AMNIOTIC FLUID CYTOLOGY IN RELATION TO OESTROGEN CONCENTRATION AND FOETAL SEX

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INTRODUCTION

Experience of vaginal cytology has shown that in the absence of circulating progesterone the proportion of mature squamous cells as assessed by the karyopyknotic index rises with the level of circulating oestrogen. It has been found that amniotic fluid oestrogen concentration rises with advancing gestation in normal pregnancy but it is not yet known whether this oestrogen has any physiological effect on the foetus. The purpose of this investigation was to determine if the rise in amniotic fluid oestrogen concentration during pregnancy is associated with a rise in the karyopyknotic index of the foetal squamous cells exfoliated into the amniotic fluid.

A relationship between the karyopyknotic index of the amniotic fluid cells and the oestrogen concentration of the amniotic fluid would have practical applications. Some workers have found a significant correlation between amniotic fluid oestrogen concentration and maternal urinary oestriol excretion and they have suggested that the amniotic fluid oestrogen concentration has advantages as an indicator of foetal well-being. A correlation between the karyopyknotic index and amniotic fluid oestrogen concentration would provide a relatively simple cytological test of the state of the foetus.

The cells of the amniotic fluid have been described by several authors. Differential counts have been reported but there is no record in the literature of karyopyknotic index estimations or of correlation between amniotic fluid cytology and amniotic fluid

oestrogen concentration.

A preliminary study was made of 57 amniotic fluid specimens, to see if there was an association between the karyopyknotic index and the oestrogen concentration. The specimens were obtained from patients who were having amniocentesis either for determination of optical density because they had rhesus antibodies, or for determination of foetal maturity, and also from patients who were having elective caesarean section. When possible, maternal 24-hour urines were collected from these patients in order that the 24-hour oestriol excretion could be compared with the amniotic fluid oestrogen concentration.

The results of the preliminary study showed a fall in karyopyknotic index with the increase in the amniotic fluid oestrogen concentration, which was associated with the later stages of pregnancy. The fall in the karyopyknotic index was unexpected, but it did correlate with reports in the literature of a fall in the maternal vaginal karyopyknotic index during the last eight weeks of pregnancy, associated with a rise in maternal 24-hour urinary oestriol excretion. Statistical analysis showed that the relationship between the karyopyknotic index and the amniotic fluid oestrogen concentration is highly significant when the foetus is female but not when the foetus is male.

The investigation was continued by obtaining further specimens of amniotic fluid for analysis to confirm the findings of the

preliminary study. The morphology of the amniotic fluid cells was studied in an attempt to account for the different results with male and female foetuses. A larger number of maternal 24-hour urinary oestriol results was analysed to exclude a difference in oestriol excretion between patients with male and female foetuses.

The cytological difference was found to depend on the presence of large numbers of cyanophilic, squamous cells in Papanicolaou-stained preparations when the foetus was female. A percentage count of the cyanophilic cells was carried out on 48 specimens and shown to be a reliable method of antenatal diagnosis of the sex of the foetus. Previous workers had reported a preponderence of cyanophilic cells in amniotic fluid from female foetuses and identified the source of the cells as the foetal vagina. In this study, further evidence was obtained, by counting the total number of cells per cu.mm. in 38 specimens, that the exfoliation from the female vagina is so abundant as to account for a significantly lower karyopyknotic index in amniotic fluid with female foetuses than with male foetuses. An increase in the total cells per cu.mm. with advancing gestation has been recorded but previous workers have not compared total cell counts from male and female foetuses. Histochemical tests were carried out in an attempt to identify the cytoplasmic substance responsible for the cyanophilia of squamous cells stained by the Papanicolaou method.

This work has been carried out in the Cytology Department of The Royal Berkshire Hospital, with the co-operation of the medical and nursing staff of the Obstetric Unit. I was personally responsible for the laboratory work, except for the oestrogen estimations, which were carried out by Searle Scientific Services to whom I am greatly indebted.

CHAPTER 1

THE PRINCIPLES INVOLVED IN THE INVESTIGATION AND A REVIEW OF THE RELEVANT LITERATURE

1.1 The Karyopyknotic Index

The karyopyknotic index is a measure of the degree of maturation of squamous epithelium. It is the percentage of superficial cells, defined as those with pyknotic nuclei, determined from a count of at least 200 superficial and intermediate squamous cells. A pyknotic nucleus is defined as being of less than 5μ diameter and uniformly hyperchromatic, without any visible chromatin structure, as opposed to the intermediate cell nucleus which is finely stippled and vesicular.

The index was first used in vaginal cytology as a measure of the action of oestrogens on the vaginal epithelium by Férin (1945). Pundel (1952), in his account of the karyopyknotic index, compared it with the eosinophilic index, which is defined as the percentage of squamous cells which take up eosinophilic cytoplasmic stain. (Pundel used Shorr's (1941) stain for eosinophilic index determinations.) He disagreed with Férin (1948) who stated that the two indices always varied in parallel with each other. The eosinophilic index has the advantage that it can be estimated with a low (x 10) microscope objective. It has disadvantages in that it varies with the pH of the material being stained, with the technique of spreading the smear on the slide and with the methods of fixation, staining and differentiation. There may also be difficulty in the interpretation of cells

with cytoplasm which has not stained either pink or blue but some intermediate colour. Pundel (1952) points out that this problem occurs less commonly with Shorr's (1941) stain than with Papanicolaou's (1942), and that, when it does occur, the cells of indeterminate colour should be excluded from the count. The karyopyknotic index has the advantage of being based on an absolute measurement since the size of the nucleus, can be measured, if necessary, micrometrically, with a higher (x 40) microscope objective. Pundel (1952) suggests that at least 200 cells should be counted and that the higher the number of cells counted the more accurate will be the index. He emphasises that the cells counted should be from different parts of the slide. He claimed that in over 6,000 vaginal smears, for which he had determined both the karyopyknotic and eosinophilic indices, the karyopyknotic index gave the more sensitive indication of oestrogenic activity.

The karyopyknotic index as a measure of oestrogen activity is only reliable in the absence of other sex hormones acting on the epithelium, as in the first half of the menstrual cycle, before the menarche and after the menopause, and in the absence of inflammatory disease, digitalis administration or liver disease. Papanicolaou and Shorr (1936) reported the changes in the vaginal smears of menopausal women after administration of ovarian follicular hormone. They described the vaginal smear qualitatively as changing progressively from the atrophic, post menopausal pattern to the mid-cycle pattern of the younger woman, consisting of "large, flat cells, ... with

small pyknotic nuclei." This would be consistent with a high karyopyknotic index if it had been estimated.

The combined action of progesterone and oestrogens inhibits the oestrogenic response of full maturation of the squamous epithelium, and results in a vaginal smear consisting predominantly of cells of the intermediate cell layer as seen in the secretory phase of the menstrual cycle and in pregnancy, with a correspondingly low karyopyknotic index. This action of oestrogen and progesterone together was demonstrated by Shorr (1940). Having induced an oestrogenic type of vaginal smear in nine postmenopausal women with oestrogen preparations, he then gave them progesterone whilst maintaining the oestrogen dosage. Within 48 hours changes appeared in the vaginal smears which were comparable with appearances in the second half of the normal menstrual cycle.

1.2 The Cytology of Amniotic Fluid

Bevis (1950) pointed out that examination of amniotic fluid obtained antenatally by amniocentesis could be used in management of cases of rhesus haemolytic disease. He suggested that the results of tests for iron (Bevis (1950)) and for bilirubin (Bevis (1956)) could provide an indication for the timing of induction of labour. Since then, with the development of amniocentesis as a safe procedure, the amniotic fluid has been studied from several aspects, including its cytology and its oestriol content.

Attention was first drawn to the cells of the amniotic fluid by Bourgeois (1942) who described foetal squamous cells derived from

the vernix in vaginal material as "contiguous translucent polygonal cells" and used their identification for the diagnosis of ruptured membranes. Hopman, Wargo and Werch (1957) followed up this method, first using Papanicolaou stain and later, in co-operation with Averette and Ferguson (1963), using pinacyanole chloride. They claimed an accuracy of 97 per cent in the diagnosis of ruptured membranes.

Kittrich (1963) described the presence of cells containing neutral fat which could be demonstrated in vaginal fluid in cases of ruptured membranes, by staining with Nile Blue Sulphate. Brosens and Gordon (1965) confirmed the findings of Kittrich and correlated the percentage of cells in amniotic fluid which stain orange with Nile Blue Sulphate with the number of weeks of gestation (Brosens and Gordon (1966)). This test for foetal maturity drew further attention to the cytology of amniotic fluid and was followed by several workers who used the same method, (Bishop and Corson (1968), Sharp (1968), Barnett and Nevin (1970), Sharma and Trussell (1970), Husain and Sinclair (1971), Doshi and Ansari (1972)). Some authors found the method less accurate than did Brosens and Gordon, (Anderson and Griffiths (1968), Chan, Willis and Woods (1969), Lind, Parkin and Cheyne (1969), Rosborg, Kristensen and Trautner (1970)).

Brosens and Gordon (1965) stated that the orange-stained cells probably originated from sebaceous glands, because these cells were found in skin scrapings of areas rich in sebaceous glands, but were absent in skin scrapings from areas such as the palms of the hands

which do not contain them. Sharp (1968) suggested that the orange stained cells were vernix cells coated with adherent lipid material which had been released by sebaceous glands. These begin to function from about three months according to Montagna (1962), and their function increases as the foetus matures. Nieland, Parmley and Woodruff (1970), using an electron microscope, did not see any cells containing lipid or vacuolated cells in sediment from specimens of amniotic fluid taken near term, which had shown between 25 and 75 per cent orange cells when stained with Nile Blue Sulphate and examined under the light microscope. They concluded that the fatty material was adherent to the outside of the cell and was lost during processing for the electron microscope. This view supports that of Parmley and Miller (1969) who found that when amniotic fluid cell preparations stained with Nile Blue Sulphate were allowed to stand for several hours, large orange-stained droplets could be seen to be floating freely in the fluid, outside the cells.

Bourne (1962) described the amniotic epithelium as consisting of a single layer of cells which were mostly cuboidal in shape, but which were flattened in some areas of the reflected amnion and were frequently columnar in the placental amnion, though none of the cell types was confined entirely to any one area. He observed that the large vacuoles in the epithelium consisted of distended intercellular canals and that some of the vacuoles contained fat, although electron-microscopic appearances suggested that the number containing lipid was relatively small. Wilt and Miller (1965) also described the placental

amnion as consisting mostly of columnar cells and the reflected or free amnion mostly of cuboidal, flattened or stratified cells. They found that amnion cells from spontaneous deliveries showed more fat-containing vacuoles than amnion cells from placentae from caesarean sections. They suggested that this fat may be the source of the vernix.

Blystad, Landing and Smith (1951) reported that the total number of squamous cells in the amniotic fluid increases up to the time of delivery. This was confirmed by Votta, Bobrow de Gagneten, Parada and Giulietti (1968), who reported that after 37 weeks of gestation the number of cells averaged 272 per cu.mm. with a standard deviation of 186.5, based on 44 observations. Several workers have reported results of differential cell counts, first Van Leeuwen, Jacoby and Charles (1965), who were followed by Huisjes (1968a, 1968b), Votta, Bobrow de Gagneten, Parada and Giulietti (1968), Bishop and Pollock (1970) and Stenback and Ojala (1970). These authors also described the morphology of the cells in the amniotic fluid and made comparisons with cells from foetal and amniotic surfaces, in order to determine the source of these cells. An account of the cell morphology and their origins has been provided by Wachtel, Gordon and Olsen (1969). The relative variation in the numbers of the different cell types at different periods of gestation has been described by these authors and also by Mandelbaum and Evans (1969) and Votta, Bobrow de Gagneten, Parada and Giulietti (1968). The terminology of vaginal cytology has been used and broad agreement is reached on four cell types found in amniotic fluid.

Anucleate cells are present in increasing numbers from as early as 22 weeks gestation until term. They are polyhedral in outline and may be eosinophilic or cyanophilic. Nucleate squamous cells also appear in the amniotic fluid between 22 and 32 weeks gestation. Their nuclei may be pyknotic, as in the superficial cells of vaginal smears, or vesicular as in intermediate cells and their cytoplasm may be eosinophilic or cyanophilic. The third type of cell is small and round, with fairly dense, blue-staining cytoplasm which is sometimes vacuolated. These small cells often occur in groups of 2-15 and may be joined by intercellular bridges which accentuate the resemblance they have to the parabasal cells of vaginal cytology. Hoyes (1968), using an electron microscope, found evidence that these cells have phagocytic properties and that they are capable of independent existence in the amniotic fluid. He referred to these as Type 2 cells, and to the larger squamous cells of foetal origin as Type 1 cells. The small cells are very similar to cells scraped from the surface of the amnion up to 32 weeks gestation, a point which led Van Leeuwen, Jacoby and Charles (1965) to suggest that this is their source of origin. Huisjes (1970) however suggested that it was unlikely that the amnion desquamates cells and he thought the small round cells were derived from the urinary tract. After approximately 32 weeks the number of small round cells decreases. This can be accounted for by differentiation of the amniotic epithelium. Wachtel, Gordon and Olsen (1969) found that between 31 and 36 weeks, tall, flask shaped cells appear in the amniotic fluid and that similar cells can be scraped from the

amnion at term and are seen in histological sections of amnion taken from freshly delivered placentae. These tall cells represent the fourth type of cell found in the amniotic fluid. They have vesicular nuclei which may be placed centrally or at either end of the cell. Huisjes (1970) also found elongated, club-shaped cells in scrapings from placental amnion.

The anucleate and nucleate squamous cells are of foetal origin. The anucleate cells exfoliate mainly from the skin and the nucleate squamous cells from the buccal mucosa and genital tract (Van Leeuwen, Jacoby and Charles (1965), Hoyes (1968), Votta, Bobrow de Gagneten, Parada and Giulietti (1968), Wachtel, Gordon and Olsen (1969), Huisjes (1970), Hilgarth, Ebmeier and Schneider (1971), Nyklicek and Hradil (1971)). These authors have examined cells obtained from the amnion and cord, and from the skin, buccal mucosa, vaginal introitus and urine of newborn infants and found them comparable with cells in the amniotic fluid. They agree that, as gestation advances there is a significant increase in the total number of cells and in the number of anucleate cells and fat-containing cells, relative to other cell types, and also that the small 'parabasal' cells decrease relatively as term approaches. Floyd, Goodman and Wilson (1969) and Mandelbaum and Evans (1969) reported a decrease in the proportion of anucleate cells after 36 weeks, which they say was unexpected, and conflicts with the findings of other authors on the subject.

Differential cell counts have been suggested as a measure of foetal maturity by Bishop and Pollock (1970) and Floyd, Goodman and

Wilson (1969) but the variation of values is so wide that other indications such as fat-cell staining and amniotic fluid creatinine concentration, (Pitkin and Zwirek (1967)), used in conjunction with differential cell counts are more reliable. Lind and Billewicz (1971) described a point-scoring system for assessment of gestational age in which they use a cytological assessment based on a differential cell count in conjunction with amniotic fluid creatinine values and the difference in urea concentration between amniotic fluid and maternal plasma. When discussing the cytology, they state that cornified cells appear at 36 weeks and come to equal the number of pre-cornified cells at 37-38 weeks gestation. Their illustrations suggest that what they refer to as 'cornified' cells are equivalent to 'superficial' squamous cells in modern terminology and their 'pre-cornified' cells are those now called intermediate cells. If this is the case, their statement, translated into terms of the karyopyknotic index, indicates a rise in this index between 36 and 38 weeks, from about 20 to 50. These conclusions will be compared with the results of this study in Chapter 5.

Huisjes and Arendzen (1970) counted the anucleate 'polygonal' cells as a measure of foetal maturity. They define these as anucleate cells which stain transparently orange in Harris-Shorr stained preparations, which have a more or less regular outline and in size are between small round 'parabasal' cells and the large nucleate squamous cells. Huisjes (1968a, 1968b) found evidence that the origin of the 'polygonal' cells was the foetal epidermis.

1.3 Prenatal Determination of Sex from Amniotic Fluid Cytology

Rosa and Fanard (1949, 1951), using Papanicolaou stain, noticed that the amniotic fluid surrounding female foetuses could be distinguished from that surrounding male foetuses by the presence of numerous squamous cells with cyanophilic cytoplasm. They did not quantify the percentage of cyanophilic cells which was diagnostic of a female foetus, but they claimed 100 per cent accuracy in prediction of the sex of the foetus in 40 cases, 27 of which were at term, 12 between seven and eight-and-a-half months and one at five-and-a-half months. They also claimed that the amount of the deposit obtained as a result of centrifuging the amniotic fluid was "generally much more" in the case of girls than boys. Rosa and Fanard (1949, 1951) made scrapings from foetal skin, buccal mucosa and amnion and examined sediment from catheterised urine of newborn infants, but only in smears from the entrance to the vagina did they find large numbers of cyanophilic squamous cells, a fact which could account for the appearance of the smeared cell deposit from amniotic fluid with female foetuses.

Langreder (1952) listed cytological criteria for prenatal diagnosis of sex from amniotic fluid cells. He described the presence of navicular cells in liquor from female foetuses and the presence of cell debris with male foetuses. (Navicular cells were first described by Papanicolaou (1925) as characteristic of the vaginal smear in pregnancy. They are a modified type of intermediate cell, oval in shape with slightly thickened borders, cyanophilic cytoplasm and an

eccentric nucleus.) In contrast to other authors, Langreder described eosinophilic cells as being most numerous with female foetuses. Ta-Jung Lin, Vasicka and Bennett (1960) correctly predicted the sex of 28 out of 30 foetuses between 36 and 40 weeks gestation by looking for nuclear chromatin in the cells of the females. They also noticed in all specimens a predominance of eosinophilic cells, but a constant and significant relative increase of cyanophilic cells from female as compared with male foetuses. This sex difference has been confirmed more recently by Huisjes (1968a), by Bennett, Morris and Davey (1972) and by Nelson (1973). Huisjes (1968a) concluded that between the 31st and 37th weeks a count of more than 21 per cent cyanophilic cells is evidence of a female foetus but he mentioned one case of a male foetus where the amniotic fluid contained large numbers of atypical cyanophilic cells. He also claimed that the dominant source of cyanophilic cells is the vaginal vestibule, the buccal mucosa yielding a mixture of cyanophilic and basophilic cells. In a further series of 37 cases, Arendzen and Huisjes (1971) diagnosed the sex of only one foetus incorrectly.

Bennett, Morris and Davey (1972) studied 31 patients, from 32 weeks gestation until term, and concluded that a count of more than 25 per cent cyanophilic squamous cells meant that the infant was female. Nelson (1973) using Shorr's stain on 93 specimens of amniotic fluid from 71 pregnancies between 12 and 40 weeks gestation diagnosed the sex of the foetus correctly in 29 out of 30 patients, between 32 weeks gestation and term.

The histochemical basis for the differential cytoplasmic staining of squamous epithelium with Papanicolaou's stain is not documented. The EA 50 stain consists of Light Green and Eosin with Bismarck Brown, phosphotungstic acid and lithium carbonate and has a pH of 4.6. Light Green is a basic stain and eosin is acidic. Generally, the superficial cells take up the eosin and the intermediate cells take up the Light Green, but the colour reaction is not specific because it can be influenced by pH, the thickness of material on the slide and by the method of fixation. With amniotic fluid cytology variation of pH can be minimised if the liquor is obtained by amniocentesis and transferred to a sterile container; fixation can be standardised and assessment confined to areas of the slide where the material is evenly spread.

