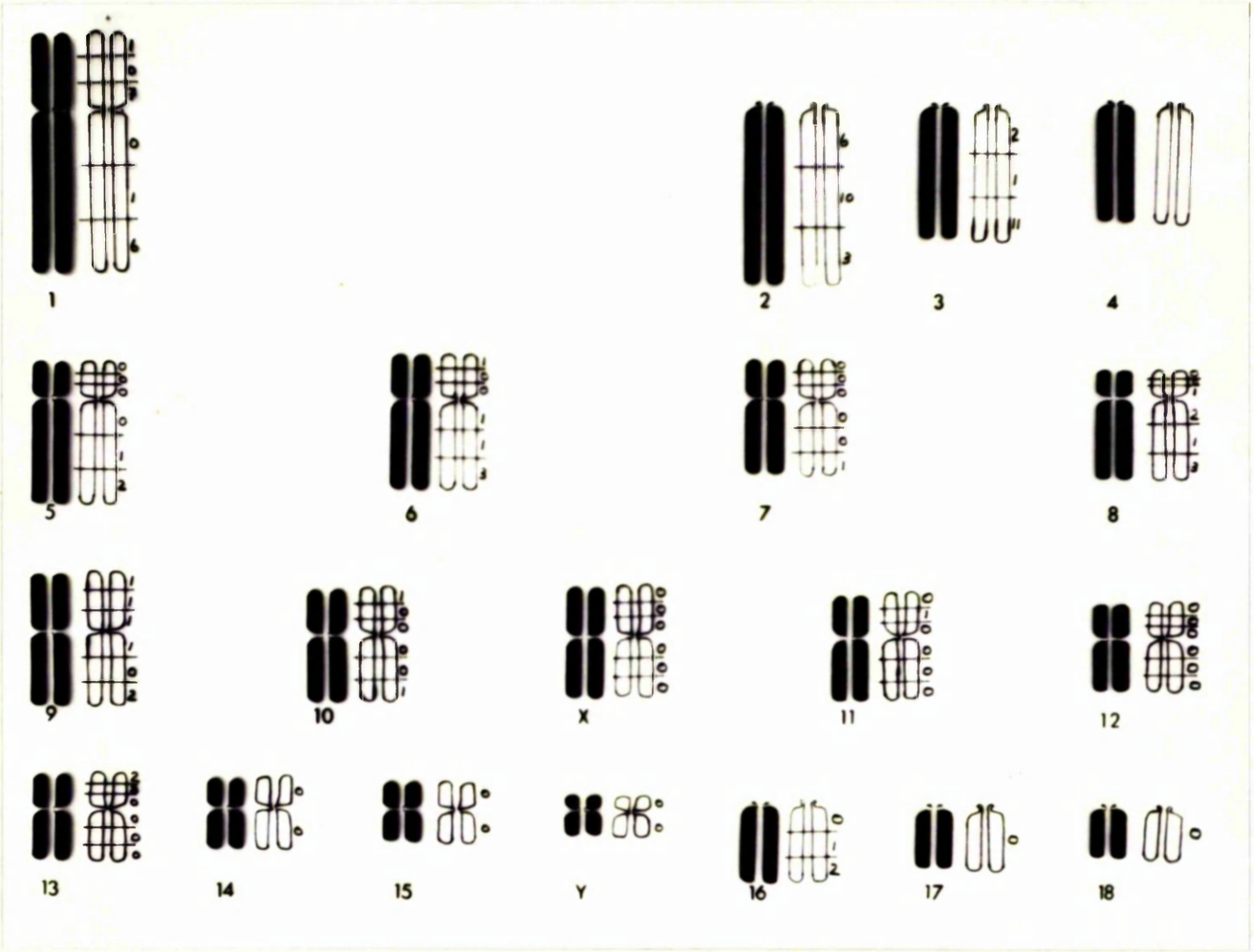


Fig. 30 f Non-staining gap

Figure 31.

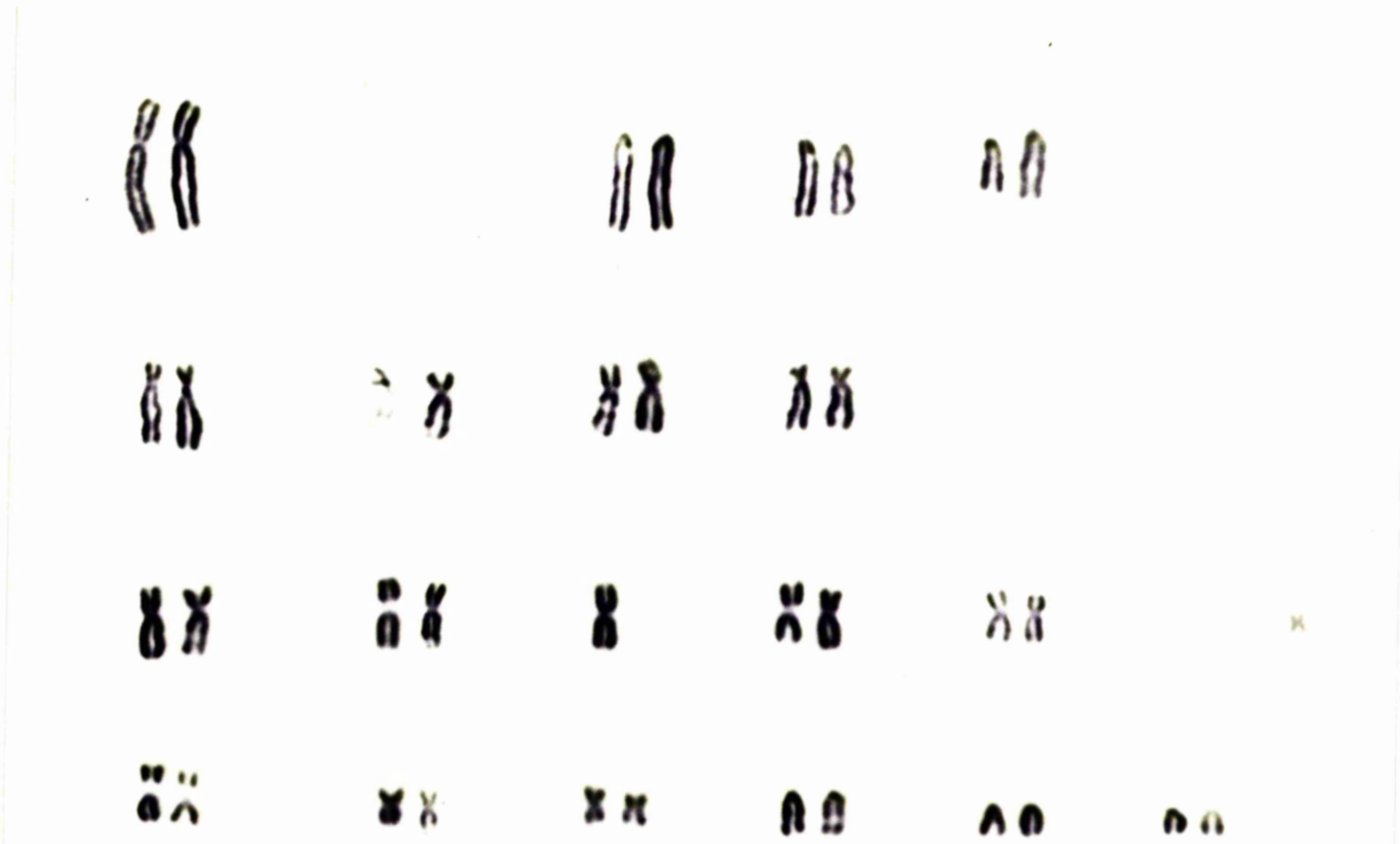


Position of breakages traced onto ghost idiogram.

Figure 32.

39,XXY karyotype from peripheral blood culture of case AE32.

Figure 33.



38,XY karyotype from peripheral blood culture of case AE32.

Figures 34-35.

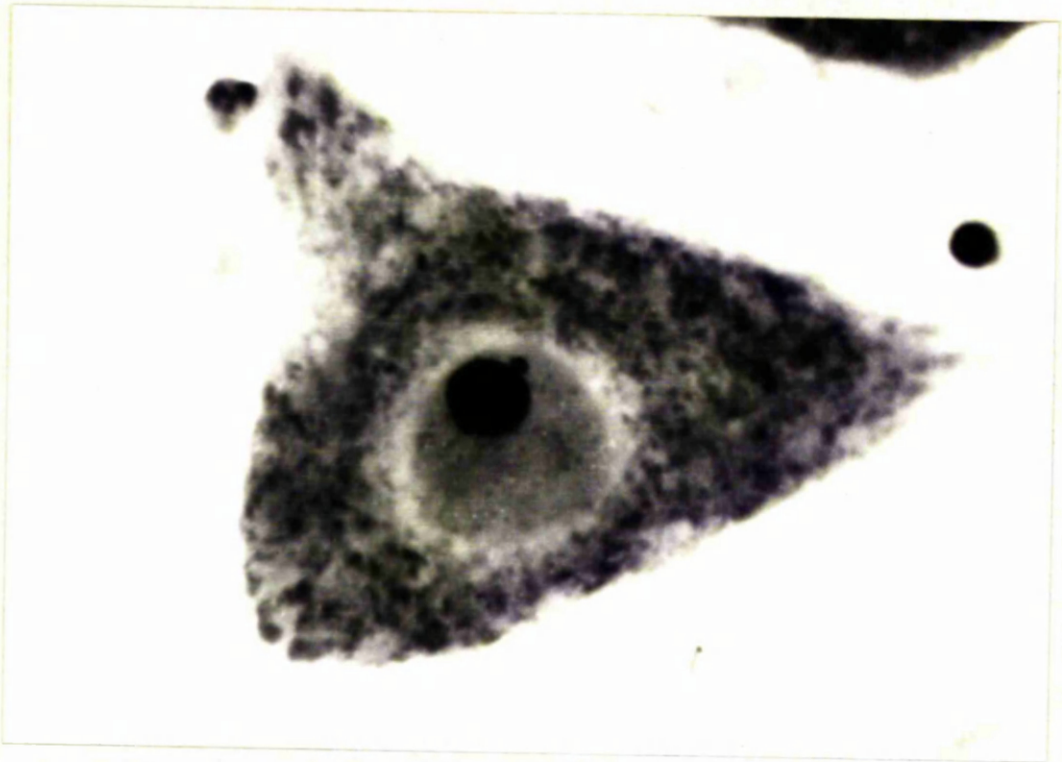


Figure 34. Sex chromatin positive neurone from case AE32

Figure 35. Sex chromatin double positive neurone from case AE32.

Tables.

Table 1 Percentage Chromosome counts of individual animals from lymphocyte cultures.