Montagna (1962), referring to sections of skin, states that the cells of the malpighian layer are intensely basophilic, the cytoplasm of the cells of the stratum granulosum is less basophilic and the stratum lucidum and corneum are very weakly basophilic or not at all. He states also that treatment of sections of skin with ribonuclease before staining with dyes buffered up to pH 5.0 abolishes all cytoplasmic basophilia which can therefore be presumed to be due to ribonucleic acid. With dyes at a pH of more than 5.0 the action of ribonuclease does not abolish all the cytoplasmic basophilia, indicating the presence of acid groups other than nucleic acids. According to Wachtel (1969), ribonucleic acid can be demonstrated in cytological specimens by fluorescence microscopy using acridine orange, and it is

present in moderate amounts in basal and parabasal cells, in small amounts in intermediate cells and absent from superficial cells. Stenback and Ojala (1969) have studied the histochemical properties of amniotic fluid cells, but they have not related their findings to the relative cyanophilia and eosinophilia seen in Papanicolaou stained preparations.

Foetal sex can be determined from amniotic fluid cells by identification of the sex chromatin. This was first described by Barr and Bertram (1949) and first applied to amniotic fluid by James (1956) who referred to the cyanophilic cells in female specimens as having nuclei in which the sex chromatin could easily be seen. He was followed by Dewhurst (1956), Fuchs and Riis (1956), Makowski, Prem and Kaiser (1956), Sachs, Serr and Danon (1956) and Shettles (1956). More recently, fluorescent light microscopy of preparations stained with atebrine have made possible the identification of the Y chromosome when the foetus is male. If termination of pregnancy is to be considered, amniotic fluid cell culture for karyotyping is the method of choice, but this takes about two weeks to complete.

There are two reports of different hormone levels in women pregnant with male and female foetuses. Rawlings and Krieger (1964) reviewed the results of 14,760 maternal urinary pregnanediol estimations, made at weekly intervals up to 34 weeks gestation, on patients with a history of three or more abortions. They found that a significantly higher proportion of male babies were born to mothers with 'above average' pregnanediol excretion curves, (24 males to 15 females), and a higher proportion of female babies were delivered to those mothers

with 'below critical' pregnanediol excretion curves, (10 males to 23 females). Brody and Carlstrom" (1965) studied serum human chorionic gonadotrophin (HCG) levels throughout pregnancy in 20 women. In the last trimester they found a considerable variation in levels between individuals, but they showed a difference between those with male and female foetuses which was statistically significant at 0.5 per cent. The women with low HCG levels had a predominance of male foetuses and those with high levels had a predominance of female foetuses.

1.4 Vaginal Cytology in Pregnancy

Favarger (1913) was the first to describe changes in the vaginal epithelium in pregnancy. Papanicolaou (1925) described navicular cells as characteristic of the vaginal smear in pregnancy. Several studies were made by Pundel and Van Meensel (1951), Pierce (1957), Wachtel (1959), Spira and MacRae (1960) and Wood, Osmond-Clarke and Murray (1961). These authors agree that as a result of the high levels of circulating oestrogen and progesterone throughout pregnancy, the squamous epithelium of the vagina thickens but it does not form a thickened superficial layer as it would in response to oestrogen alone. The thickening occurs partly in the parabasal layer, but most markedly in the intermediate cell layer. Consequently the vaginal smear in normal pregnancy consists almost exclusively of intermediate cells, many of them in the form of navicular cells. As pregnancy progresses these cells tend to form clumps. According to Wachtel (1969) if the karyopyknotic index is calculated from a vaginal smear in normal pregnancy, it should not be higher than ten. A karyopyknotic index

higher than ten is an indication that there is horomonal imbalance (progesterone deficiency) as seen in certain cases of threatened abortion and sometimes with an antepartium haemorrhage.

Lichtfus (1959) and Pundel (1959) claimed that in 90 per cent of pregnancies the appearance of the smear changed during the last two weeks of gestation, indicating the onset of labour within the next 48 to 72 hours. They described the 'at term' smear as showing a decrease in navicular cell clumping followed by the appearance of superficial squamous cells. Other authors, (Birtch (1961), Abrams and Abrams (1962), Hindman, Schwalenberg and Efstation (1962) and Wachtel (1969)) have not been able to corroborate these claims. The changes probably appear near term in the vaginal smears of a number of pregnant women, but they do not give a reliable forecast of the onset of labour and many pregnancies never show this vaginal smear pattern.

It is generally accepted by cytologists that the vaginal smear in pregnancy reflects the balance between oestrogens and progesterone. M.J. Smith (1927) reported that in normal pregnancy maternal urinary oestriol levels increase until the onset of labour. Pregnanediol excretion, which according to Shearman (1959) is proportional to progesterone production, rises during pregnancy until about 37 weeks of gestation when it levels off and may fall slightly one to two weeks before term, as Russell, Paine, Coyle and Dewhurst (1957) found in 25 normal pregnancies. The appearance of the 'at term' smear may be due to a change in the oestrogen-progesterone balance or there may be as yet unknown factors, hormonal or otherwise, acting on the vaginal

epithelium. A fall in progesterone relative to the still rising oestriol near term might be expected to account for the 'at term' smear. This is not corroborated by Spira and MacRae (1960) or by Leeton (1963), who found a fall in the vaginal karyopyknotic index towards term whereas an 'at term' smear indicates an increase in superficial cells which would result in a rise in karyopyknotic index. The explanation suggested by Aubry and Nesbitt (1970) is that the karyopyknotic index has poor sensitivity as an indicator, resulting in inconsistent results. They compared the vaginal karyopyknotic index with oestriol, pregnanediol and human chronionic gonadotrophin excretion, in 144 pregnancies, 93 of which were abnormal cases. They found that a karyopyknotic index above ten was related to falling oestriol excretion, but only 10 to 15 per cent of all their cases of depressed hormonal excretion in the last trimester of pregnancy showed this raised karyopyknotic index. Nesbitt, Aubry, Goldberg and Jacobs (1965) had already shown that administration of oestrogen, in the presence of adequate progesterone, lowered the karyopyknotic index, or produced no effect if the karyopyknotic index was already low. Stamm, Rawyler and Riotton (1959) had reported an association between a raised maternal vaginal karyopyknotic index and a low oestriol excretion.

Mandelbaum and Evans (1969) took maternal vaginal smears from 59 patients, whose amniotic fluid they examined. They report a "remarkable relationship between the oestrogenic activity in the vaginal smears and the number of cornified squamous cells present in the amniotic fluid". This appears to be the only report of comparison

between maternal vaginal smears and amniotic fluid cytology in the literature.

Leeton (1963) took vaginal smears at weekly intervals, during the last ten weeks of pregnancy, from ten women with normal pregnancies. He estimated the karyopyknotic index and found that it fell with time until the onset of labour, and that by 36 weeks all his ten patients had a karyopyknotic index below ten. This confirmed the findings of Spira and MacRae (1960) who reported a maximum karyopyknotic index of 25 at 20 weeks, and a maximum of 12 at term, with a linear fall as gestation progressed. These findings conflict with those of Pundel and Van Meensel (1951) who found a rise of karyopyknotic index prior to the onset of labour. Leeton (1963) also determined the maternal urinary oestriol, by collecting 24-hour urine specimens from his ten patients within 48 hours of taking each vaginal smear. He found an inverse relationship between the vaginal karyopyknotic index and the maternal urinary oestriol excretion. The oestriol levels of all his ten normal patients rose until delivery.

Present knowledge of the relationship between vaginal cytology and oestriol excretion in pregnancy has been discussed because it is the closest parallel that can be drawn with the present investigation into amniotic fluid cytology and the amniotic fluid oestrogen concentration.

1.5 Oestrogens in Pregnancy

The rise in oestrogen activity in pregnancy and the fall after delivery was observed by M.J. Smith (1927) in the United States,

while similar work was being carried out by Fels (1926) and Ascheim (1926) in Europe. Since then improved chemical methods of estimating oestrogens and further understanding of the process of synthesis of oestrogens in the mother, placenta and foetus have shown that oestriol production and excretion reflect closely the well-being of the foetus.

That the placenta is the main source of oestriol in pregnancy was demonstrated by the high urinary oestrogen levels maintained when pregnant women were oophorectomised (Waldstein (1929), Guldberg (1935)) or hypophysectomised, (Little, Smith, Jessiman, Selenkow, Van't Hoff, Eglin and Moore (1958)), or have Addison's disease, (Knowlton, Mudge and Jailer (1949)). Further evidence was provided by Cassmer (1959), who found that following separation of the foetus from the placenta in the uterus during induction of therapeutic abortion there was a fall in oestrogen excretion, thus establishing that a foetal-placental circulation is necessary to maintain the raised maternal oestriol excretion.

Diczfalusy and his co-workers, (Wilson, Eriksson and Diczfalusy (1964) and Bolté, Mancuso, Eriksson, Wiqvist and Diczfalusy (1964)) have described the synthesis of oestrogens in pregnancy. This involves the synthesis of pregnenolone and progesterone, which are C-21 steroids, in the placenta and their conversion to C-19 neutral steroids, chiefly in the form of dehydroepiandrosterone sulphate, which takes place in the foetus. The foetus provides the main source of 17-20 desmolase, which is necessary to split off the side chain of the C-21 steroids, to convert them to C-19 steroids, and to sulphurylate them.

The foetus is also essential for the 16α hydroxylation of dehydroepiandrosterone, (Villee, Engel, Loring and Villee (1961)). The 16-hydroxylase is present in the foetal liver. 16α-hydroxy-dehydroepiandrosterone was isolated from foetal cord-blood and was converted to oestriol by perfusion through placental tissue by Bolté and his co-workers (1964), demonstrating the completion of oestriol synthesis by hydrolysis and aromatisation in the placenta. The low maternal urinary oestriol excretion in pregnancies with an anencephalic foetus, where the adrenal cortex is atrophic, provides evidence of the essential role of the foetal adrenal in oestrogen production. Small amounts of oestrogens are produced by chemical pathways other than the main one described. The placenta is able to convert androgens, such as dehydroiso-androsterone to androstenedione and thence to oestrone and oestradiol. These do not act as oestriol precursors in the placenta because of the absence of the enzyme 16α-hydroxylase.

It has been shown by Klausner and Ryan (1964) that there is a gradient for free and total blood oestriol levels, which is highest at the intervillous spaces of the placenta and descends to the maternal arm vein. This supports the theory that oestriol is transferred to the mother from the placenta. Oestriol is quickly removed from the maternal circulation by the kidney. A high renal clearance for oestriol as opposed to oestrone and oestradiol-17β has been demonstrated by Brown, Saffan, Howard and Preedy (1964) and Sandberg and Slaumthwaite (1965). Thus the determinations of maternal 24-hour

excretion of oestriol in the urine provides a means of assessing the function of both foetus and placenta.

Amniotic fluid oestrogen concentration has been investigated more recently by Diczfalusy and Magnusson (1958), whose results suggest that it is related to foetal oestriol metabolism as a result of part of the oestriol circulating between the foetus and placenta being conjugated in the foetal liver and then partially excreted into the amniotic fluid with the foetal urine. Troen, Nilsson, Wiqvist and Diczfalusy (1961) found that the conjugates of oestriol in the amniotic fluid at term correlate with those in the newborn infant's urine. Diczfalusy, Tillinger, Wiqvist, Levitz, Condon and Dancis (1963) and Katz, Dancis and Levitz (1965) studied the passage of oestriol through the membranes, and found that free oestriol passes through readily. The sulphate is hydrolysed by the membranes and then transferred through the amnion and chorion, but oestriol glucosiduronate cannot be hydrolysed, so it passes only slowly through the membranes. According to Klopper (1972), 55 per cent of the oestriol in the amniotic fluid is glucosiduronate, 35 per cent is in the form of oestriol sulphate, and 10 per cent is free oestriol. Berman, Kalchman, Chattoraj and Scommegna (1968) found a significant correlation ($r = 0.69$) between maternal urinary oestriol excretion and oestriol concentration in the amniotic fluid in the 24 patients they studied. Klopper (1972) reported a similar correlation ($r = 0.72$), but Michie and Livingstone (1969) could not confirm this. They investigated 22 normal patients and found the correlation to be $r = 0.301$. Bolognese, Corson,

Touchstone and Lakoff (1971), with 20 cases, found a generally positive correlation ($p < 0.05$), but could show no close statistical relationship. Oestriol concentrations are estimated in preference to the total amount of oestriol in the amniotic fluid. When amniotic fluid volumes were determined to give the total oestriol content of the amniotic fluid, by Berman and his co-workers (1968) no advantage in accuracy was achieved. They found that the correlation between amniotic fluid oestriol concentration and maternal urinary oestriol ($r = 0.69$) was more significant than that between the total amniotic fluid oestriol and the maternal urinary oestriol ($r = 0.51$) and there was a complete lack of correlation between amniotic fluid volume and its oestriol concentration. Although amniotic fluid oestriol concentration has been shown to rise during pregnancy, most notably in the last four weeks, the range of values in normal pregnancies at any one stage of gestation show a wide variation. Biggs and Klopper (1969) observed that day-to-day and hour-to-hour variation in the same patient are relatively small.

Maternal urinary oestriol levels are a useful and widely used guide to foetal well being but there are several drawbacks to its determination and interpretation. It is inconvenient for ambulatory patients to collect 24-hour specimens of urine and it is possible that what is thought to be a 24-hour specimen is in fact incomplete. There is considerable variation of normal values at any one period of gestation between individual patients and there may be as much as 50 per cent variation from one day to another in the same pregnancy,

(Frandsen, (1953)), so a series of values from any one patient over a period of time is necessary for optimal interpretation. Furthermore, in pathological conditions involving the maternal kidney such as toxaemia and pyelonephritis, when renal function may be impaired, and in rhesus iso-immunisation, the maternal 24-hour urinary oestriol excretion may not be a true reflection of foetal-placental function, (Roy, Harkness and Kerr (1963), Klopper and Stephenson (1966), Berman, Kalchman, Chattoraj and Scommegna (1968)).

Urinary oestriol estimations were carried out by the method of Brown (1955), modified by Brown, Bulbrook and Greenwood (1957) and accepted as the standard method until increased demand led to the development of less specific but more rapid methods. The method of Oakey, Bradshaw, Eccles, Stitch and Heys (1967) measures the ether soluble Kober chromogen excretion in acid-hydrolysed pregnancy urine and it is claimed that one technician can make 15 estimations per day. Brown, MacLeod, MacNaughtan, Smith and Smyth (1968) developed a semi-automatic method, also dependent on the Kober-Ittrich colour reaction, whereby one technician can make 24 estimations per day. The results are expressed in terms of oestriol because an oestriol standard is used but it is a mixture of oestrogens which is measured. Fully automated methods are now in use, by which 120 samples can be analysed per day. (Barnard and Logan (1970), Campbell and Gardner (1971), Hainsworth and Hall (1971), Craig, Leek and Palmer (1973)). These have followed the work of Strickler, Holt, Acevedo, Saier and Grower (1967) who first described a fully automated fluorimetric method.

Amniotic fluid oestriol concentrations have been suggested as an alternative to maternal urinary oestriol values as a means of monitoring foetal well-being, by the following authors; Schindler and Herrmann (1966), Berman, Kalchman, Chattoraj and Scommegna (1968), Aleem, Pinkerton and Neill(1969), Michie and Livingstone (1969), Klopper and Biggs (1970), Bolognese, Corson, Touchstone and Lakoff (1971) and Montserrat de M., Alonso and Alba (1972). Their estimations were made by gas chromatography except for those of Michie and Livingstone (1969) who used the standard method for urine analysis as modified by Brown, Bulbrook and Greenwood (1957), and of Montserrat de M., Alonso and Alba (1972) who used the semi-automatic method of Brown and his co-workers (1968). These authors agree fairly closely about the range and averages of amniotic fluid concentrations in normal pregnancies. They do not all agree that there is a significant correlation between maternal urinary oestriol and amniotic fluid oestriol concentration. However, the numbers of cases studied so far are too few on which to base any final conclusion and the large day-to-day variation in maternal urinary oestriol excretion could contribute to the discrepancies. Although the factors regulating the two fluids may not be completely understood, and may be different in each case, it is generally agreed that they both derive their oestriol from the foetal-placental unit. There is little information available yet on oestriol values in amniotic fluid in abnormal pregnancy although some studies have been made particularly with regard to rhesus isoimmunisation, (Schindler, Ratanasopa, Lee and Herrmann (1967), Berman, Kalchman,

Chattoraj, and Scommegna (1968), Aleem, Pinkerton and Neill (1969), Michie and Robertson (1971), Montserrat, Alonso and Alba (1972)). The oestriol composition of amniotic fluid and foetal urine in the later weeks of pregnancy is so similar that estimations of oestriol concentration from amniotic fluid probably reflect the excretion from the foetal kidneys, (Troen, Nilsson, Wiqvist and Diczfalusy (1961)).

It was shown by Biggs (1969) that storage of amniotic fluid at $4^{\circ}C$ and at $-16^{\circ}C$ for a period of nine weeks, without preservative, has no appreciable effect on the oestriol concentration. Similar results for the effects of storage at $-10^{\circ}C$ on urinary oestriol had already been reported by Leon, Bulbrook and Greenwood (1959). This allows specimens to be stored and transported under refrigeration, and oestriol estimations to be made in batches at convenient times.

CHAPTER 2

MATERIALS AND METHODS

One hundred and eight specimens of amniotic fluid were obtained from 93 patients. It was obtained from patients who were having amniocentesis either for determination of optical density because they had rhesus antibodies, or for estimation of foetal maturity by Nile Blue Sulphate staining for fatty cells and creatinine concentration. Amniotic fluid was also obtained at elective caesarean section. One specimen of amniotic fluid was taken from an 11 week pregnancy terminated by hysterectomy.

In as many cases as possible, 59 out of the 108, a 24-hour urine specimen was collected within 48 hours of amniocentesis or before caesarean section, for estimation of the maternal 24-hour oestriol excretion. The volume was measured and an aliquot deep frozen until oestriol estimations were carried out.

The amniotic fluid specimens were either sent straight to the laboratory for processing or kept in a refrigerator at approximately $4^{\circ}C$, until required. The specimens were then centrifuged at 2,000 r.p.m. for five minutes. The supernatant was poured off and frozen to await oestriol estimation which was done in batches of 20-30 specimens at a time. The deposit, which contained the cell content of the amniotic fluid, was spread on three glass slides, using a Pasteur pipette, and fixed immediately to avoid drying, with carbowax, acetic acid and methanol fixative, in exactly the same way as for cervical smears. At least 15 minutes were allowed for fixation. Two of the slides were

stained by Papanicolaou's (1942) method in a 24 station Columbia Hendrey, Type E 7751 staining machine. The third slide was kept unstained as a spare until the project was nearly completed. Later it was decided to stain this by the Harris-Shorr (trichrome) method, (Pundel (1952), Osmond-Clarke and Murray (1958)), which some workers consider gives better differential cytoplasmic staining than Papanicolaou's method. It was then compared with the Papanicolaou preparation in order to make a critical assessment of the work of the authors who prefer the Harris-Shorr stain.

To confirm that there was no significant change in the cell nuclei if the fluid was kept under refrigeration, two specimens of amniotic fluid, one from a female foetus and one from a male, were kept for four days in a domestic type refrigerator at $4^{\circ}C$. Two Papanicolaou-stained slides were made from the cell deposit on each day. After making the slides, the supernatant amniotic fluid was poured back onto the cell deposit and returned to the refrigerator until the next day. Estimations of the karyopyknotic index were made from slides prepared on each of the four days.

2.1 Amniocentesis

This procedure was carried out in the antenatal clinic in the ward or theatre. The standard technique was used as described by Gordon (1969). No cases of infection, accidental haemorrhage, or premature labour occured following amniocentesis. The possibility of minor placental haemorrhage, as a result of which foetal blood from a

rhesus positive foetus may be transferred to a rhesus negative mother, initiating rhesus iso-immunisation in the current pregnancy, presents a possible serious complication of amniocentesis, (Peddle (1968)). Amniotic fluid was only obtained by antenatal amniocentesis for this investigation when it was being performed as a necessary diagnostic procedure. Amniocentesis at elective caesarean section was carried out after the abdomen had been opened, by needle aspiration through the lower segment.

2.2 Karyopyknotic Index

The karyopyknotic index was calculated by counting 300 superficial and intermediate type cells from each of the two Papanicolaou-stained slides. Of the 300 cells counted on each slide each 100 was taken from a different area of the slide. The mean of the percentages of superficial cells from the two slides gave the final figure for the karyopyknotic index for that specimen. Anucleate cells and the small, round or oval cells which probably originate from the amnion, and the long flask shaped cells when seen, were not counted. In order to minimise subjective error a micrometric eyepiece was used when assessing the cell nuclei for pyknosis. This eyepiece had divisions equivalent to 3.3μ. One and a half divisions was therefore equivalent to 4.95μ. This was used in a x 10 eyepiece, with a x 40 objective on a Wild M30 microscope. Superficial cells were recorded as having pyknotic nuclei if their longest diameter occupied one and a half divisions of the micrometer or less, and if their chromatin was uniformly hyperchromatic and without any visible chromatin structure.

As most of the nuclei were uniformly round, any error arising from not taking the average diameter of the nucleus was considered negligible. The cell counts were made when no more than the patient's name and number were known so that the karyopyknotic index could not be influenced by knowledge of the time of gestation or oestrogen concentration in the amniotic fluid or urine.

2.3 Clinical Details

After delivery of the patient, details of the last normal menstrual period, date of delivery and the infant's sex and birth weight were recorded. Weeks of gestation were counted to the nearest number of completed weeks from the first day of the last menstrual period. Five cases, where the date of the last menstrual period was unknown, were excluded from the correlation of karyopyknotic index and oestrogen values with length of gestation. Birth weights were compared with the tables of average birth weights at different weeks of gestation, published by Thompson, Billewicz and Hytten (1968). Any infant with a birth weight below the fifth percentile for its sex and maturity was assessed to find a reason for the low birth weight; for example, maternal hypertension. This was defined as 140/90 or more, recorded on at least two occasions before 28 weeks. If there was nothing abnormal about the pregnancy the recordings of clinical, radiological, cytological and biochemical assessments of maturity were checked for correlation with the stage of gestation as calculated from the last menstrual period.

2.4 Preliminary Study

A preliminary study was carried out to see if there was a correlation between the oestrogen concentration in the amniotic fluid and the karyopyknotic index of the amniotic fluid cells. Fifty seven specimens of amniotic fluid were obtained for this purpose, and from 29 of these patients, 24-hour specimens of urine were collected within 48 hours of amniocentesis or within the 48 hours before caesarean section. The same clinical and laboratory methods were used as have been described for the rest of the project. A preliminary analysis of the results showed a fall in the karyopyknotic index with advancing gestation as the amniotic fluid oestrogen concentration increased. Although this inverse relationship was not the one that was expected, it was considered to be of sufficient importance to merit completion of the study by obtaining further specimens to bring the total to approximately 100.

The preliminary analysis had drawn attention to the possibility that specimens of amniotic fluid from pregnancies with female foetuses might have a more significant correlation between the oestrogen concentration and karyopyknotic index than the specimens from male foetuses. Therefore in the second half of the series attention was paid to the cytological difference between the specimens from pregnancies with male and with female foetuses and to the sources of the different cell types in the amniotic fluid.

2.5 Maternal Urinary Oestriol

Because of the closer correlation between the amniotic fluid oestrogen concentration and karyopyknotic index with female than with male foetuses, a larger number of maternal urinary oestriol results than were available from this series was obtained from records at the Institute of Obstetrics at Hammersmith Hospital. These results were divided into those from patients with male and with female foetuses at the same stage of gestation and compared for a significant sex difference in maternal urinary oestriol excretion.

2.6 Determination of Foetal Sex

Twenty four of the amniotic fluid specimens were examined for determination of foetal sex, before this was known. One specimen was of 26 weeks gestation, the remaining 23 were between 32 and 41 weeks gestation. Counts of 200 squamous cells were made, including large anucleate forms but excluding the 'polygonal' cells from the vernix which usually appear in clumps and do not take up the stain and also excluding the small 'parabasal' type cells of amniotic origin. The percentage of cells with cyanophilic cytoplasm was recorded. This count was made first from the Papanicolaou stained preparation, and then repeated from the Harris-Shorr stained slide for comparison. The cells counted were taken from areas of the slide where the cells were well spread out since thick clumps of cells do not give consistent results of differential cytoplasmic staining.

Total cell counts were made on a further 38 specimens of amniotic fluid, before the sex of the foetus was known, to determine

whether there was a significantly higher concentration of cells in amniotic fluids from female foetuses than from male foetuses. After gently shaking the fluid to distribute the cells, two drops were put in a Neubauer counting chamber. The cells in all the nine large squares were counted in both sides of the chamber, the result giving the number of cells per cu.mm. The pH of the fluid was also measured in a Radiometer 28 pH meter.

Slides were made of the centrifuged cell deposit of 24 of these specimens and stained by Papanicolaou's method. Counts of the percentage of cyanophilic foetal squamous cells were made before the sex of the foetus was known, as a further test of the reliability of this method of determination of foetal sex.

2.7 Identification of the Sources of Amniotic Fluid Cells

The source of the cells in the amniotic fluid was investigated by comparison with cells scraped from the buccal mucosa, axilla and vaginal introitus of newborn infants. Cell preparations from specimens of urine from male and female infants, collected within 24 hours of birth, were made by means of a Shandon Elliott Cytocentrifuge. Scrapings were also taken from the amniotic surface of a freshly delivered placenta, both from the amnion over the placenta and from the free amniotic membrane, and from the umbilical cord. These specimens were stained by Papanicolaou's method. Histological sections were made of amnion and umbilical cord from a full term placenta. The sections were stained with haematoxylin and eosin, and with Papanicolaou's stain and examined to confirm the morphology of the surface cells. The cells from the infant's surface epithelia and the

surface cells. The cells from the infant's surface epithelia and urine and from the amnion were examined microscopically and their morphology compared with that of the cells seen in the amniotic fluid preparations.

A further series of buccal smears was taken from ten male and ten female infants within 24 hours of delivery and vulval smears were made from the ten female infants. Two slides were made from each site and stained by the Papanicolaou (1942) method. The same method of fixation was used for all the cytological specimens as had been used for the amniotic fluid cytology. Counts of 200 cells were made from all these specimens to determine the karyopyknotic index and the percentage of squamous cells with cyanophilic cytoplasm was determined from a count of 200 cells from each smear.

Photographs were taken with a Leitz 35 mm photomicrographic camera using a Leitz Orthoplan microscope and Kodachrome II film with a Wratton 80A filter. One set of colour prints were made from the transparencies by Kodak Limited. (Owing to an industrial dispute further copies had to be obtained from another firm who were unable to match the quality of the Kodak prints.)

2.8 Histochemistry

To determine the substance responsible for the basophilia of intermediate squamous cells when stained by the Papanicolaou method tests were carried out on amniotic fluid cells from female foetuses and on vulval smears from newborn female infants. These were fixed with the same methanol, acetic acid and carbowax fixative used throughout this study. Methyl green and pyronin stain, buffered at pH 4.8 was used to demonstrate the presence of ribonucleic acid. Other slides

were treated with ribonuclease at 40° for two hours to remove the ribonucleic acid and were then stained by the Papanicolaou method to see if the removal of ribonucleic acid reduced the percentage of cyanophilic cells. One control slide was stained by the Papanicolaou method without ribonuclease treatment. Another control slide was stained with methyl green and pyronin after ribonuclease treatment to confirm the removal of ribonucleic acid. Periodic acid-Schiff (PAS) staining was also applied to amniotic fluid cells and vulval smears, with and without digestion by saliva.

2.9 Estimation of Oestrogens

The frozen specimens of amniotic fluid and urine were sent to Searle Scientific Services for estimation according to the following methods.

The rapid method of estimating oestrogens in non-pregnancy urine of Brown, MacLeod, MacNaughtan, Smith and Smyth (1968) was used for determining amniotic fluid levels. This involves the use of a semi-automatic extractor, which processes 12 samples at a time to a stage where total oestrogens can be measured fluorimetrically using the Kober-Ittrich reaction. The oestrogens in pregnancy urine were estimated using the fully automated method of Craig, Leek and Palmer (1973).

2.9.1 Extraction of Oestrogens from Amniotic Fluid

The hydrolysis is carried out after checking the specimen for protein and glucose by diluting 1 ml with 6 ml of 16% HCl and

heating in an autoclave at 15 lbs pressure for 15 minutes (120°C). The urine is then cooled and transferred to the semi-automatic extractor. 1 g of sodium chloride is added to improve the extraction of oestriol and the sample is rocked mechanically until it is dissolved. 6 ml of ether is added and, after extraction, the lower aqueous layer is discarded. 2 ml carbonate buffer solution is added to remove the acidic fraction which is also discarded. 6 ml of petroleum ether (b.p. 40-60°) is added to improve the extraction of oestrone and the phenolic steroids in this mixture are extracted into 6 ml of N sodium hydroxide. The upper layer containing the organic fraction is discarded. This leaves the alkali fraction to which 0.8 g of sodium bicarbonate is added and dissolved by shaking. The oestrogens are extracted with 6 ml of ether and the aqueous (carbonate) layer is discarded. The ether layer is poured into another tube with an anti-bumping granule and evaporated to dryness at 60°C.

A standard containing 100 ng oestriol in ethanol is evaporated to dryness and subjected to fluorimetry. To the dry residue is added 1 ml of 66% Kober reagent, followed by 1 mg of powdered hydroquinone and this is heated to 125°C in an oil bath for 10 minutes. After cooling in ice, 1.5 ml of deionised water is added to the contents, mixed, and poured into a fluorimeter tube containing 1.75 ml Ittrich reagent. The fluorescence is extracted by vigorous shaking and the layers separated by centrifugation at 2,000 r.p.m. for five minutes at -4°C.

Fluorimetry is performed using an Aminco Bowman spectrophotofluorimeter with a mercury-xenon lamp. The wavelengths used are:-

(a) Excitation 546 nm Emission 565 nm

(b) Excitation 490 nm Emission 520 nm.

Fluorescence due to oestrogen is calculated by the formula:-

$$\text{Corrected fluorescence} = F_{546/565} - K \times F_{490/520}$$

(Corrected fluorescence is corrected for fluorescent substances other than oestrogens).

The oestrogen content is calculated by direct comparison with the corrected fluorescence reading of the oestriol standard.

The oestrogens measured by the method described are oestriol, oestrone, and oestradiol, together with minor Kober-chromogenic and alkali stable oestrogens. It is acknowledged that some specificity is lost in this method. The recovery of added oestriol from non-pregnancy urine is 70-74 per cent, and the coefficient of variation between batches for daily quality controls is of the order of 10 per cent.

2.9.2 Automated Method for the Determination of Oestrogens in Pregnancy Urine

The method is based on a simplified Auto Analyser technique, which has been developed and evaluated at Searle Scientific Services by Craig, Leek and Palmer (1973). They have compared the results of 2,500 samples analysed by their automated method with the results using the colorimetric method of Brown, MacLeod, MacNaughtan, Smith and Smyth (1968), and found a correlation coefficient of 0.912 between

the methods. Slightly higher values were obtained with the automated method which they attribute to the better recovery of the automated procedure and the measurement of oestrogens other than oestriol.

REAGENTS USED:

Kober reagent	66 per cent v/v sulphuric acid containing 2 per cent quinol.
Ittrich reagent	p - nitrophenol in tetrachlorethane.
Extraction reagent	5 per cent w/v trichloracetic acid in chloroform.
Standards	10, 20, 30 and 40 mg/l oestriol standards in water prepared from a stock solution of 1 mg/ml ethanol.

APPARATUS:

This is illustrated in Figure 1 (page 48).

The oil bath containing a standard 40 foot Technicon coil is set at a temperature of 120°C. The fluorimeter used is a Locarte Mark IV, Model LFM/5, equipped with a mercury light source. The excitation wavelength of 533 nm is obtained with a Balzer filter and the monochromator in the emission path is set at 580 nm and includes a 575 nm cut-off filter. The flow cell supplied with this fluorimeter proved unsatisfactory and a U tube of pyrex glass was substituted.

The fluorimeter response was measured with a Leeds and Northrup 10 mV Speedomax recorder. All other items of equipment were standard Technicon parts.

METHOD:

The Kober reagent, water and extraction reagent are connected, and pumping commenced about 30 minutes prior to beginning the run, to allow sufficient time for the system to equilibrate. The run is commenced with a 40 mg/l standard, followed by the standard curve. Each sixth sample cup contains a urine control prepared daily from freeze dried urine and is used for identification and quality control purposes. All urine specimens are checked for the presence of glucose which interferes with the oestrogen determination. Glucose is removed by a modification of the method of Bradlow (1968) for the extraction of steroid conjugates. The oestrogens are extracted by applying 5 ml of urine to a 10 mm diameter column containing 5 g of Amberlite XAD-2 resin and washing the column with sufficient water until the eluate is glucose-free (usually 20-30 ml). The conjugates are eluted with 20 ml methanol and after removal of the solvent the residue is reconstituted to the original volume with water.

A mean recovery of 95 per cent of oestriol and of oestriol -16α monoglucuronide added to male urine is obtained by this method, compared with 75 per cent using the Brown method at the same laboratory. The coefficient of variation for within-batch analysis of a urine sample is 1.98 per cent at a concentration of 23 mg/l, using the Auto Analyser technique, and between batches, 4.3 per cent for the daily control.

Figure 2 (page 49) illustrates the normal range using this automated procedure, as determined by Craig, Leek and Palmer (1973) from 2,500 samples.

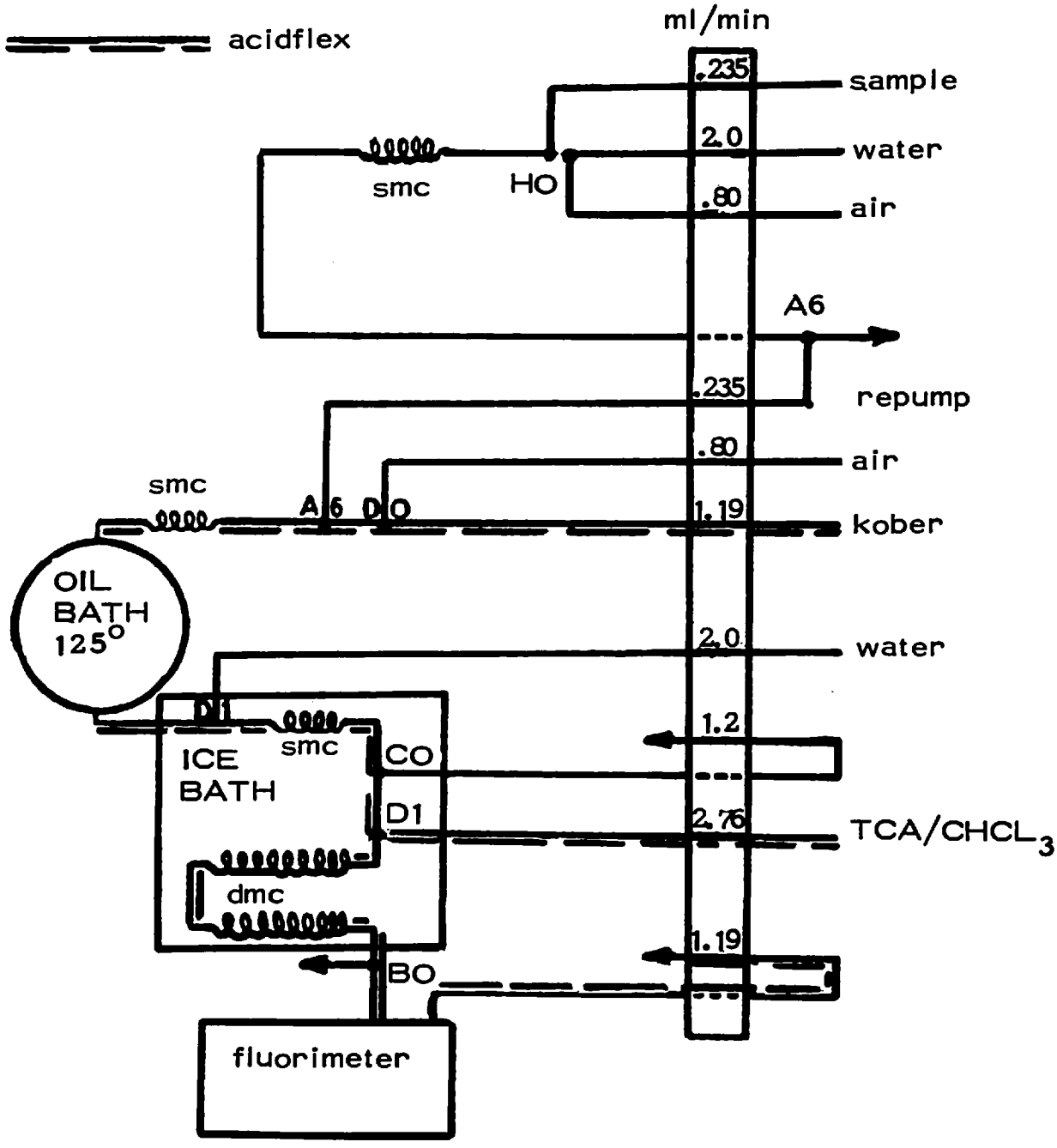


Figure 1. Flow diagram of the Auto Analyser method of determination of œstrogens in pregnancy urine. (Craig, Leek and Palmer, 1973)

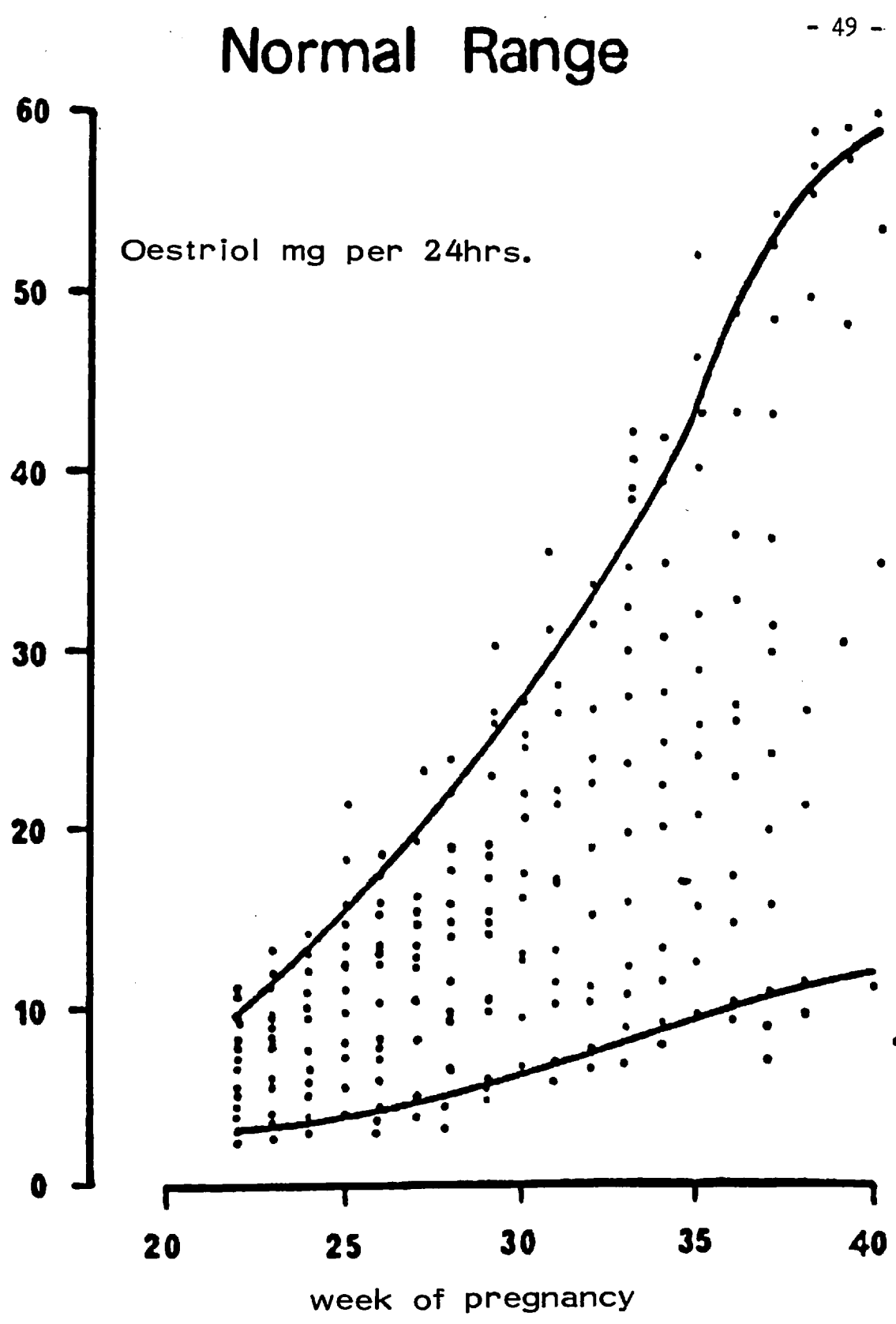


Figure 2. Normal range of pregnancy urine œstriol results Using the Auto Analyser method.
(Craig, Leek and Palmer, 1973)

CHAPTER 3

RESULTS

TABLE I

RESULTS IN CHRONOLOGICAL ORDER

Specimen Number	Normal Pregnancy or Complications	Gestation in Weeks	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	Sex of Foetus
1	Normal	39	7.0	43.5	7.6	F
2	Normal	40	17.5	59.0	-	M
3	Normal	40	25.0	29.2	8.3	F
4	Normal	38	23.0	11.8	17.0	F
5	Normal	40	9.0	70.2	-	F
6	Normal	37	34.0	38.4	-	M
7	Normal	40	C	42.2	9.3	M
8	Rhesus Antibodies	32	42.0	9.8	8.2	M
9	Normal	39	14.5	32.0	11.6	M
10	Normal	39	13.0	85.8	21.6	M
11	Normal	38	18.0	38.4	-	F
12	Normal	42	27.5	12.0	9.3	F
13	Diabetes	37	38.0	16.0	23.3	F
14	Normal	41	47.0	56.0	-	M
15	Diabetes	33	46.0	16.4	25.4	F
16	Hypertension	38	34.5	64.2	-	M
18	Normal	35	36.6	18.8	-	M
19	Normal	41	C	28.2	15.4	M
20	Diabetes	39	22.0	10.6	-	F
21	Rhesus Antibodies	32	C	285.8	-	M
22	Hypertension Hydramnios	36	29.5	21.0	17.4	F

C	Amniotic fluid too heavily contaminated with blood for accurate K.P.I. estimation.
K.P.I.	Karyopyknotic Index.
N.D.	Not determined.
?	Date of last menstrual period unknown.