Chromosome Number	< 36	36	37	38	39	40	> 40	Tetra ploid	Total Counted
1 1	0	2.53	10.13	79.74	5.06	0	1.27	1.27	79
2	0	0	7.89	84.21	2.63	5.26	0	0	38
3	0	0	4.17	95.83	0	0	0	0	24
4	2.63	2.63	7.89	73.68	7.89	2.63	2.63	0	38
5	0	2.04	8.16	85.71	2.04	0	0	2.04	49
6	3.17	6.35	0	79.37	7.94	1.59	1.59	0	63
7	0	0	4.17	83.33	4.17	4.17	4.17	0	24
8	0	0	12. 0	76.00	4. 0	4. 0	4. 0	0	25
9	0	3.85	11.54	75.00	5.77	3.85	0	0	52
10	0	0	9.38	87. 5	3.13	0	0	0	32
11	0	0	0	100. 0	0	0	0	0	20
12	3.92	5.88	9. 8	78. 4	1.96	0	0	0	51
13	0	6. 0	6. 0	86. 0	2. 0	0	0	0	50
14	2. 0	4. 0	4. 0	90. 0	0	0	0	0	50
15	0	0	2. 0	88. 0	10. 0	0	0	0	50
16	0	4. 0	8. 0	86. 0	2. 0	0	0	0	50
17	2. 5	2. 5	2. 5	92. 5	0	0	0	0	40
18	0	0	4.55	88.64	4.55	2.27	0	0	44
19	0	0	4. 0	86. 0	4. 0	4. 0	0	2	50
20	0	0	7. 5	92. 5	0	0	0	0	40
21	0	0	0	97.30	2.70	0	0	0	37
22	0	0	7.32	85.37	7.32	0	0	0	41
23	0	2.94	7.35	83.79	2.94	2.94	0	0	68
24	0	3	8	84	5	0	0	0	100
25	1.09	3.26	6.52	84.79	3.26	0	0	1.09	92
26	1.18	2.35	2.35	87.02	7.06	0	0	0	85
27	1.25	1.25	2.50	88.75	6.25	0	0	0	80
28	1.82	7.27	5.45	80.00	3.64	0	0	1.82	55
29	0	2. 5	7. 5	87. 5	2. 5	0	0	0	40
30	1.43	0	2.86	85.72	7.14	1.43	0	1.43	70
31	0	0	4.29	88.57	5.71	1.43	0	0	70
32	0	0	5.66	84.91	9.43	0	0	0	53
33	0	0	8	84	8	0	0	0	25
34	0	0	0	100	0	0	0	0	27
35	0	4.35	10.87	78.26	6.52	0	0	0	46

Table 1a

Chromosome Number	< 36	36	37	38	39	40	> 40	Tetraploid	Total
Number Counted	12	39	102	1,500	78	16	5	6	1,758
Percentage	0.68	2.22	5.80	85.32	4.44	0,91	0.28	0.34	100,00
Percentage Range	0.00 to 3.92	0.00 to 7.27	0.00 to 11.54	73.68 to 100.00	0.00 to 9.43	0.00 to 5.26	0.00 to 4.17	0.00 to 2.04	

Total distribution of Chromosome Counts from Lymphocyte Cultures

Table 2.

CELL NUMBER

Chrm.	1	2	3	4	5	6	7	8	9	10	Mean
1.	108.06	115.33	103.93	114.97	104.58	111.22	115.81	104.20	118.26	127.23	112.36
2.	78.52	82.24	92.43	82.12	76.85	90.79	86.69	82.43	86.94	84.64	84.46
3.	64.56	57.50	62.14	63.99	64.62	71.39	61.00	62.21	58.72	61.63	62.78
4.	59.96	53.82	56.38	55.09	57.42	53.54	54.13	61.28	55.79	53.68	56.11
5.	66.68	70.03	72.77	59.88	66.37	66.22	70.06	65.48	63.29	66.06	66.68
6.	62.79	62.00	59.75	62.28	63.97	67.25	64.71	58.94	59.70	63.46	62.48
7.	56.59	52.68	51.20	50.98	51.52	51.73	55.50	55.52	56.60	54.14	53.65
8.	54.47	49.64	53.16	50.98	54.58	48.11	54.68	52.10	50.24	52.91	52.09
9.	57.84	59.28	57.22	68.78	61.46	57.42	59.35	61.74	62.31	52.76	59.82
10.	51.82	53.65	56.10	54.75	53.71	53.03	52.20	53.66	55.13	52.30	53.64
X.	50.58	62.68	51.33	51.33	52.18	50.18	54.54	55.37	53.34	53.22	52.47
11.	50.58	48.03	52.17	47.39	51.52	48.89	47.67	48.68	49.42	46.64	49.10
12.	42.44	45.78	44.88	40.72	43.45	38.28	42.04	39.19	41.43	40.83	41.90
13.	41.74	45.30	39.83	44.48	41.92	41.39	40.94	41.99	37.35	42.97	41.79
14.	33.25	33.90	30.29	35.93	37.01	34.14	29.67	36.39	31.97	31.66	33.42
15.	29.19	30.69	27.49	26.35	30.46	29.23	24.45	30.02	23.26	27.99	27.91
16.	38.55	36.94	34.65	36.95	37.44	33.37	37.92	36.39	38.66	36.09	36.70
17.	29.00	27.47	30.29	28.74	28.93	28.97	24.73	30.79	31.16	28.75	28.88
18.	23.34	22.98	23.99	24.29	22.05	24.83	23.90	23.64	26.42	22.02	23.75
Y.	19.10	-	-	19.16	-	11.38	-	20.22	-	18.96	17.76

Idiogram:- CHROMOSOMAL LENGTHS OF 10 HAPLOID CELLS EXPRESSED AS PARTS OF THE TOTAL CHROMOSOMAL LENGTH OF THE CELL (1000).

TABLE 3

CHR'M	CELL NUMBER 1	2	3	4	5	6	7	8	9	10	MEAN
1	2.34	2.01	2.03	2.05	2.17	2.00	1.88	1.90	2.40	1.99	2.08
2	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
3	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
4	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
5	2.48	2.91	2.95	2.61	3.00	2.45	3.32	2.69	3.31	2.76	2.85
6	1.83	2.08	1.65	1.69	1.85	2.08	1.60	1.95	2.09	2.05	1.89
7	1.71	1.85	1.76	2.07	1.83	2.05	1.83	1.52	1.78	1.92	1.83
8	3.00	3.05	2.76	3.47	2.69	3.08	3.38	2.56	3.00	3.21	3.02
9	1.24	1.65	1.47	1.40	1.57	1.13	1.53	1.32	1.44	1.20	1.39
10	1.40	1.46	1.50	1.30	1.43	1.27	1.48	1.20	1.42	1.51	1.40
X	1.28	1.09	1.19	1.05	1.17	1.00	1.09	1.12	1.19	1.00	1.12
11	1.41	1.32	1.48	1.22	1.47	1.57	1.38	1.59	1.64	1.51	1.46
12	1.46	1.65	1.49	1.87	1.28	1.26	1.32	1.21	1.32	1.26	1.41
13	1.13	1.19	1.16	1.21	1.12	1.42	1.21	1.37	1.13	1.25	1.22
14	1.00	1.18	1.42	1.18	1.40	1.06	1.30	1.13	1.04	1.19	1.19
15	1.24	1.29	1.19	1.10	1.16	1.07	1.22	1.14	1.41	1.17	1.20
16	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
17	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
18	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞
Y	1.5	-	-	1.00	-	2.00	-	1.10	-	1.25	1.37

IDIOGRAM:-

IDIOGRAM:- ARM RATIOS OF HAPLOID CELLS (Long Arm/Short Arm)

Table 4

CHR'M	CELL NUMBER 1	2	3	4	5	6	7	8	9	10	MEAN
1	29.94	33.23	33.00	32.79	31.55	33.33	34.72	33.44	29.41	34.49	32.59
2	o	o	o	o	o	o	o	o	o	o	o
3	o	o	o	o	o	o	o	o	o	o	o
4	o	o	o	o	o	o	o	o	o	o	o
5	28.73	25.57	25.31	27.71	25.00	28.98	23.15	26.60	23.19	27.11	26.13
6	35.34	32.47	37.74	37.17	35.09	32.46	38.46	32.79	32.36	33.90	34.78
7	36.90	35.08	36.23	32.58	35.33	32.79	35.33	34.24	35.97	39.68	35.41
8	25.00	24.70	26.60	22.36	27.10	24.51	22.82	23.76	25.00	28.08	24.99
9	44.64	37.74	40.49	41.67	38.89	46.95	39.53	45.45	40.99	43.10	41.95
10	41.66	40.65	40.00	43.47	41.15	44.05	40.33	39.85	41.32	45.45	41.79
X	43.85	47.85	45.67	48.78	46.09	50.00	47.85	50.00	45.67	47.17	47.29
11	41.50	43.10	40.33	45.05	40.49	38.90	42.02	39.84	37.88	38.62	40.77
12	40.65	37.75	40.15	34.85	43.87	44.31	43.10	44.26	43.11	45 24	41.73
13	46.96	45.65	46.30	45.26	47.16	41.31	45.24	44 45	46.96	42.20	45.15
14	50.00	45.87	41.33	45.87	41.66	48.54	43.48	45.67	49.01	46.96	45.84
15	44.64	43.66	45.65	47.63	46.29	48.31	45.08	46.09	41.49	46.74	45.55
16	o	o	o	o	o	o	o	o	o	o	o
17	o	o	o	o	o	o	o	o	o	o	o
18	o	o	o	o	o	o	o	o	o	o	o
Y	40.00	-	-	50.00	-	33.30	-	44.46	-	47.63	43.08

Table 4a

Chr'm	Haploid Chromosome Length	Standard Deviation	Arm Ratio	Standard Deviation	Centromeric Index	Standard Deviation
1	112.36	7.08	2.08	0.15	32.59	1.68
2	84.46	4.73	∞		0	
3	62.78	3.63	∞		0	
4	56.11	2.60	∞		0	
5	66.68	3.46	2.85	0.29	26.13	1.96
6	62.48	2.44	1.89	0.18	34.78	2.23
7	53.65	2.15	1.83	0.11	35.41	1.94
8	52.09	2.16	3.02	0.28	24.99	1.73
9	59.82	3.99	1.39	0.16	41.95	2.86
10	53.64	1.31	1.40	0.10	41.79	1.80
X	52.47	1.62	1.12	0.07	47.29	1.90
11	49.10	1.73	1.46	0.12	40.77	2.08
12	41.90	2.23	1.41	0.20	41.73	3.14
13	41.79	2.13	1.22	0.09	45.15	1.90
14	33.42	2.40	1.19	0.13	45.84	2.82
15	27.91	2.41	1.20	0.09	45.55	1.88
16	36.70	1.59	∞		0	
17	28.88	1.74	∞		0	
18	23.45	1.24	∞		0	
Y	17.76	3.22	1.37	0.36	43.08	5.92

Meaned Chromosome length, Arm ratio and Centrometric Index together with the respective standard deviation.

Chromosome segment	Number of secondary constrictions observed	% of chromosomes displaying the secondary constriction
Proximal segment of shortarm of 13	570	95.00%
Proximal segment of shortarm of 9	25	4.16%
Proximal segment of shortarm of 11	18	3.00%

FREQUENCIES OF SEGMENTS DISPLAYING SECONDARY CONSTRICTION (3% or over).