Numbers 17, 43, 49 and 55 are not recorded because after collection of 24-hour urine, amniotic fluid could not be obtained at elective caesarean section.

TABLE I

TABLE I (continued)

Specimen Number	Normal Pregnancy or Complications	Gestation in Weeks	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	Sex of Foetus
23	Rhesus Antibodies	33	52.0	8.2	7.5	M
24	Diabetes	38	34.5	19.2	-	F
25	Normal	42	23.0	28.8	-	F
26	Normal	41	28.5	50.0	-	M
27	Twins	26	60.5	6.6	-	Mx2
28	Rhesus Antibodies	35	41.0	14.2	-	F
29	Rhesus Antibodies	33	35.0	7.6	-	F
30	Rhesus Antibodies	36	49.5	12.0	-	M
31	Normal	38	23.0	21.2	17.5	F
32	Normal	40	17.6	28.6	16.6	F
33	Normal	40	13.0	60.0	16.8	F
34	Normal	39	13.0	30.4	12.4	F
35	Rhesus Antibodies	38	54.5	14.4	-	M
36	Normal	39	25.0	30.4	14.8	F
37	Rhesus Antibodies	37	22.3	10.8	-	F
38	Rhesus Antibodies	37	39.0	5.8	-	M
39	Rhesus Antibodies	25	41.0	4.8	-	M
40	Rhesus Antibodies	38	34.0	47.6	24.3	M
41	Normal	39	47.7	88.6	26.3	M
42	Rhesus Antibodies	40	44.0	26.8	12.3	M
44	Hypertension	40	21.0	54.4	10.3	M
45	Normal	39	29.8	28.0	6.9	M

TABLE I (continued)

TABLE I (continued)

Specimen Number	Normal Pregnancy or Complications	Gestation in Weeks	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	Sex of Foetus
46	Rhesus Antibodies	29	21.6	5.2	9.6	M
47	Rhesus Antibodies	31	27.0	13.2	15.9	F
48	Rhesus Antibodies	36	22.6	21.2	-	F
50	Normal	38	21.9	16.6	-	F
51	Rhesus Antibodies	34	33.3	17.4	-	M
52	Rhesus Antibodies	35	26.8	28.0	-	F
53	Normal	39	C	87.8	18.6	M
54	Normal	42	5.6	60.8	-	F
56	Rhesus Antibodies	34	20.6	9.8	-	M
57	Normal	39	23.6	201.4	29.8	M
58	Rhesus Antibodies	34	24.6	23.0	18.0	M
59	Normal	40	17.4	63.8	15.2	F
60	Rhesus Antibodies	31	25.1	20.0	-	M
61	Rhesus Antibodies	30	13.9	27.0	-	F
62	Normal	38	21.9	12.4	18.6	M
63	Normal	37	16.4	10.1	16.8	F
64	Normal	38	14.0	32.1	16.7	F
65	Normal	39	14.6	24.4	11.8	F
66	Normal	38	15.0	64.7	9.9	M
67	Achondroplasia	38	9.4	35.8	24.9	F
68	Normal	39	17.3	18.6	8.5	M
69	Normal	42	9.0	16.9	33.5	M

TABLE I (continued)

TABLE I (continued)

Specimen Number	Normal Pregnancy or Complications	Gestation in Weeks	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	Sex of Foetus
70	Normal	40	25.6	43.7	28.5	M
71	Termination	11	-	0.4	-	N.D.
72	Normal	41	23.0	94.0	-	M
73	Normal	36	6.8	19.9	17.4	F
74	Anencephalic	36	25.8	3.0	3.2	F
75	Normal	38	20.7	9.9	13.8	F
76	Diabetes	37	14.4	12.4	-	M
77	Rhesus Antibodies	34	14.8	8.0	-	M
78	Normal	41	15.6	10.6	-	M
79	Hypertension	40	10.1	8.4	14.8	F
80	Rhesus Antibodies	37	5.3	13.2	-	M
81	Normal	39	3.8	25.5	22.3	F
82	Rhesus Antibodies	32	C	3.2	-	F
83	Normal	42	4.0	47.4	11.8	M
84	Normal	38	11.0	40.0	-	F
85	Normal	40	16.1	75.3	-	M
86	Normal	36	9.8	51.9	34.0	M
87	Normal	39	11.1	25.5	20.4	M
88	Normal	39	11.0	26.4	15.0	M
89	Normal	38	11.5	28.0	-	F
90	Hydramnios	35	7.8	23.3	-	F
91	Normal	40	4.8	34.5	-	M

TABLE I (continued)

TABLE I (continued)

Specimen Number	Normal Pregnancy or Complications	Gestation in Weeks	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	Sex of Foetus
92	Normal	39	10.4	26.8	13.5	F
93	Hydramnios	39	10.1	38.6	25.2	F
94	Normal	41	5.6	41.2	-	F
95	Normal	39	7.8	45.9	18.2	F
96	Normal	40	4.3	220.9	12.0	F
97	Normal	39	5.6	78.9	20.7	M
98	Rhesus Antibodies	37	10.0	25.4	-	M
99	Rhesus Antibodies	?	13.0	12.6	11.7	M
100	Normal	34	24.3	27.2	12.4	M
101	Normal	40	20.3	6.4	17.3	M
102	Normal	?	11.1	28.9	16.2	F
103	Diabetes	37	24.3	62.2	14.8	F
104	Normal	39	20.0	24.7	19.1	M
105	Normal	41	10.6	51.5	6.8	M
106	Rhesus Antibodies	?	12.0	12.5	-	M
107	Normal	?	11.0	10.5	-	M
108	Normal	40	19.0	54.5	-	M
109	Normal	38	16.3	41.2	-	M
110	Normal	?	6.6	8.4	-	M
111	Normal	40	10.3	40.2	-	M
112	Normal	38	12.3	7.0	-	F

TABLE I (continued)

TABLE II

RESULTS ACCORDING TO GESTATIONAL AGE
MALE AND FEMALE FOETUSES SEPARATELY

Weeks of Gestation	Specimen Number	MALE FOETUSES			FEMALE FOETUSES			Abnormality
		K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	
11	71							Termination
25	39	41.0	4.8	-				Rhesus Antibodies
26	27	60.5	6.6	-				Rhesus Antibodies
29	46	21.6	5.2	9.6				Rhesus Antibodies
30	61				13.9	27.0	-	Rhesus Antibodies
31	60	25.1	20.0	-				Rhesus Antibodies
	47				27.0	13.2	15.9	Rhesus Antibodies
32	21	C	285.8	-				Rhesus Antibodies
	82				C	3.2	-	Rhesus Antibodies
	8	42.0	9.8	8.2				Rhesus Antibodies
Mean		42.0	147.8	8.2				
33	15				46.0	16.0	25.4	Diabetes
	23	52.0	8.2	7.5				Rhesus Antibodies
	29				35.0	7.6	-	Rhesus Antibodies
Mean					40.5	12.8	25.4	

K.P.I. Karyopyknotic Index.

( ) Excluded from calculation of mean.

C Amniotic fluid too heavily contaminated with blood for accurate K.P.I. estimation.

TABLE II

TABLE II (continued)

Weeks of Gestation	Specimen Number	MALE FOETUSES			FEMALE FOETUSES			Abnormality
		K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	
34	77	14.8	8.0	-				Rhesus Antibodies
	100	24.3	27.2	12.4				
	51	33.3	17.4	-				Rhesus Antibodies
	56	20.6	9.8	-				Rhesus Antibodies
	58	24.6	23.0	18.0				Rhesus Antibodies
Mean		23.5	17.1	15.2				
35	18	36.6	18.8	-				
	28				41.0	14.2	-	Rhesus Antibodies
	52				26.8	28.0	-	Rhesus Antibodies
	90				7.8	23.3	-	
Mean					25.2	21.8	-	
36	22				29.5	21.0	17.4	
	30	49.5	12.0	-				Rhesus Antibodies
	48				22.6	21.2	-	Rhesus Antibodies
	73				6.8	19.9	17.4	
	74				25.8	(3.0)	(3.2)	Anencephalic
	86	9.8	51.9	34.0				
Mean		29.6	31.9	34.0	21.2	20.7	17.4	
37	6	34.0	38.4	-				
	13				38.0	16.0	23.3	Diabetes
	37				22.3	10.8	-	Rhesus Antibodies
	38	39.0	5.8	-				Rhesus Antibodies
	63				16.4	10.1	16.8	
	76	14.4	12.4	-				Diabetes
	80	5.3	13.2	-				Rhesus Antibodies
	98	10.0	25.4	-				Rhesus Antibodies
	103				24.3	62.2	14.8	Diabetes
Mean		20.5	19.0	-	25.2	24.8	18.3	

TABLE II (continued)

TABLE II (continued)

Weeks of Gestation	Specimen Number	MALE FOETUSES			FEMALE FOETUSES			Abnormality
		K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	
38	4				23.0	11.8	17.0	
	11				18.0	38.4	-	
	16	24.5	64.2	-				
	24				34.5	19.2	-	Diabetes
	31				23.0	21.2	17.5	
	35	54.5	14.4	-				Rhesus Antibodies
	40	34.0	47.6	24.3				Rhesus Antibodies
	50				21.9	16.6	-	
	62	21.9	12.4	18.6				
	64				14.0	32.1	16.7	
	66	15.0	64.7	9.9				
	67				9.4	35.8	24.9	
	75				20.7	9.9	13.8	
	84				11.0	40.0	-	
	89				11.5	28.0	-	
	109	16.3	41.2	-				
	112				12.3	7.0	-	
Mean		29.4	40.8	17.6	18.1	23.6	18.0	
39	1				7.0	43.5	7.6	
	9	14.5	32.0	11.6				
	10	13.0	85.8	21.6				
	20				22.0	10.6	-	Diabetes
	34				13.0	30.4	12.4	
	36				25.0	30.4	14.8	
	41	47.7	88.6	26.3				
	45	29.8	28.0	6.9				
	53	C	87.8	18.6				
	57	23.6	201.4	29.8				
	65				14.6	24.4	11.8	
	68	17.3	18.6	8.5				
	81				3.8	25.5	22.3	
	87	11.1	25.5	20.4				
	88	11.0	26.4	15.0				
	92				10.4	26.8	13.5	
	93				10.1	38.6	25.2	
	95				7.8	45.9	18.2	
	97	5.6	78.9	20.7				
	104	20.0	24.7	19.1				
Mean		19.4	54.3	18.0	12.6	30.7	15.7	

TABLE II (continued)

TABLE II (continued)

Weeks of Gestation	Specimen Number	MALE FOETUSES			FEMALE FOETUSES			Abnormality
		K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	
40	2	17.5	59.0	-				
	3				25.0	29.2	8.3	
	5				9.0	70.2	-	
	7	C	42.2	9.3				
	32				17.6	28.6	16.6	
	33				13.0	60.0	16.8	
	42	44.0	26.8	12.3				Rhesus Antibodies
	44	21.0	54.4	10.3				
	59				17.4	63.8	15.2	
	70	25.6	43.7	28.5				
	79				10.1	8.4	14.8	
	85	16.1	75.3	-				
	91	4.8	34.5	-				
	96				4.3	220.9	12.0	
	101	20.3	6.4	17.3				
	108	19.0	54.5	-				
	111	10.3	40.2	-				
	Mean	19.8	43.7	15.5	13.8	68.7	13.9	
41	14	47.0	56.0	-				
	19	C	28.2	15.4				
	26	28.5	50.0	-				
	72	23.0	94.0	-				
	78	15.6	10.6	-				
	94				5.6	41.2	-	
	105	10.6	51.5	6.8				
	Mean	24.9	48.7	11.1				
42	12				27.5	12.0	9.3	
	25				23.0	28.8	-	
	54				5.6	60.8	-	
	69	9.0	16.9	33.5				
	83	4.0	47.4	11.8				
	Mean	6.5	32.1	22.6	18.7	33.8	9.3	

TABLE II (continued)

TABLE III

ABNORMAL PREGNANCIES

Specimen Number	Complication	Weeks of Gestation	K.P.I. Per Cent	Amniotic Fluid Oestrogen g/100 mls	Urinary Oestriol mg/24 hrs	Sex
39	Rhesus Antibodies	25	41.0	4.8	-	M
27	Rhesus Antibodies - Twins	26	60.5	6.6	-	Mx2
46	Rhesus Antibodies	29	21.6	5.2	9.6	M
61	Rhesus Antibodies	30	13.9	27.0	-	F
47	Rhesus Antibodies	31	27.0	13.2	15.9	F
60	Rhesus Antibodies	31	25.1	20.0	-	M
8	Rhesus Antibodies	32	42.0	9.8	8.2	M
21	Rhesus Antibodies	32	C	285.8	-	M
82	Rhesus Antibodies	32	C	3.2	-	F
23	Rhesus Antibodies	33	52.0	8.2	7.5	M
29	Rhesus Antibodies	33	35.0	7.6	-	F
51	Rhesus Antibodies	34	33.3	17.4	-	M
56	Rhesus Antibodies	34	20.6	9.8	-	M
58	Rhesus Antibodies	34	24.6	23.0	18.0	M
77	Rhesus Antibodies	34	14.8	8.0	-	M
28	Rhesus Antibodies	35	41.0	14.2	-	F
52	Rhesus Antibodies	35	26.8	28.0	-	F
30	Rhesus Antibodies	36	49.5	12.0	-	M
48	Rhesus Antibodies	36	22.6	21.2	-	F
37	Rhesus Antibodies	37	22.3	10.8	-	F
38	Rhesus Antibodies	37	39.0	5.8	-	M

K.P.I. Karyopyknotic Index.

C Amniotic fluid too heavily contaminated with blood for K.P.I. estimation.

? Date of last menstrual period not known.

TABLE III

TABLE III (continued)

Specimen Number	Complication	Weeks of Gestation	K.P.I. Per Cent	Amniotic Fluid Oestrogen µg/100 mls	Urinary Oestriol mg/24 hrs	Sex
80	Rhesus Antibodies	37	5.3	13.2	-	M
98	Rhesus Antibodies	37	10.0	25.4	-	M
35	Rhesus Antibodies	38	54.5	14.4	-	M
40	Rhesus Antibodies	38	34.0	47.6	24.3	M
42	Rhesus Antibodies	40	44.0	26.8	12.3	M
99	Rhesus Antibodies	?	13.0	12.6	11.7	M
106	Rhesus Antibodies	?	12.0	12.5	-	M
15	Diabetes	33	46.0	16.4	25.4	F
13	Diabetes	37	38.0	16.0	23.3	F
76	Diabetes	37	14.4	12.4	-	M
103	Diabetes	37	24.3	62.2	14.8	F
24	Diabetes	38	34.5	19.2	-	F
20	Diabetes	39	22.0	10.6	-	F
90	Hydramnios	35	7.8	23.3	-	F
22	Hydramnios - Hypertension	36	29.5	21.0	17.4	F
74	Anencephaly - Hydramnios	36	25.8	3.0	3.2	F
93	Hydramnios	39	10.1	38.6	25.2	F
16	Hypertension	38	34.5	64.2	-	M
44	Hypertension	40	21.0	54.4	10.3	M
79	Hypertension	40	10.1	8.4	14.8	F
67	Achondroplasia affecting mother and foetus	38	9.4	35.8	24.9	F

TABLE III (continued)

TABLE IV

Indications for Amniocentesis or Caesarean Section in 93 patients from whom 108 specimens of amniotic fluid were obtained.

Number of Specimens	Indications for Amniocentesis
28	for optical density assessment of bilirubin concentration in rhesus negative mothers with antibodies.
1	for optical density in rhesus negative mother without antibodies.
28	for foetal maturity.
1	for foetal maturity in a diabetic mother.
1	amniocentesis on mother with hydramnios and an anencephalic foetus.
	Indications for Caesarean Sections
34	for disporoportion.
6	for breech presentation.
4	in diabetics.
2	for placenta praevia.
1	after failed surgical induction at 41 weeks.
1	because of infertility and maternal age.

One specimen of amniotic fluid was obtained from a termination by hysterectomy at 11 weeks.

TABLE V

CASES WITH INFANTS' BIRTH WEIGHT BELOW THE FIFTH PERCENTILE ACCORDING TO DATA OF THOMPSON, BILLEWICZ AND HYTTEN (1968) OBTAINED FROM RECORDS OF BIRTHS IN THE CITY OF ABERDEEN.

Specimen Number	Weeks Gestation at Amniocentesis	K.P.I. Per Cent	Amniotic Fluid Oestrogens µg/100 mls	Urinary Oestriol mg/24 hrs	Birth Weight g	Weeks Gestation at Delivery	Sex	Fifth Percentile According to Sex g	Complication
12	42	27.5	9.3	12.0	2610	42	F	2700	Small for dates.
20	39	22.0	10.6	-	2380	40	F	2620	Diabetes.
46 56	29 34	21.6 20.6	5.2 9.8	9.6) -)	2090	38	M	2510	Rhesus Antibodies.
63	37	16.4	10.1	16.8	2300	39	F	2540	Small for dates.
74	36	25.8	3.0	3.2	1600	36	F	2090	Anencephaly.
79	40	10.1	8.4	14.8	2370	41	F	2680	Hypertension.
87	39	11.1	25.5	20.4	2590	40	M	2740	Small for dates.
102	38-40	11.1	28.9	16.2	2060	38-40	F	>2420	Small for dates.
104	39	20.0	24.7	19.1	2520	42	M	2840	Small for dates.