Table 6

Chromosome Number	1	2	3	4	5	6	7	8	9	10	X	11	12	13	14	15	16	17	18	Y
Observed Frequency of Secondary Constrictions (%)	0.43	0.29	0.14	0.0	0.0	1.44	0.86	0.14	4.03	1.59	1.59	2.59	1.3	82.13	1.15	1.59	0.72	0.0	0.0	0.0
Expected Frequency Related to Length (%)	11.24	8.45	6.28	5.61	6.67	6.25	5.37	5.21	5.98	5.36	5.25	4.91	4.19	4.18	3.34	2.79	3.67	2.89	2.37	1.78

Frequencies of secondary constrictions as percentages of total number compared with the expected frequency, based on length.

x^2 test = $P < 0.01$: Highly significant

Table 7

Animal	Number of Sex Chromatin - Negative Cells	Sex Chromation Positive Cells				Sex Chromatin Double Positive	Total
		Posn. (1)	Position (2)	Posn. (3)	Total		
206	37	23	35	5	63	0	100
247	38	25	35	2	62	0	100
387	36	22	41	1	64	0	100
388	35	21	43	1	65	0	100
389	43	23	34	0	57	0	100
391	39	45	16	0	61	0	100
$571A_1$	49	21	28	2	51	0	100
$571B_1$	28	45	27	0	72	0	100
$573A_1$	35	20	41	4	65	0	100
$573B_1$	42	27	29	2	58	0	100
Total (%)	38.2	27.2	32.9	1.7	61.8	0	100
		44.0%	53.2%	2.8%	100%		

ADULT SPINAL CORD - FEMALE/CRESYL VIOLET

DISTRIBUTION OF BODY IN NUCLEI OF NEURONES

Table 8

Animal	Number of Sex Chromatin Negative Cells	Sex Chromatin Positive				Sex Chromatin Double Positive	Total
		Posn. (1)	Posn. (2)	Posn. (3)	Total		
192	98	0	2	0	2	0	100
193	98	0	2	0	2	0	100
194	98	2	0	0	2	0	100
254	94	5	1	0	6	0	100
303	100	0	0	0	0	0	100
36988	98	2	0	0	2	0	100
36989	100	0	0	0	0	0	100
$572A_1$	100	0	0	0	0	0	100
$572B_1$	100	0	0	0	0	0	100
$572C_1$	100	0	0	0	0	0	100
Total (%)	98.6	0.9	0.5	0.0	1.4	0	100

ADULT SPINAL CORD - MALE/CRESYL VIOLET

DISTRIBUTION OF BODY IN NUCLEI OF NEURONES.

Table 9.

Animal	Number of sex chromatin negative cells	Sex Chromatin Positive Cells				Sex Chromatin Double Positive Cells	Total	Non-Sex Specific Clumps			
		Posn. 1	Posn. 2	Posn. 3	Total			Total	Posn. 1.	Posn. 2.	Posn. 3.
391	37	33	24	5	62	1	100	4	1	2	1
386C	39	31	22	8	61	0	100	12	5	5	2
254E	36	18	40	3	61	3	100	7	3	4	0
573A	41	33	23	3	59	0	100	10	4	6	0
571A	35	39	20	5	64	1	100	13	10	3	0
Total %	37.6	30.8	25.8	4.8	61.4	1	100	9.2	4.6	4.0	0.6
		50.2%	42.0%	7.8%	100.0%			100%	50	43.5	6.5

ADULT SPINAL CORD - FEMALE/FEULGEN.

Distribution of body in Nuclei of Neurones.

Table 10

Animal	Number of sex chromatin negative cells	Sex Chromatin Positive Cells				Sex Chromatin Double Positive Cells	Total	Non-Sex Specific Clumps			
		Posn. 1	Posn. 2	Posn. 3	Total			Total	Posn. 1.	Posn. 2.	Posn. 3.
192	98	1	1	0	2	0	100	8	3	5	0
572C$_{\ell}$	97	2	1	0	3	0	100	5	2	3	0
572B$_{\ell}$	100	0	0	0	0	0	100	10	5	5	0
572A$_{\ell}$	96	1	3	0	4	0	100	12	6	6	0
194	97	2	1	0	3	0	100	7	4	3	0
Total%	97,6	1,2	1,2	0,0	2,4	0,0	100	8,4	4,0	4,4	0,0
		50,0%	50,0%	0,0%	100%			100%	47,6%	52,4%	0,0%

ADULT SPINAL CORD - MALE/FEULGEN

DISTRIBUTION OF BODY IN NUCLEI OF NEURONES

Table 11.

Animal	Number of sex chromatin negative cells	Sex Chromatin Positive Cells				Sex Chromatin Double Positive Cells	Total	Non-sex Specific Clumps			
		Posn. 1	Posn. 2	Posn. 3	Total			Total	Posn. 1	Posn. 2	Posn. 3
41	39	56	3	1	60	1	100	20	20	0	0
42	35	58	3	4	65	0	100	23	22	1	0
43	34	62	0	0	62	4	100	12	11	1	0
47	37	59	2	0	61	2	100	14	11	3	0
570E	31	66	1	1	68	1	100	13	13	0	0
570K	33	65	0	1	66	1	100	13	13	0	0
Total %	34.8	61.0	1.5	1.2	63.7	1.5		15.8	15.0	0.8	0.0
		95.8%	2.4%	1.8%	100%			100%	94.8%	5.2%	0.0%

FOETAL SPINAL CORD - FEMALE/CRESYL VIOLET

Distribution of body in Nuclei of Neurones.