TABLE V

TABLE VI

STATISTICAL ANALYSIS. CORRELATIONS
Total 107. 48 Female and 59 Male Foetuses

Female Foetuses

	Weeks of Gestation (G)	Amniotic Fluid Oestrogens (AFO)
Amniotic Fluid Oestrogens (AFO)	r = +0.42 Significant 0.01	
Karyopyknotic Index (KPI)	r = -0.42 Significant 0.01	r = -0.43 Significant 0.01

Male Foetuses

	Weeks of Gestation (G)	Amniotic Fluid Oestrogens (AFO)
Amniotic Fluid Oestrogens (AFO)	r = +0.50 Significant 0.001	
Karyopyknotic Index (KPI)	r = -0.43 Significant 0.01	r = -0.13 Not significant

Maternal Urinary Oestriol
59 Observations. 30 Male and 29 Female Foetuses

	Weeks of Gestation (G)	Amniotic Fluid Oestrogens (AFO)
Urinary Oestriol (UO)	r = +0.04 Not significant	r = +0.20 Not significant

TABLE VII

STATISTICAL ANALYSIS. PARTIAL CORRELATIONS

Partial Correlation of KPI and AFO at a Constant Value for G		
Female foetuses	r = -0.31	Significant 0.05
Male foetuses	r = -0.15	Not significant

The difference between these is significant 0.05.

Partial Correlation of KPI and G at a Constant Value for AFO		
Female foetuses	r = -0.29	Significant 0.05
Male foetuses	r = -0.43	Significant 0.01

TABLE VIII

STATISTICAL ANALYSIS. MATERNAL URINARY OESTRIOL

Male/Female Foetus Comparison.
Data from The Institute of Obstetrics, Hammersmith Hospital

A. Comparison of 45 maternal urinary oestriol results at 38 weeks gestation.

	Male Foetuses	Female Foetuses
Number of results	19	26
Mean maternal urinary oestriol mg/24 hrs	26.84	26.23

$t = 0.225$ No significant difference.

B. Comparison of 82 maternal urinary oestriol values for 38 weeks gestation, comprising 45 estimations at 38 weeks (as in A. above) and 37 values estimated by interpolation or extrapolation from the results of patients who had more than one oestriol estimation made, although none at 38 weeks.

	Male Foetuses	Female Foetuses
Number of results	42	40
Mean maternal urinary oestriol mg/24 hrs	29.90	25.92

Difference 3.98 ± 2.83 which is not significant.

These 82 values for maternal urinary oestriol at 38 weeks gestation do not show any significant difference between male and female foetuses.

TABLE IX

Determination of Foetal Sex from the Percentage of Cyanophilic Cells in Amniotic Fluid. First Series

13 Male Foetuses				11 Female Foetuses			
Specimen Number	Weeks of Gestation	Per Cent Cyanophilic Cells		Specimen Number	Weeks of Gestation	Per Cent Cyanophilic Cells	
		Papanicolaou Stain	Harris-Shorr Stain			Papanicolaou Stain	Harris-Shorr Stain
27	26	17	14	15	33	44	42
8	32	19	17	29	33	54	53
60	32	10	9	28	35	52	48
77	34	4	3	22	36	58	55
40	37	16	20	63	37	52	48
41	39	20	18	64	38	49	48
87	39	4	4	93	39	46	51
88	39	2	2	95	39	51	64
97	39	7	8	79	40	39	37
42	40	7	12	96	40	63	68
91	40	3	2	94	41	47	45
78	41	8	7				
99	?	10	9				

Highest Male Count 20 Per Cent

Lowest Female Count 37 Per Cent

TABLE IX

TABLE X

Determination of Foetal Sex from the Percentage of Cyanophilic Cells in Amniotic Fluid. Second Series

11 Male Foetuses			13 Female Foetuses		
Specimen Number	Weeks of Gestation	Cyanophilic Cells Per Cent	Specimen Number	Weeks of Gestation	Cyanophilic Cells Per Cent
204	32	3	225	34	79
203	35	8	222	36	51
202	36	10	231	37	63
200	38	14	232	37	71
201	38	12	220	38	73
207	38	15	221	38	68
205	39	11	223	38	55
199	40	15	228	38	53
206	40	6	229	38	79
209	40	8	224	39	48
208	42	10	227	39	64
			230	39	39
			226	40	40

TABLE X

TABLE XI

KARYOPYKNOTIC INDEX (KPI) AND PERCENTAGE OF CYANOPHILIC SQUAMOUS CELLS IN BUCCAL AND VULVAL SMEARS FROM 10 MALE AND 10 FEMALE NEWBORN INFANTS.

Infant Number	Buccal Smears		Vulval Smears	
	K.P.I.	Percentage Cyanophilic Cells	K.P.I.	Percentage Cyanophilic Cells
Female				
1	2	32	2	98
2	-	44	3	97
3	2	41	1	98
4	1	50	<1	96
5	1	82	4	92
6	3	84	1	97
7	1	72	4	99
8	1	52	5	96
9	4	30	2	95
10	1	43	<1	99
Mean	1.6	53.0	2.2	96.7
Male				
11	1	45		
12	1	25		
13	2	62		
14	3	68		
15	4	40		
16	2	54		
17	2	37		
18	1	36		
19	2	42		
20	1	36		
Mean	1.7	44.5		

t = 1.14 (18 degrees of freedom) for the percentage of cyanophilic cells in buccal smears from male and female infants. This is not significant.

(A value of t = 2.11 is needed for significance at 5 per cent).

TABLE XII

Amniotic Fluid. Total Cells per cu.mm. and pH

17 Male Foetuses				21 Female Foetuses			
Specimen Number	Weeks of Gestation	Total Cells per cu. mm.	pH	Specimen Number	Weeks of Gestation	Total Cells per cu. mm.	pH
200	38	130	8.3	220	38	185	7.8
201	38	110	7.8	221	38	310	7.7
199	40	1630	7.6	222	36	130	7.5
202	36	68	7.7	223	38	99	7.7
203	35	56	7.6	224	39	220	7.8
204	36	90	8.1	225	38	190	8.0
205	39	103	8.4	226	40	530	-
206	40	180	7.7	227	40	230	-
207	38	210	7.8	228	39	350	7.5
208	42	210	7.4	229	38	240	7.7
209	40	280	7.8	230	39	190	7.5
238	39	70	7.5	231	37	325	7.6
239	29	130	8.1	232	38	145	7.9
240	36	90	-	233	40	660	7.8
242	34	470	8.1	234	40	330	-
244	33	190	7.9	235	36	500	7.5
245	38	240	7.8	236	40	230	7.4
				237	40	300	7.5
				241	42	1530	8.4
				243	36	530	7.9
				246	40	920	8.0
	MEAN	250	7.8		MEAN	383	7.7

Notes: - insufficient fluid for pH estimation.
The average cell counts are significantly (P = 0.05) higher for female foetuses.
Average female (log) cell count, 2.251. - Average male (log) cell count, 1.747 = 0.504.
Standard error of difference 0.246. (35 degrees of freedom).