Table 12

Animal	Number of sex chromatin negative cells	Sex Chromatin Positive Cells				Sex Chromatin Double Positive Cells	Total	Non-sex Specific Clumps			
		Posn. 1	Posn. 2	Posn. 3	Total			Total	Posn. 1	Posn. 2	Posn. 3
41	46	52	1	0	53	1	100	13	11	1	1
42	41	58	0	0	58	1	100	15	15	0	0
43	40	58	2	0	60	0	100	13	13	0	0
570E	28	64	2	2	68	4	100	15	14	1	0
570K	29	63	2	3	68	3	100	11	11	0	0
Total%	36,8	59,0	1,4	1,0	61,4	1,8	100	13,4	12,8	0,4	0,2
		96,1%	2,3%	1,6%	100%			100,0%	95,5%	3,0%	1,5%

FOETAL SPINAL CORD - FEMALE/FEULGEN

DISTRIBUTION OF BODY IN NUCLEI OF NEURONES

Table 13

Animal	Number of sex chromatin negative cells	Sex Chromatin Positive Cells				Sex Chromatin Double Positive Cells	Total	Non-Sex Specific Clumps			
		Posn. 1	Posn. 2	Posn. 3	Total			Total	Posn. 1.	Posn. 2.	Posn. 3.
570C	98	2	0	0	2	0	100	5	3	2	0
570D	97	3	0	0	3	0	100	6	6	0	0
570F	98	2	0	0	2	0	100	0	0	0	0
570G	96	4	0	0	4	0	100	7	7	0	0
570H	98	2	0	0	2	0	100	5	5	0	0
570I	98	2	0	0	2	0	100	3	3	0	0
570J	93	7	0	0	7	0	100	4	3	1	0
570L	96	3	0	1	4	0	100	3	2	1	0
Total %	96.8	3.1	0.0	0.1	3.2	0.0	100	4.1	3.6	0.5	0
		96.1%	0.0%	3.9%	100.0			100.0	87.9%	12.1%	0%

Table 14.

Animal	Number of sex chomatin negative cells	Sex Chromatin Positive Cells				Sex Chromatin Double Positive Cells	Total	Non-sex Specific Clumps			
		Posn. 1	Posn. 2	Posn. 3	Total			Total	Posn. 1	Posn. 2	Posn. 3.
570C	96	4	0	0	4	0	100	9	7	1	1
570G	96	4	0	0	4	0	100	1	1	0	0
570I	98	2	0	0	2	0	100	9	8	1	0
570J	97	2	1	0	3	0	100	4	4	0	0
570L	95	5	0	0	5	0	100	5	5	0	0
Total %	96,4	3,4	0,2	0,0	3,6	0,0	100	5,6	5,0	0,4	0,2
		94,4%	5,6%	0,0%	100%			100%	89,3%	7,1%	3,6%

FOETAL SPINAL CORD - MALE/FEULGEN

DISTRIBUTION OF BODY IN NUCLEI OF NEURONES

Table 15

Results of Amnion Preparations

Foetus	% Incidence Sex Chromation	Sex Diagnosis	External Morpholo-gical Sex	Gonadal Sex	Crown-Rump Length (mm.)	Age post-service (Days)
1	0	M	M	M	-	51
2	61	F	F	F	-	51
3	1	M	M	M	-	51
11	1	M	M	M	180	79
12	54	F	F	F	210	79
13	0	M	M	M	170	79
14	0	M	M	M	-	79
15	0	M	M	M	190	79
16	0	M	M	M	180	79
17	0	M	M	M	190	79
18	62	F	F	F	-	79
19	0	M	M	M	190	79
21	3	M	M	-	250	95
22	3	M	M	-	250	95
23	0	M	M	-	-	95
24	0	M	M	-	225	95
25	0	M	M	-	235	95
26	53	F	F	-	230	95
27	0	M	M	-	245	95
31	0	M	-	M	26	30
33	43	F	-	F	25	30
41	40	F	F	-	210	83
42	46	F	F	-	210	83
43	44	F	F	-	180	83
44	56	F	F	-	200	83
45	1	M	M	-	200	83
46	0	M	M	-	180	83
47	52	F	F	-	200	83
49	2	M	M	-	210	83
51	25	F	-	F	25	30
52	41	F	-	F	28	30
57	0	M	-	M	25	30
58	40	F	-	F	28	30
59	1	M	-	M	25	30
60	0	M	-	M	28	30

Foetus	% Incidence Sex Chromation	Sex Diagnosis	External Morphological Sex	Gonadal Sex	Crown-Rump Length (mm.)	Age post-service (Days)
71	33	F	F	-	65	42
72	0	M	M	-	65	42
74	46	F	F	-	70	42
75	0	M	M	-	65	42
76	43	F	F	-	65	42
77	0	M	M	-	65	42
78	0	M	M	-	65	42
79	0	M	M	-	65	42
80	42	F	F	-	70	42
91	39	F	F	-	160	72
92	0	M	M	-	160	72
93	0	M	M	-	165	72
94	27	F	F	-	170	72
95	0	M	M	-	170	72
96	36	F	F	-	150	72
97	40	F	F	-	170	72
98	38	F	F	-	150	72
99	37	F	F	-	160	72
100	31	F	F	-	170	72
Total		30 M 24 F				
MEAN	M:- 0.40% F:- 42.87%					