TABLE XII

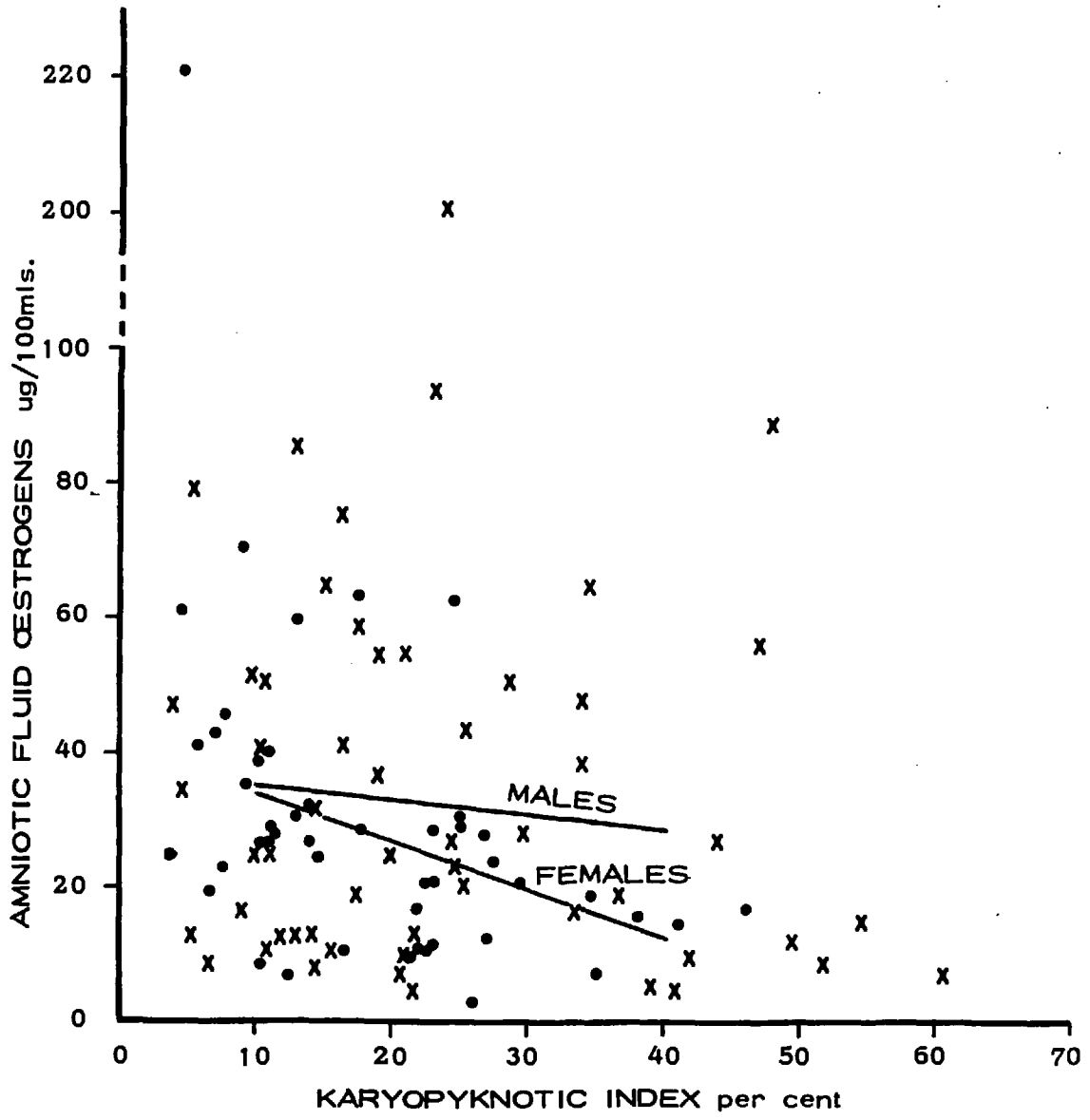


Figure 3 AMNIOTIC FLUID ŒSTROGENS and KARYOPYKNOTIC INDEX

x 55 MALE FŒTUSES r = -0.13

AFO = 37.3 - 0.22 KPI not significant

• 47 FEMALE FŒTUSES r = -0.43

AFO = 40.9 - 0.70 KPI significant 0.01

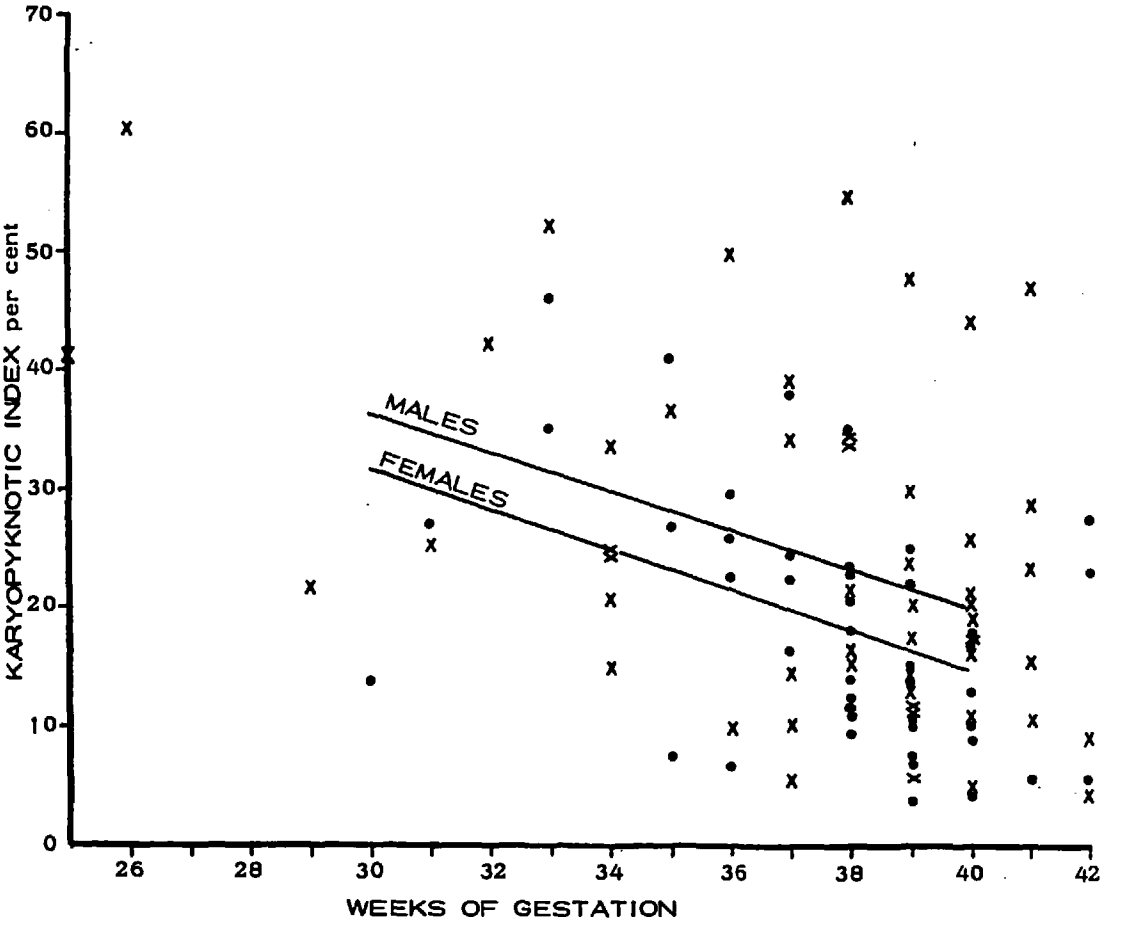


Figure 4 KARYOPYKNOTIC INDEX and WEEKS OF GESTATION

x 51 MALE FŒTUSES r = -0.43
KPI = 85.7 - 1.64G significant 0.01

• 46 FEMALE FŒTUSES r = -0.42
KPI = 81.8 - 1.67G significant 0.01

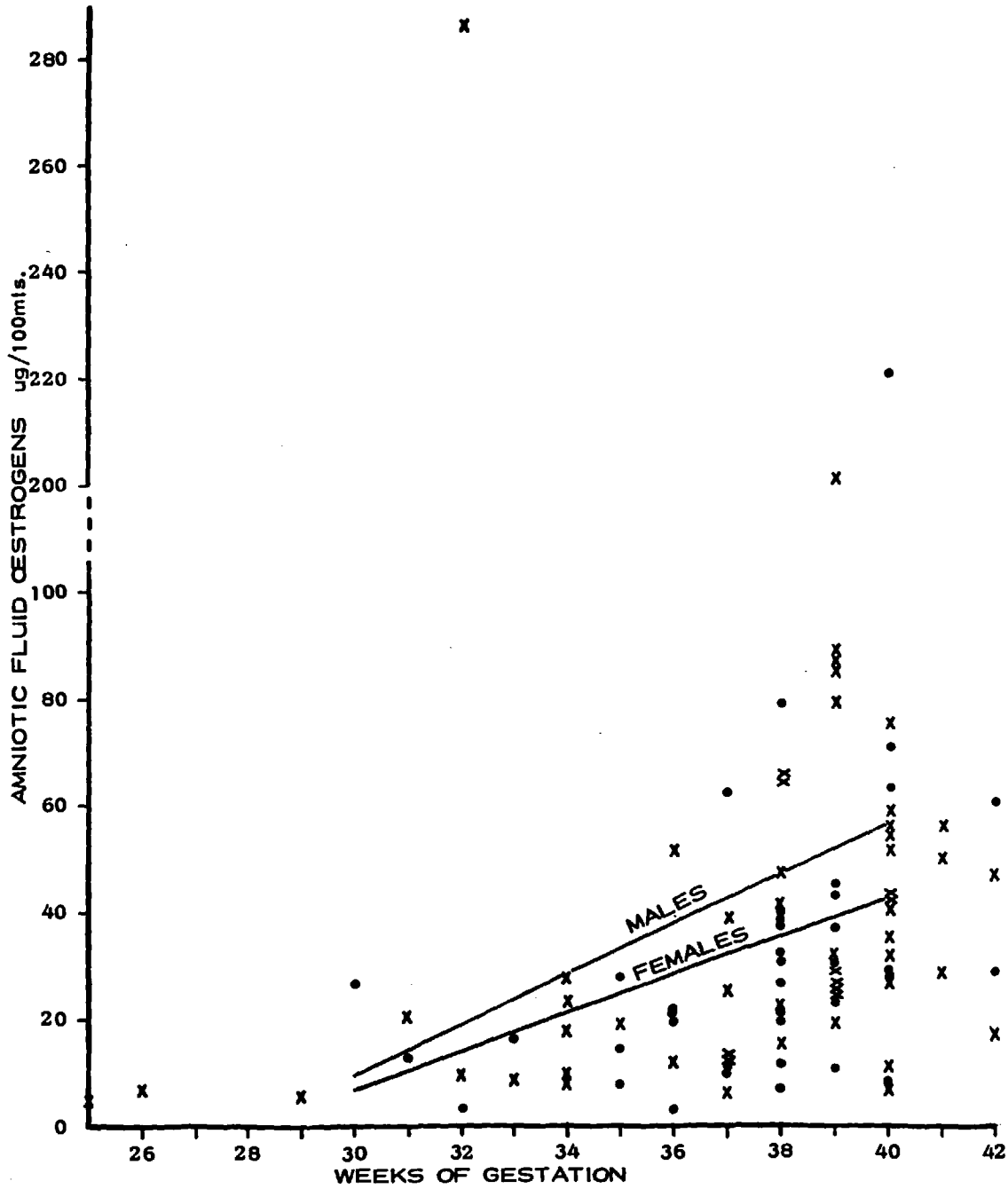


Figure 5 AMNIOTIC FLUID OESTROGENS and WEEKS OF GESTATION

x 54 MALE FŒTUSES r = +0.50 AFO = -90.1 + 3.34G
significant 0.001

• 47 FEMALE FŒTUSES r = +0.42 AFO = -69.7 + 2.58G
significant 0.01

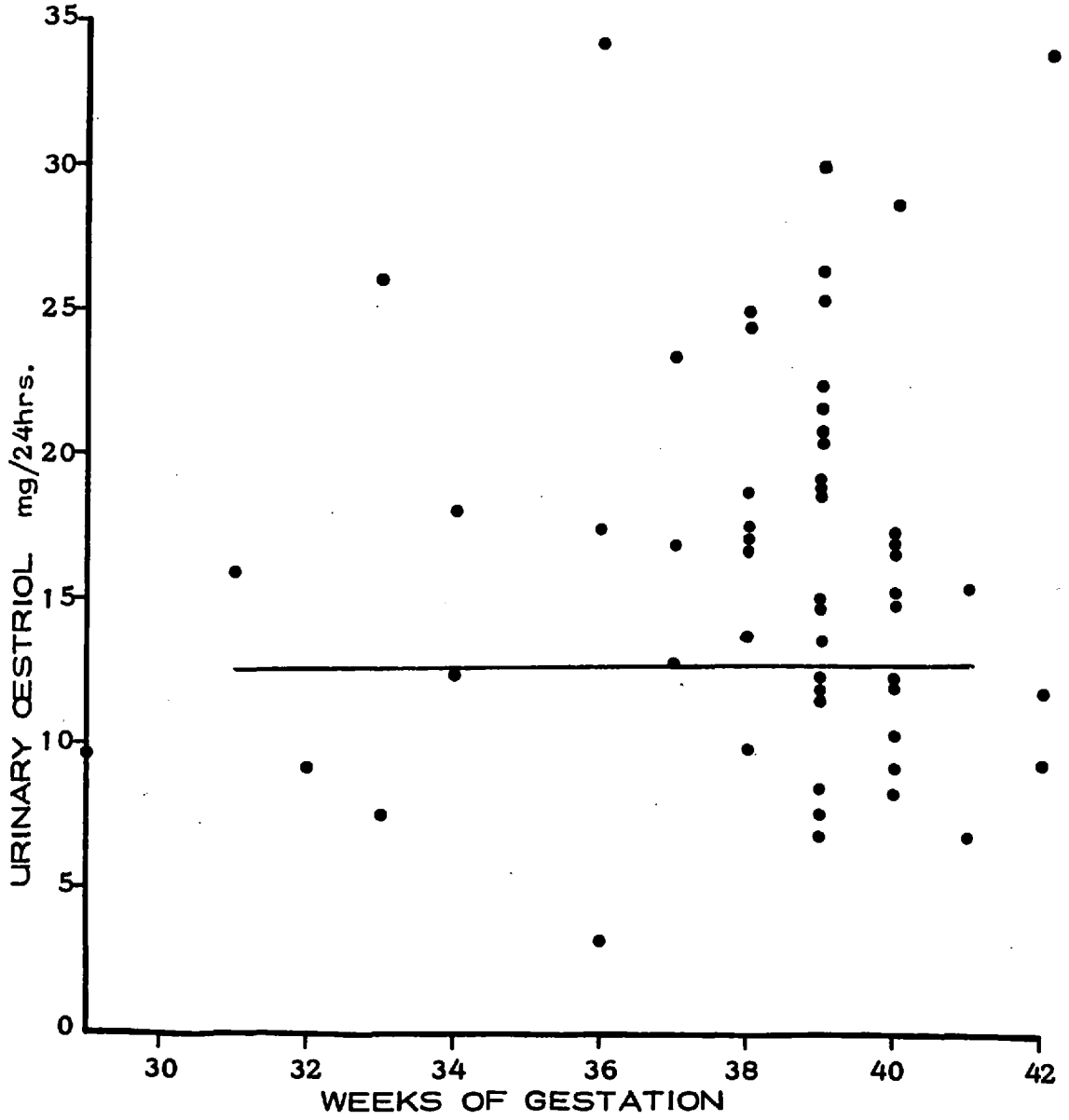


Figure 6 MATERNAL URINARY ŒSTRIOL and WEEKS OF GESTATION

56 RESULTS r = 0.04
UO = 12.50 + 0.106G

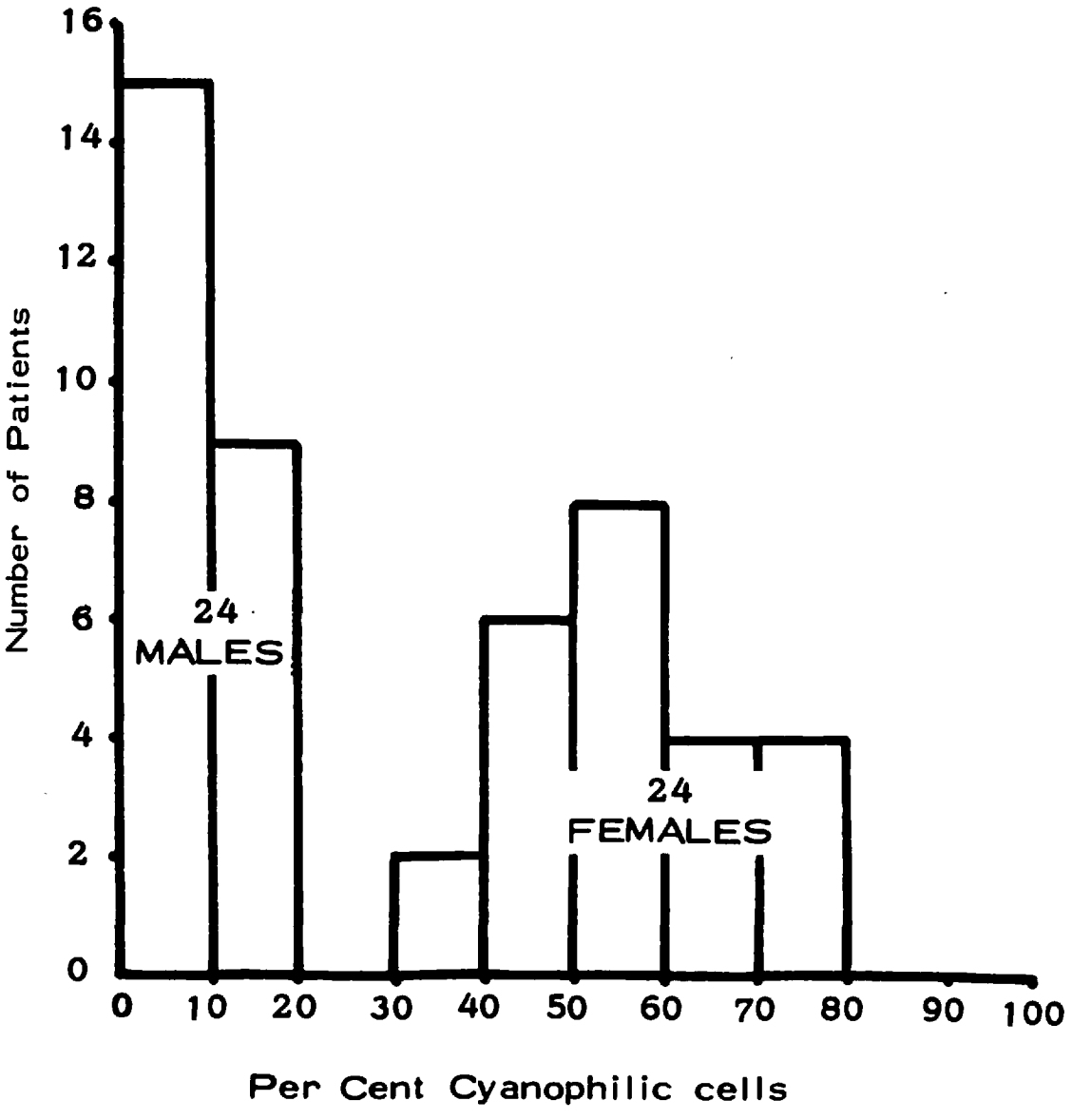


Figure 7.

Determination of Foetal Sex from the Percentage of Cyanophilic cells in amniotic fluid, using Papanicolaou stain.

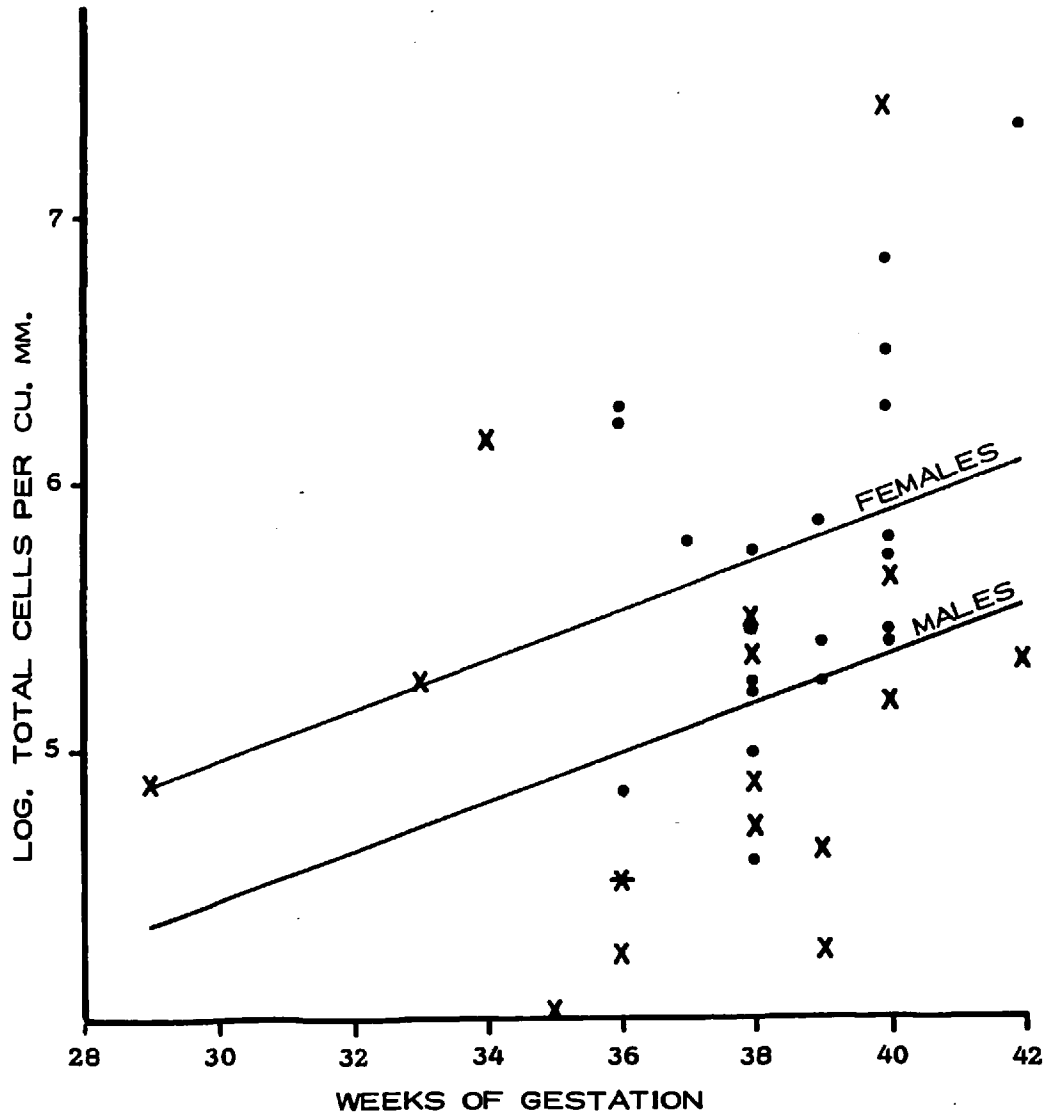
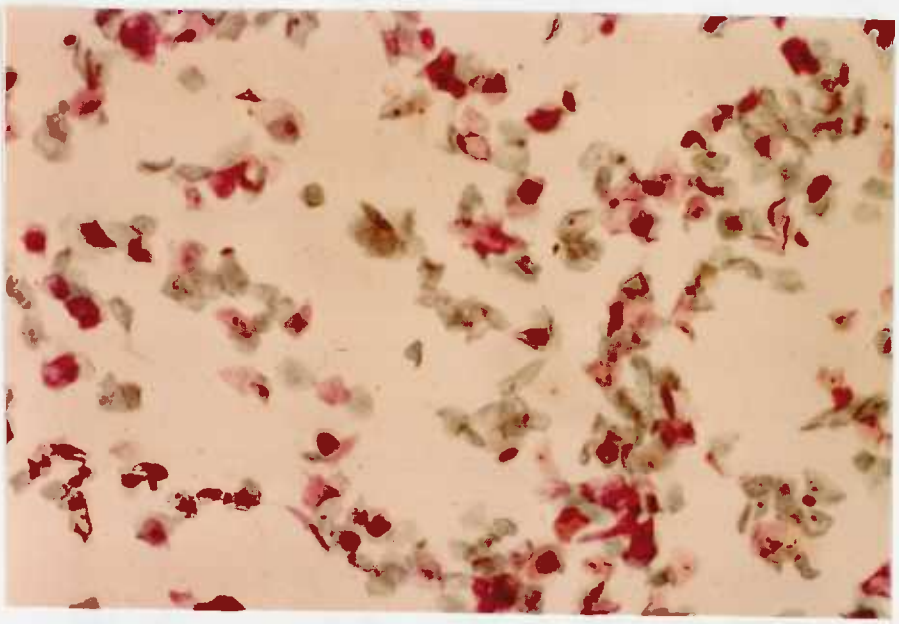


Figure 8 TOTAL CELLS per cu. mm. AND WEEKS OF GESTATION

X 17 MALE FOETUSES $y = 1.747 + 0.090x$

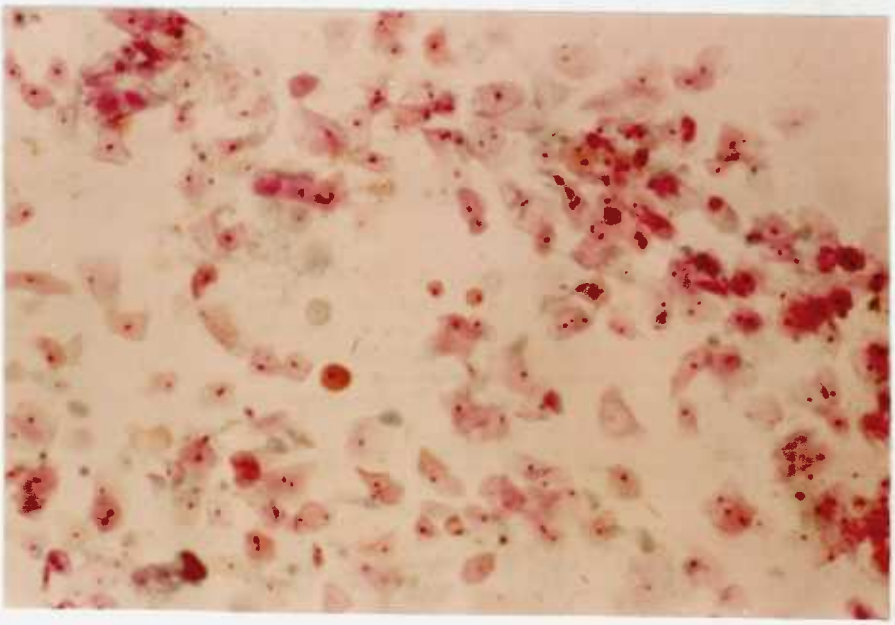
• 21 FEMALE FOETUSES $y = 2.251 + 0.090x$

Figure 9



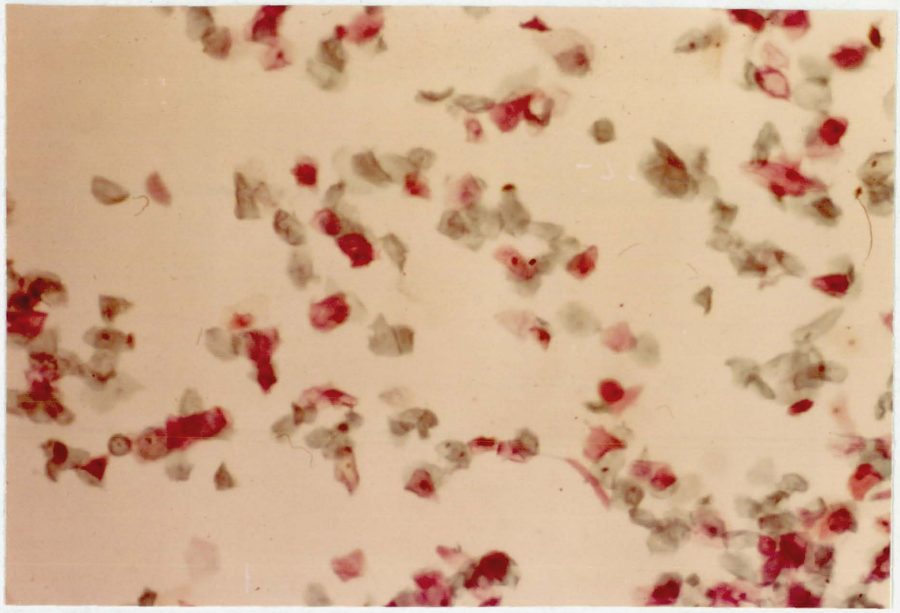
Amniotic Fluid. Female Foetus. 36 Weeks. Papanicclaou x 32. Cyanophilic Squamous Cells Predominate.

Figure 10



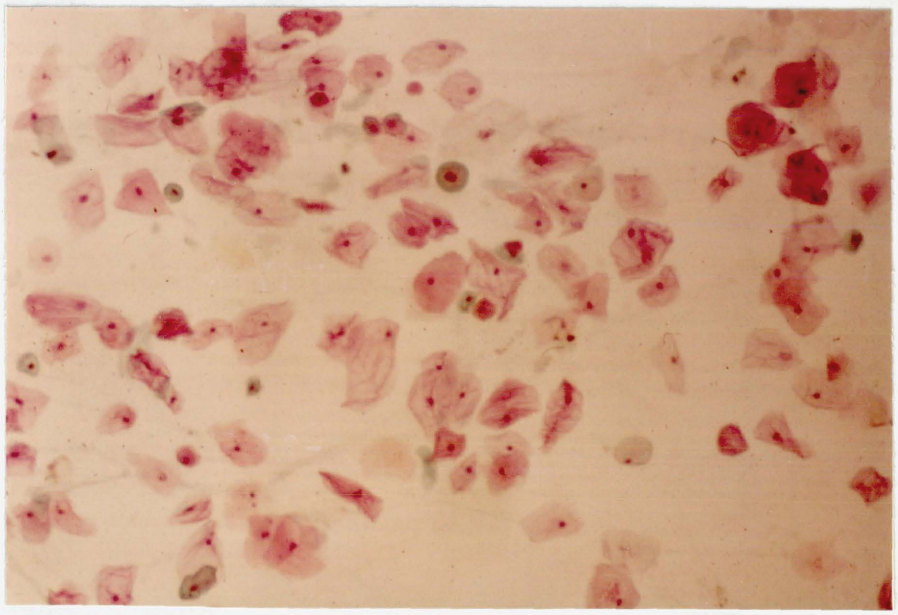
Amniotic Fluid. Male Foetus. 36 Weeks. Papanicolaou x 32. Eosinophilic Squamous Cells Predominate.

Figure 11



Amniotic Fluid. Female Foetus. 36 Weeks. Papanicolaou x 32.

Figure 12

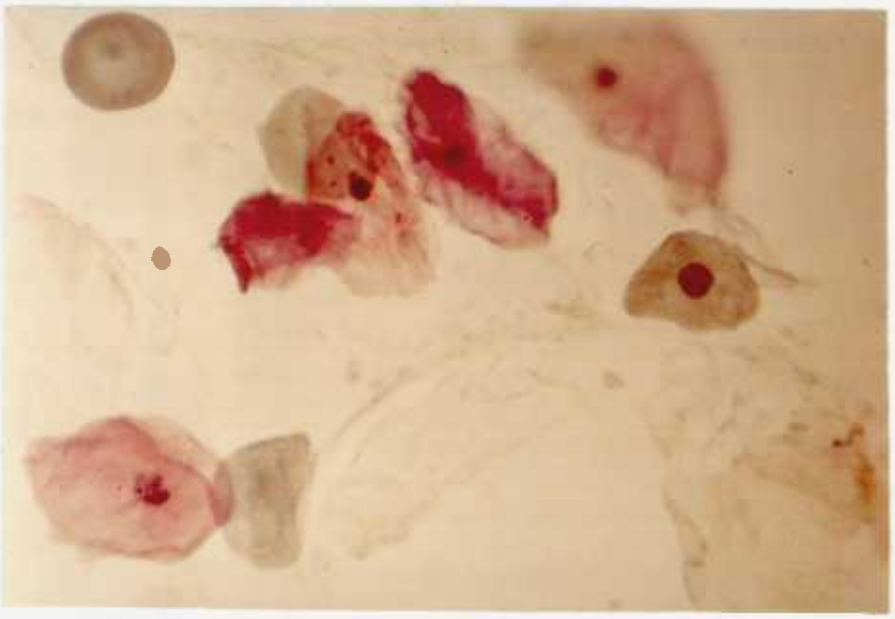


Amniotic Fluid. Male Foetus. 40 Weeks. Papanicolaou x 32.

Figure 13

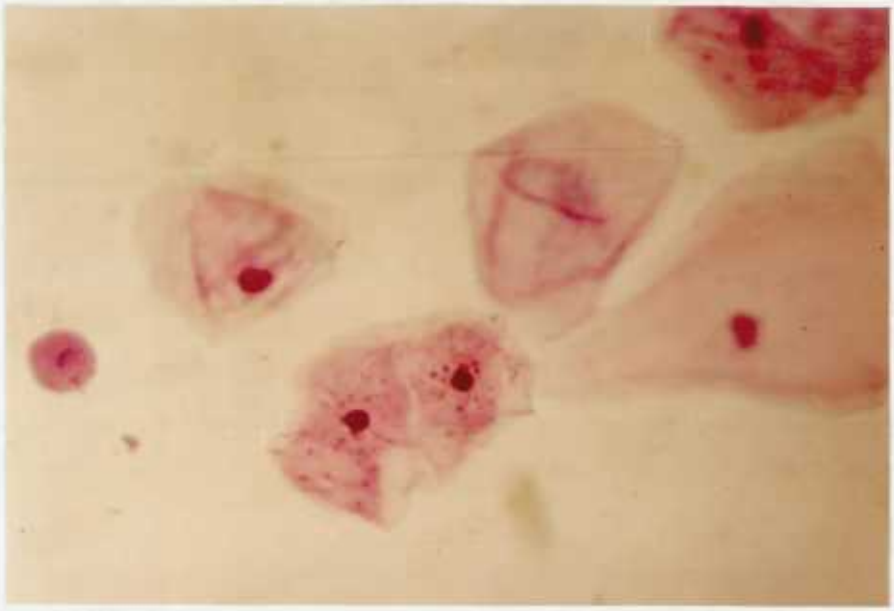
Amniotic Fluid. Female Foetus. 38 Weeks. Papanicolaou x 128. Cyanophilic, Anucleate Cells, Intermediate Cell and One Navicular Cell.

Figure 14



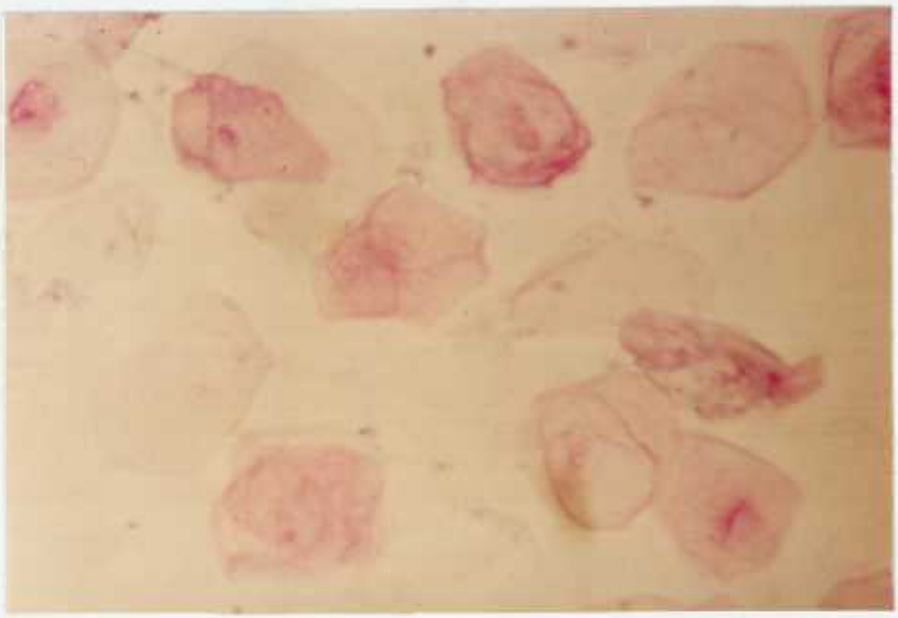
Amniotic Fluid. Male Foetus. 39 Weeks. Papanicolaou x 128. Eosinophilic, Anucleate and Nucleate Squamous Cells. Cyanophilic, 'Parabasal' Cells and Unstained, Anucleate, 'Vernix' Cells.

Figure 15



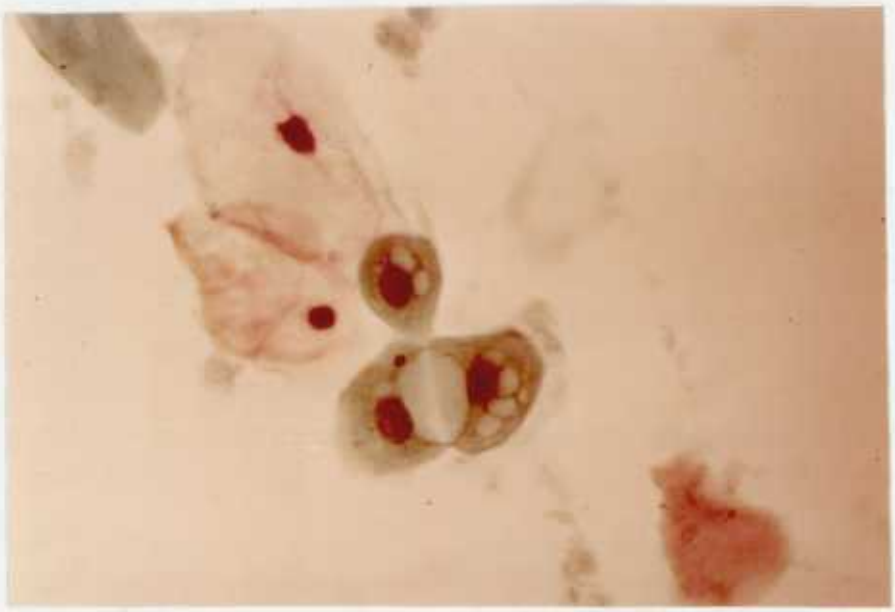
Amniotic Fluid. Male Foetus. 40 Weeks. Papanicolaou x 128. Eosinophilic Cells. Nucleate Squamous Cells Showing Pyknotic and Intermediate Type Nuclei and One Large and One Small Anucleate Cell.

Figure 16



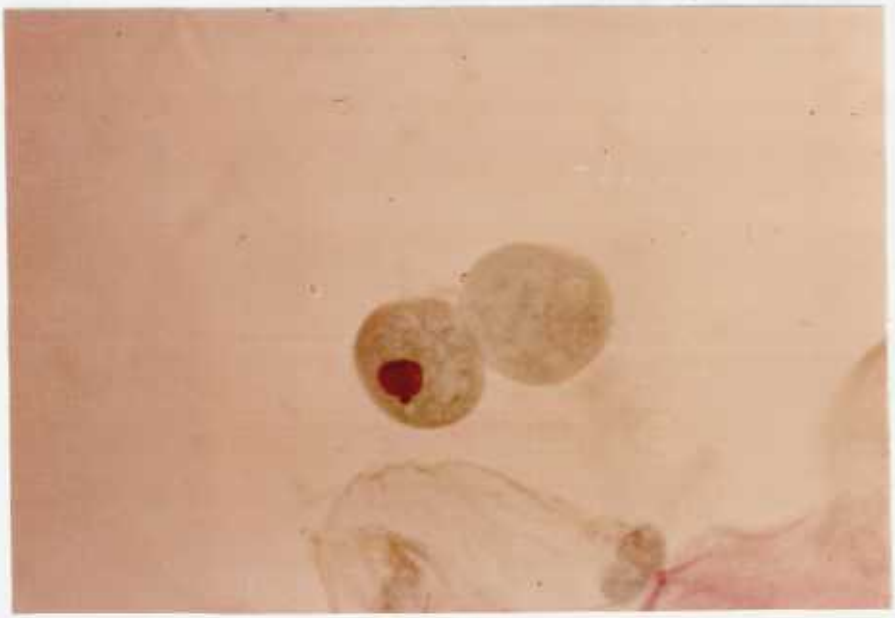
Amniotic Fluid. Female Foetus. 42 Weeks. Haematoxylin and Eosin x 128.
Anucleate 'Vernix' Cells.

Figure 17



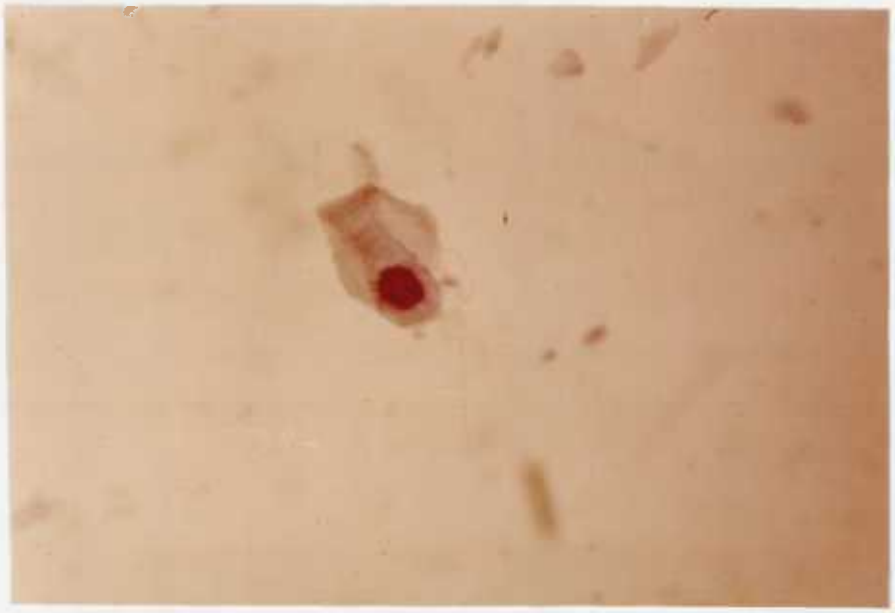
Amniotic Fluid. Male Foetus. 40 Weeks. Papanicolaou x 172. Group of Three Vacuolated, 'Parabasal' Cells, also Nucleate Squamous Cells and Debris.

Figure 18



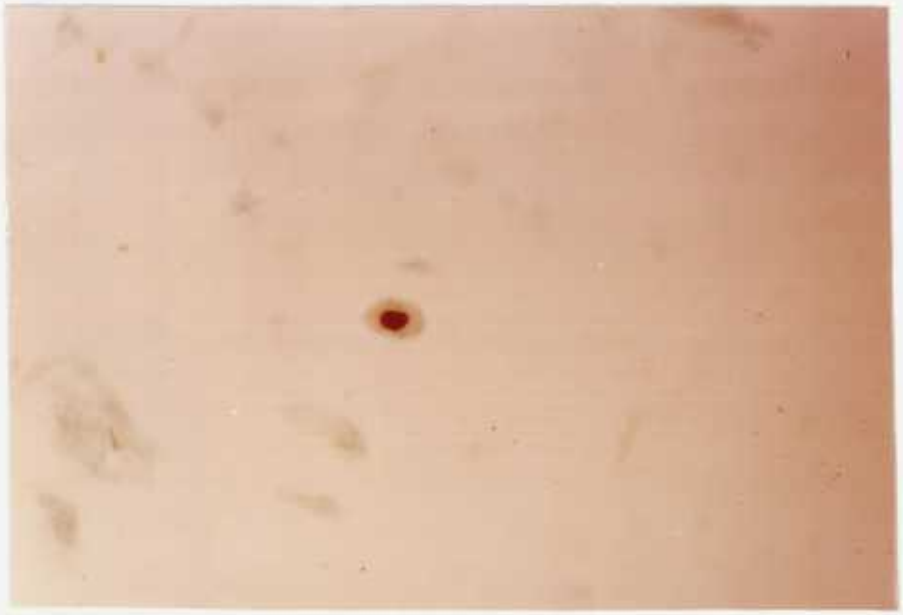
Amniotic Fluid. Male Foetus. 40 Weeks. Papanicolaou x 172. Nucleate and Anucleate, 'Parabasal' Cells.

Figure 19



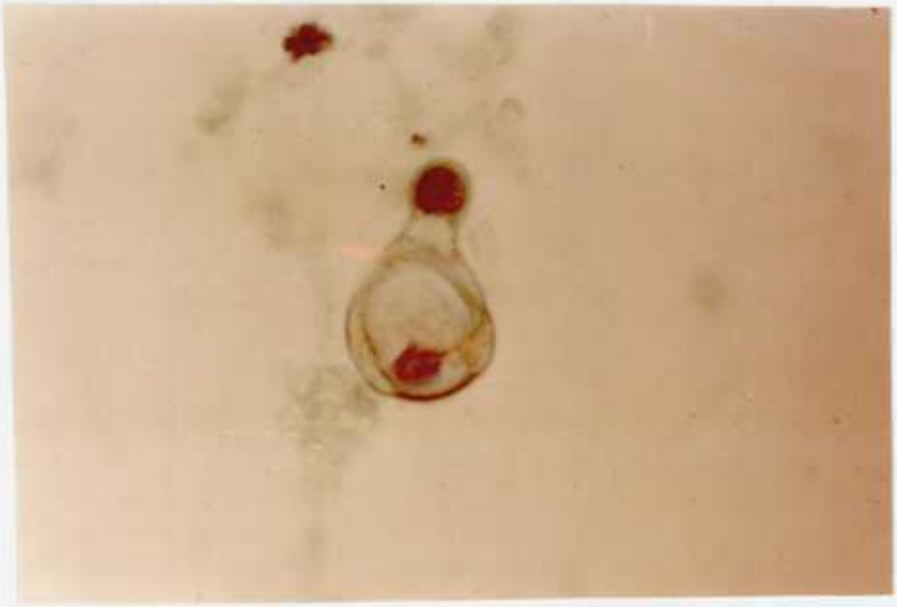
Amniotic Fluid. Male Foetus. 38 Weeks. Papanicolaou x 172. Columnar Cell.

Figure 20



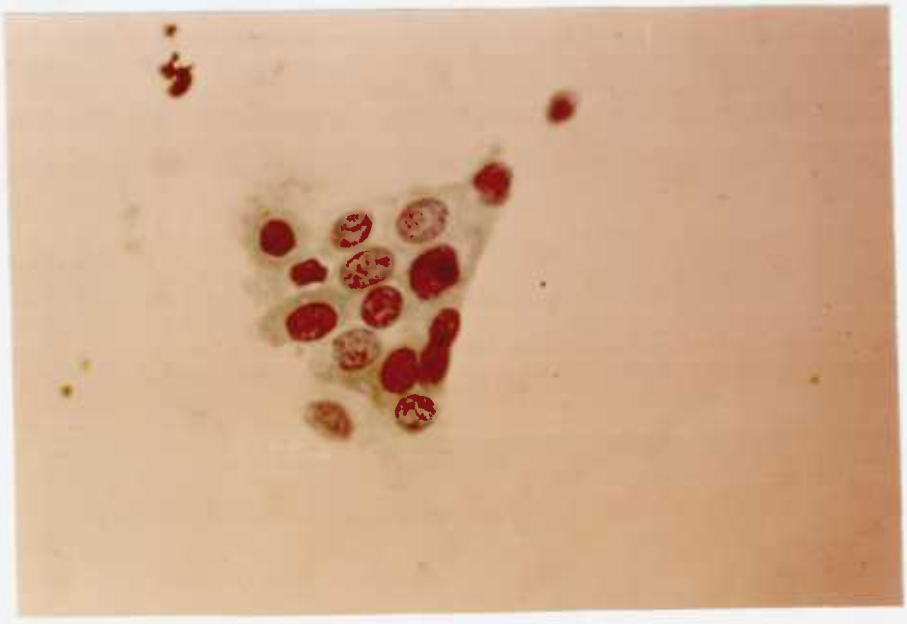
Amniotic Fluid. Male Foetus. 38 Weeks. Papanicolaou x 172. Small Cell, probably from Urinary Tract.

Figure 21



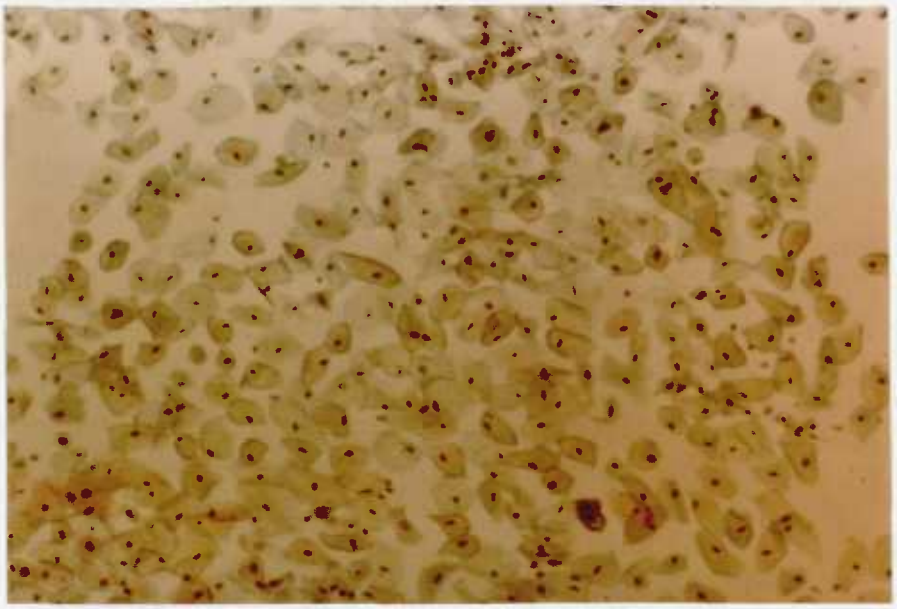
Amniotic Fluid. 11 Weeks. Papanicolaou x 172.
Vacuolated Amnion Cells.

Figure 22



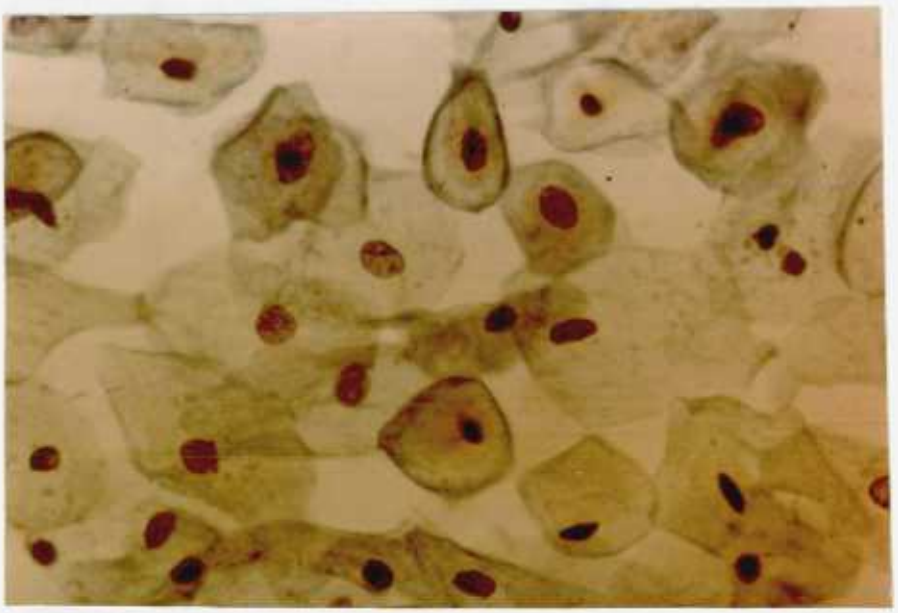
Amniotic Fluid. 11 Weeks. Papanicolaou x 172.
Sheet of Amnion Cells.