Table 16

Chromosome	Total	Proportion	Chromosome	Total	Porportion
1. D.S.A.	1326	1.32	10. S.A.	1805	1.80
P.S.A.	2211	2.20	P.L.A.	2063	2.06
P.L.A.	1403	1.40	D.L.A.	1547	1.54
M.L.A.	1403	1.40			
D.L.A.	1988	1.98	X. S.A.	1629	1.62
D.L.A.	2129	2.12	P.L.A.	2327	2.32
			D.L.A.	3490	3.48
2. P.	1977	1.97			
M.	1376	1.37	LATE		
D.	1661	1.66	X. S.A.	3143	3.13
			P.L.A.	6242	6.22
3. P.	3892	3.89	D.L.A.	5611	5.60
M.	2119	2.11			
D.	1271	2.27	11. S.A.	1939	1.93
			P.L.A.	1913	1.91
4. P.	2587	2.58	D.L.A.	1695	1.69
M.	1250	1.25			
D.	1121	1.12	12. S.A.	1345	1.34
			L.A.	1284	1.28
5. S.A.	1506	1.50			
P.L.A.	360	0.36	13. S.A.	2827	2.82
D.L.A.	1797	1.79	L.A.	1966	1.96
6. S.A.	2289	2.28			
P.L.A.	1984	1.98	14. S.A.	3290	3.28
D.L.A.	1429	1.42	L.A.	3140	3.13
7. S.A.	1395	1.39	15. S.A.	750	0.75
P.L.A.	1248	1.24	L.A.	387	0.39
D.L.A.	1294	1.29			
			16. P.	3516	3.51
8. S.A.	1369	1.37	D.	1758	1.75
P.L.A.	1073	1.07			
D.L.A.	1648	1.64	17. P.	3574	3.56
			D.	2569	2.56
9. S.A.	1934	1.93			
P.L.A.	2456	2.45	18. P.	2377	2.37
D.L.A.	2318	2.31	D.	1290	1.29
			Y. S.A.	13,350	13.06
			L.A.	10.954	10.71

Autoradiography. Segmental scores and proportions Grain scores 0-100

Code: D (distal) M (middle) P (proximal) S.A. (Shortarm) L.A. (Longarm)

Table 17.

Chromosome	Total	Proportion	Chromosome	Total	Porportion
1. D.S.A.	2655	1.45	10. S.A.	2587	1.42
P.S.A.	6425	3.52	P.L.A.	3564	1.95
P.L.A.	2800	1.53	D.L.A.	2797	1.53
M.L.A.	2883	1.58			
D.L.A.	3542	1.94	X. S.A.	2609	1.43
D,L.A.	3157	1.73	D.L.A.	4584	2.51
			P.L.A.	4510	2.49
2. P.	3442	1.88			
M.	2350	1.29	Late		
D.	5199	2.85	X. S.A.	4602	2.52
			P.L.A.	8856	4.85
3. P.	4753	2.60	D.L.A.	8010	4.42
M.	2877	1.55			
D.	2862	1.57	11. S.A.	3262	1.79
			P.L.A.	3825	2.09
4. P.	6100	3.34	D.L.A.	3324	1.79
M.	3676	2.01			
D.	2980	1.63	12. S.A.	3145	1.72
			L.A.	2252	1.23
5. S.A.	2977	1.63			
P.L.A.	866	0.49	13. S.A.	3792	2.08
D.L.A.	4686	2.56	L.A.	2902	1.59
6. S.A.	3174	1.74	14. S.A.	5406	2.96
P.L.A.	3025	1.66	L.A.	5212	2.85
D.L.A.	2589	1.42			
			15. S.A.	1752	0.96
7. S.A.	2730	1.49	L.A.	1592	0.87
P.L.A.	3194	1.75			
D.L.A.	2805	1.54	16. P.	5251	2.87
			D.	3463	1.90
8. S.A.	2814	1.54			
P.L.A.	2317	1.27	17. P.	5050	2.76
D.L.A.	2716	1.49	D.	3391	1.86
9. S.A.	3413	1.87	18. P.	4431	2.43
P.L.A.	4161	2.28	D.	3027	1.66
D.L.A.	3772	2.07			
			Y. S.A.	14,857	7.13
			L.A.	11,240	5.39

Autoradiography. Segmental Scores and proportions: Grain scores 101-200

D (distal) M (middle) P (proximal) S.A. (Shortarm) L.A. (Longarm)

'able 18.

hromosome		Total	Proportion	Chromosome		Total	Proportion
1.	D.S.A.	6493	2.00	X	S.A.	4743	1.46
	P.S.A.	7330	2.25		P.L.A.	8000	2.46
	P.L.A.	5787	1.78		D.L.A.	7843	2.41
	M.L.A.	6360	1.96				
	D.L.A.	7506	2.31	Late			
	D,L.A.	6246	1.92	X	S.A.	7304	2.25
					P.L.A.	12320	3.79
2	P.	5443	1.67		D.L.A.	10353	3.18
	M.	5367	1.65				
	D.	5135	1.58	11	S.A.	6154	1.89
					P.L.A.	7609	2.34
3.	P.	7220	2.22		D.L.A.	6117	1.88
	M.	5817	1.79				
	D.	6335	1.95	12	S.A.	5750	1.77
					L.A.	4746	1.46
4.	P.	8313	2.56				
	M.	6102	1.88	13	S.A.	6700	2.06
	D.	6102	1.88		L.A.	5300	1.63
5.	S.A.	5774	1.76	14	S.A.	9337	2.87
	P.L.A.	2689	0.83		L.A.	8661	2.66
	D.L.A.	5813	1.79				
				15	S.A.	3967	1.22
6.	S.A.	5965	1.83		L.A.	3257	1.00
	P.L.A.	5563	1.71				
	D.L.A.	5670	1.74	16	P.	7937	2.44
					D.	6041	1.86
7.	S.A.	5526	1.70				
	P.L.A.	5480	1.69	17	P	6926	2.13
	D.L.A.	4922	1.51		D	5570	1.71
8.	S.A.	6961	2.14	18	P	6867	2.11
	P.L.A.	5278	1.62		D	5587	1.71
	D.L.A.	5113	1.57				
				Y	S.A.	18224	5.60
9.	S.A.	6037	1.86		L.A.	10402	3.20
	P.L.A.	6935	2.13				
	D.L.A.	7309	2.25				
0.	S.A.	4863	1.50				
	P.L.A.	6533	2.01				
	D.L.A.	6046	1.86				

toradiography. Segmental scores and proportion. Grain Scores > 200

(distal) M (middle) P (proximal) S.A. (shortarm) L.A. (Longarm)

Table 19

	2 LARGE	1 LARGE 1 SMALL	2 SMALL	NO DIFFERENCE	TOTAL
LATE LABELLING CHROMOSOMES 14 - 15	103 (206)	29 (29)	3 (0)	3	138 (235)
	74.6	21.0	2.2	2.2	100% (85.1%)

Relationship between size and labelling patterns of chromosomes 14-15

Figures in parenthesis denote number of larger chromosomes in each cell which were late replicating.

Table 20

Chromosome Number	1	2	3-4	5	6	7	8	9	10	X	11	12	13	14-15	16	17-18	Y	
Observed Frequency of Breakages	18.5	23.5	17.3	3.7	7.4	1.2	8.6	7.4	2.5	0.0	1.2	0.0	4.9	0.0	3.7	0.0	0.0	100% (81 counted)
Expected Frequency Related to Length	11.2	8.5	11.9	6.7	6.3	5.4	5.2	6.0	5.4	5.3	4.9	4.2	4.2	6.1	3.7	5.3	1.8	100%

Correlation between observed and expected frequency of Tritium-induced breakages, both expressed as percentages. (x^2 test : $P < 0.01$: Highly significant.)

Table 21.

A. Percentage distribution of Chromosome counts from cultures with tritium added 5 hours pre-fixation

Cultures	Chromosome Numbers							Total
	< 36	36	37	38	39	40	> 40	
1	0	6	6	86	2	0	0	100%
2	0	12	4	82	2	0	0	100%
3	2	4	4	90	0	0	0	100%
Mean	0.66	7.33	4.66	86.00	1.33	0	0	100%

B. Percentage distribution of chromosome counts from cultures with tritium added 6 hours pre-fixation

Cultures	Chromosome Numbers							Total
	< 36	36	37	38	39	40	> 40	
1	2	4	6	86	2	0	0	100%
2	0	4	8	86	2	0	0	100%
3	0	4	6	88	2	0	0	100%
Mean	0.66	4.00	6.66	86.66	2.0	0	0	100%

C. Percentage distribution of chromosome counts from pooled control cultures

	Chromosome numbers							Total
	< 36	36	37	38	39	40	> 40	
Pooled Cultures	0.83	0.66	3.83	90.5	2.66	1.50	0	100%

Table 22

A. Frequency of breakages in experiment A (Tritium added 5 hours pre-fixation)

Culture	Single Breaks	Double Breaks	Single Fragments	Double Fragments	Complexes (Number of breaks in parenthesis)	Total	Frequency per cell	Non-staining Gaps (Frequency per cell)
1	3	-	-	1	-	5	0.10	12 (0.24)
2	7	-	2	-	-	9	0.18	7 (0.14)
3	7	-	1	-	-	8	0.16	5 (0.10)
Mean	5.66	-	1.00	0.33	-	7.33	0.14	8.0 (0.16)

B. Frequency of breakages in experiment A (Tritium added 6 hours pre-fixation)

1	21	1	-	1	-	25	0.50	14 (0.28)
2	4	6	-	-	-	16	0.32	4 (0.08)
3.	21	5	-	-	1 (4)	35	0.70	20 (0.40)
Mean	15.33	4.00	-	0.33	0.33 (1.33)	25.33	0.51	12.66 (0.25)

C. Frequency of breakages in control cultures.

Control Cultures	-	-	-	-	-	-	-	9 (1.50)
Mean	-	-	-	-	-	-	-	1.5 (0.03)

Results of students 't' test.

Breakages:- Controls/a : $P < 0.01$ - Significant
Controls/b : $P < 0.01$ - Significant
a/b : $P < 0.05$ - Probably significant

Non staining gap:- Control/a $P < 0.01$
Control/b $P < 0.01$
a/b > 0.5 N.S.

Table 23.

Experiment	Number of breaks labelled (Percentages in parenthesis)	Number of breaks unlabelled (Percentages in parenthesis)	Total (Percentages)
A	8 (50)	8 (50)	16 (100)
B	49 (94.2)	3 (5.8)	52 (100)
Total	57 (83.8)	11 (16.2)	68 (100)

Association of labelling patterns and tritium-induced breakages.

TABLE 24

CHROMOSOME NUMBER	1	2	3-4	5	6	7	8	9	10	x	11	12	13	14	15	16	17	18	y
OBSERVED FREQUENCY OF GAPS (%)	29.0	16.1	17.7	11.3	4.8	1.6	9.7	1.6	4.8	1.6	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0
EXPECTED FREQUENCY RELATED TO LENGTH (%)	11.2	8.4	11.9	6.7	6.2	5.4	5.2	6.0	5.4	5.2	4.9	4.2	4.2	3.3	2.8	3.7	2.9	2.4	1.8

PERCENTAGE FREQUENCY OF NON-STAINING GAPS IN TRITIUM ADDED CULTURES COMPARED WITH THE EXPECTED FREQUENCY, BASED ON LENGTH

x^2 test : $P < 0.01$: Highly significant.

Table 25

	Number of Chromosomes per cell							Total
	36	36	37	38	39	40	~~40~~ 41	
Number of cells examined	1	3	4	11	120	30	3	172
% of cells examined	0.58	1.74	2.33	6.40	69.77	17.44	1.74	100,00

Chromosome distribution in lymphocyte culture cells from case AE 32

Table 26

	Number of sex chromatin bodies per cell			Total
	0	1	2	
Number of cells examined	37	104	9	150
Percentage of cells examined	24.67	69.33	6.00	100%

Sex Chromatin distribution in neurones from case number AE 32