Figure 23



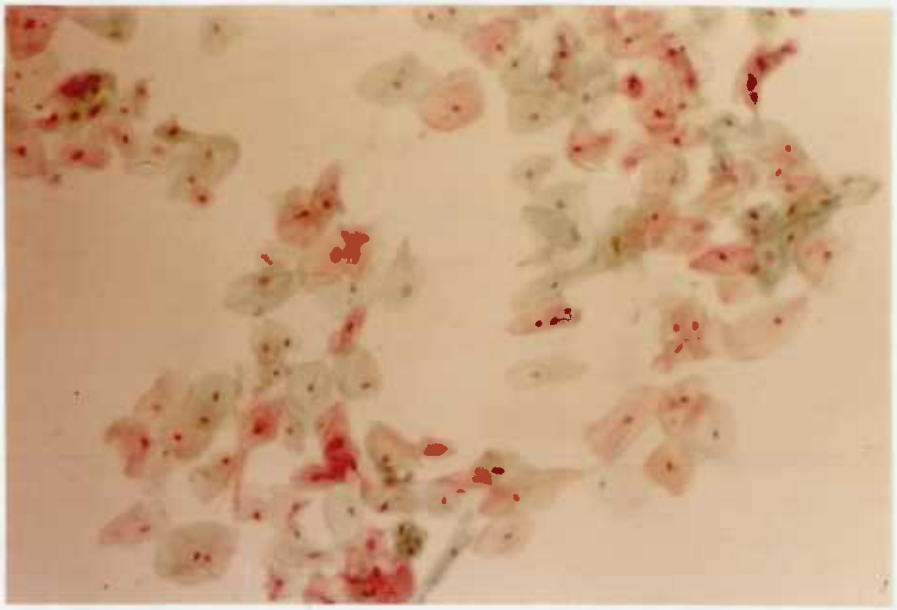
Smear from Newborn, Female Infant's Vulva.
Papanicolaou x 32.
Cyanophilic, Squamous Cells.

Figure 24



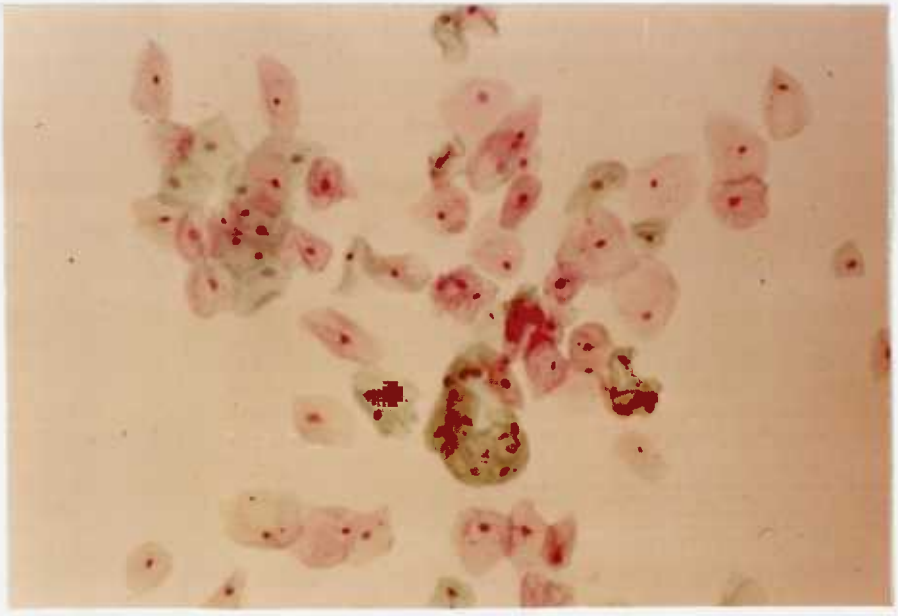
Smear from Newborn, Female Infant's Vulva.
Papanicolaou x 128.
Cyanophilic Intermediate Cells, Showing Some in Navicular Form.

Figure 25



Buccal Smear from Newborn Female Infant. Papanicolaou x 32. Cyanophilic and Eosinophilic Squamous Cells.

Figure 26

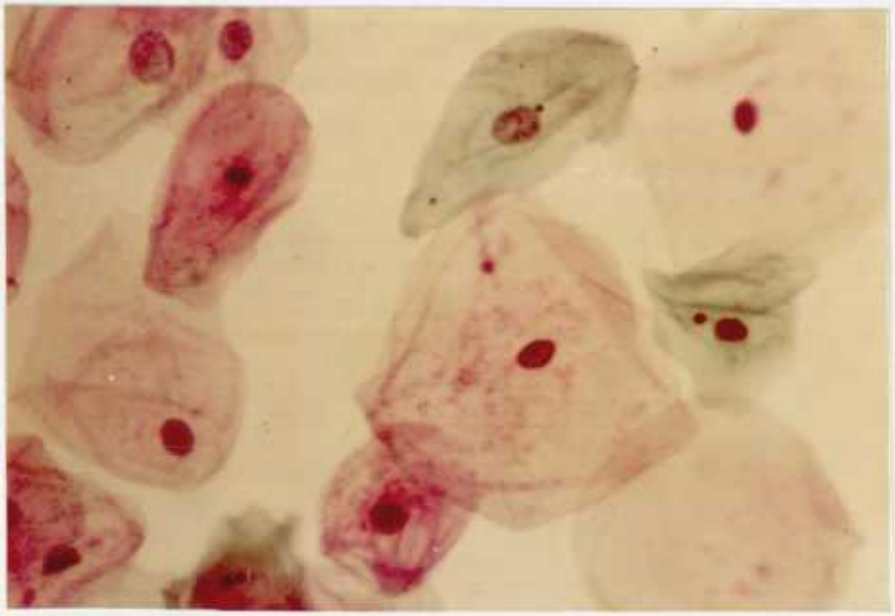


Buccal Smear from Newborn Male Infant. Papanicolaou x 32. Cyanophilic and Eosinophilic Squamous Cells.

Figure 27

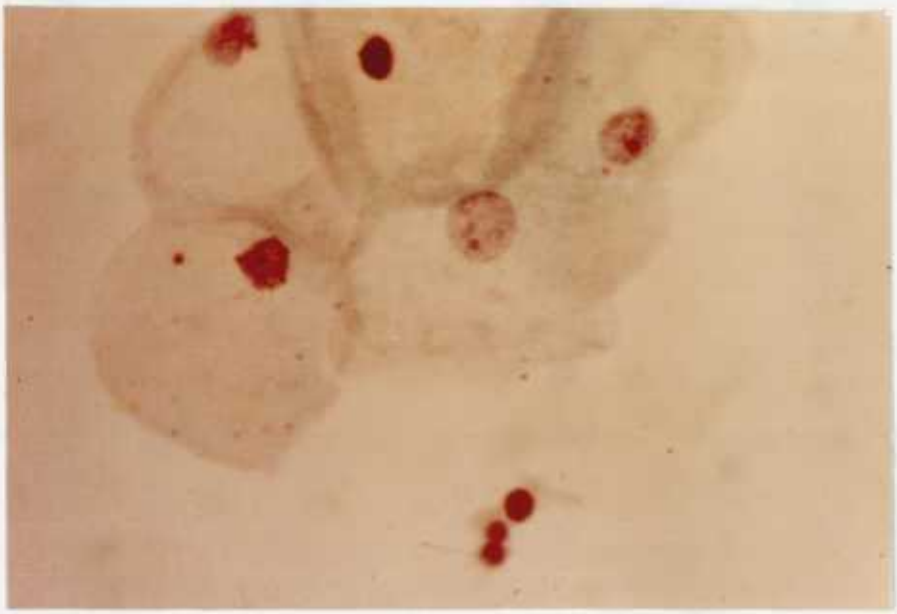
Buccal Smear from Newborn Female Infant. Papanicolaou x 128.

Figure 28



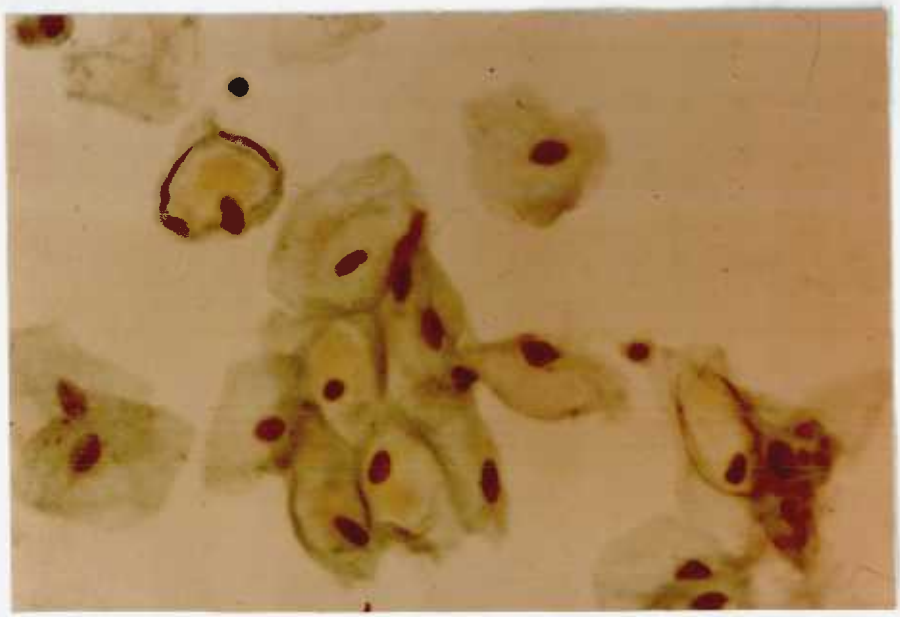
Buccal Smear from Newborn Male Infant. Papanicolaou x 128.

Figure 29



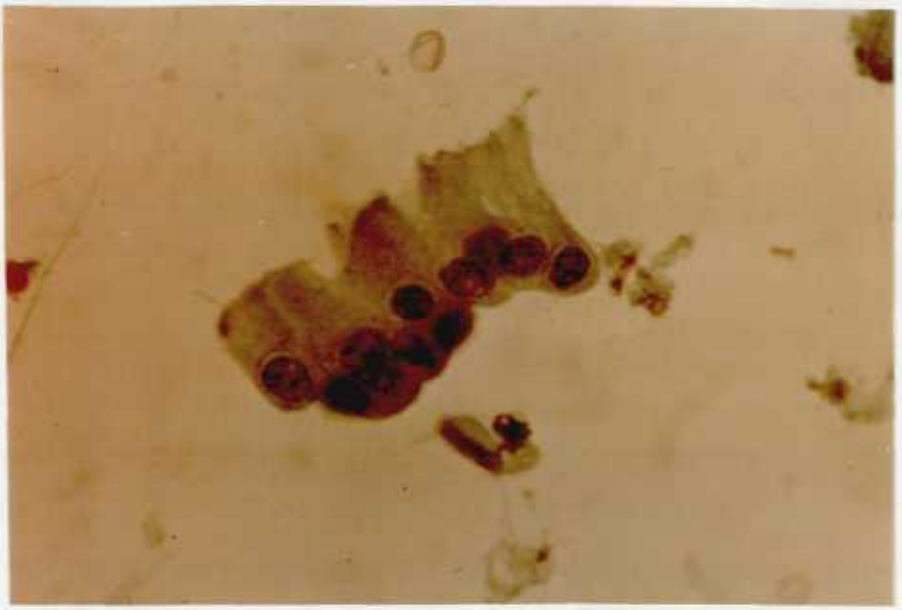
Cells from Newborn Male Infant's Urine. Papanicolaou x 172. Squamous Cells and Small Cells from Urinary Tract.

Figure 30



Cells from Newborn Female Infant's Urine. Papanicolaou x 128. Cyanophilic Intermediate Cells, some in Navicular Form.

Figure 31

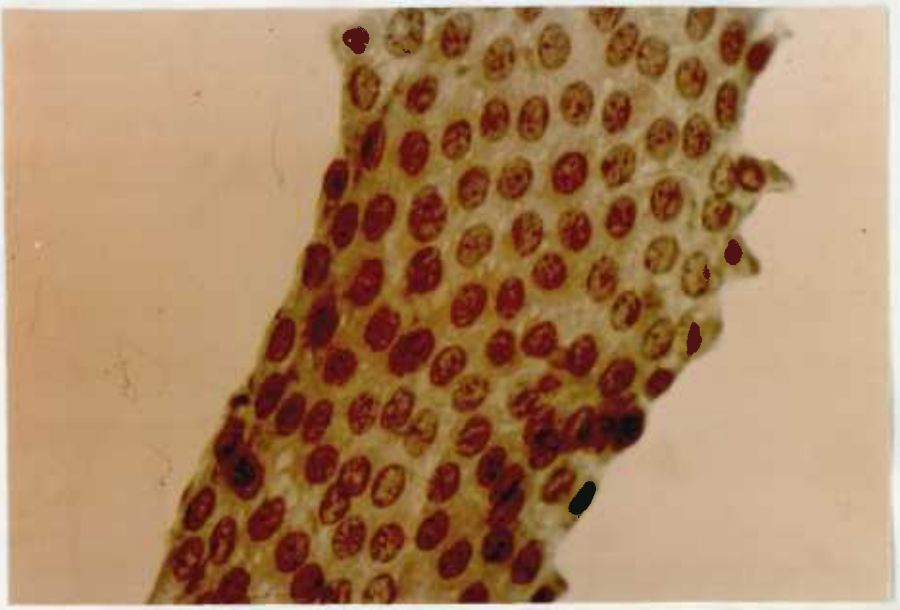


Cells Scraped from Placental Amnion. Papanicolaou x 128. Columnar Cells.

Figure 32

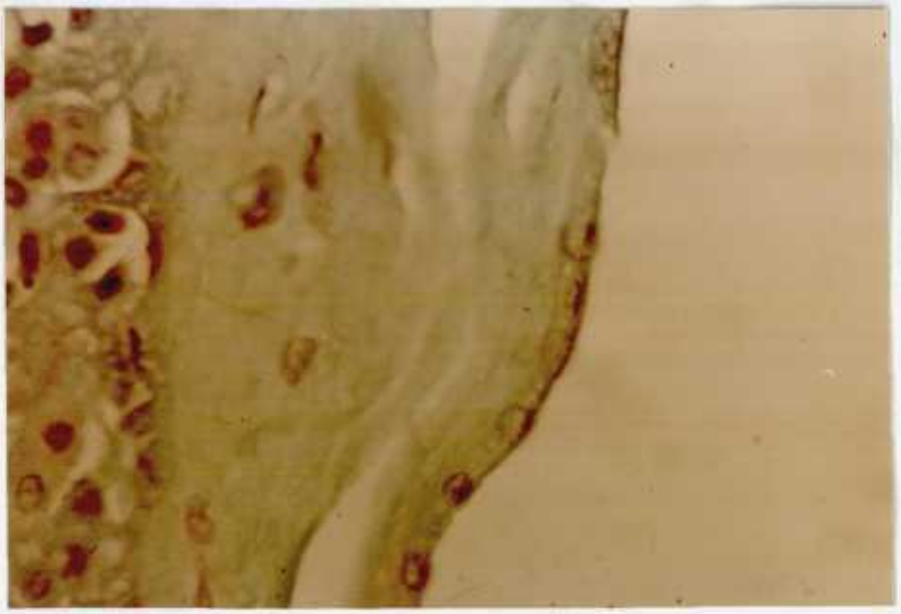
Section of Placental Amnion. Papanicolaou x 128.

Figure 33



Cells Scraped from Free Amnion. Papanicolaou x 128.

Figure 34



Section of Free Amnion. Papanicolaou x 128.

Figure 35

Cells Scraped from Amnion on Umbilical Cord. Papanicolaou x 128.

Figure 36

Section of Umbilical Cord. Papanicolaou x 128.

CHAPTER 3 (continued)

RESULTS (continued)

3.1 Karyopyknotic Index

It was found that keeping amniotic fluid specimens at 4°C for up to four days did not have any significant effect on the karyopyknotic index. Estimations on two specimens on four consecutive days showed a variation of up to 3 per cent. This compared favourably with the variation of the estimation between the two slides used from each amniotic fluid specimen in the investigation. Out of 102 results for the karyopyknotic index, there was a variation of 3 per cent or less between the two slides of each sample in 77 instances. The variation was between 4 and 7 per cent in 22 specimens and between 8 and 9 per cent in three specimens. Four samples of amniotic fluid were too heavily contaminated with blood for accurate estimation of the karyopyknotic index.

3.2 Determination of Foetal Sex

Low power, (x 10), examination of slides of amniotic fluid cells gave an immediate indication of the sex of the foetus (figures 9, 10, 11 and 12). Twenty-four good preparations were re-examined for this purpose. Female foetuses usually showed a predominance of cyanophilic, nucleate, squamous cells and male foetuses always showed a predominance of eosinophilic, nucleate, squamous cells. The percentage of cyanophilic cells from counts of 200 squamous cells are shown in Table IX. Of the 24 specimens from different patients, 23 were taken between 32 and 41 weeks and one at 26 weeks. Thirteen were from patients with male foetuses and 11 from patients with

female foetuses. The male foetuses gave percentages of cyanophilic cells between 2 and 20 per cent. The female foetuses yielded counts between 37 and 68 per cent. The correlation between the results from the Papanicolaou and Harris-Shorr stained slides was very close and no advantage of one staining method over the other was observed. Moderate contamination with blood did not interfere with the result. Examination of 24 more unselected specimens to test the method further did not alter the criteria established for diagnosis of foetal sex, although cyanophilic counts for female foetuses went up to 79 per cent (Table X).

It was noticed that amniotic fluid specimens from female foetuses were more cellular than those from male foetuses. When the cell deposit was small and the material on the slide scanty, the specimen usually turned out to be from a male foetus. The greater abundance of cyanophilic squamous cells in the female specimens resulted in a relative scarcity of the less numerous cell types. When looking for the 'parabasal' type cells, these were more easily found in the slides prepared from male foetuses, at all gestational ages studied. It was also noticed that the squamous cell nuclei in specimens from male foetuses more often showed signs of degeneration, i.e. shrinkage and indefinite nuclear margins, than those from female specimens. Confirmatory evidence in favour of a female foetus was the presence of navicular forms of cyanophilic cells. Amorphous debris was a notable feature of some specimens from male foetuses.

3.2.1 Total Cell Counts and Hydrogen Ion Concentration

The mean value for total cell counts is significantly higher when the foetus is female than when it is male, for any one week of gestation (Table XII). In Figure 8 the logarithm of the total cell counts is used to accommodate graphically the large numbers and the wide variation between specimens. The slope (+ 0.90), which is the same for males and females, has a standard error of 0.049 (35 degrees of freedom) and is therefore not quite significant. Specimen number 199 in which the total cells were 1,630 per cu.mm. was from a patient who had a fresh stillborn male infant two days after amniocentesis. The amniotic fluid showed more than 50 per cent fatty cells with the Nile Blue Sulphate test. The placenta had a large retroplacental blood clot and numerous infarcted areas. Stillbirth was attributed to asphyxia as a result of post-maturity and minor pelvic disproportion. Specimen number 241 also had an exceptionally high total count of 1,530 cells per cu.mm. at 42 weeks gestation by her dates. The fat cell count was 75 per cent and the foetus was female.

3.3 Description of Cell Types in Amniotic Fluid and their Derivation from the Foetus

The morphological characteristics of the cells in the amniotic fluid samples were observed and there was general agreement with the four cell types described by previous authors as related in Chapter 1.2. Each of the four cell types will be described in turn and its source identified by comparison with the results of investigation of foetal tissues.

3.3.1 Anucleate Cells

Anucleate cells were seen most frequently in the last month of gestation. They took up the Papanicolaou or Shorr stain with only very faint eosinophilia or cyanophilia or none at all, but they stained pink with pinacyanole and with haematoxylin and eosin (Figures 14 and 16). They often appeared in clumps or sheets. In some, a 'shadow' of the nucleus could be seen. These are the 'polygonal' cells described by Huisjes (1970). Similar plaques of anucleate cells were obtained by scraping vernix from the axilla or groin of newborn infants. Larger anucleate cells which were eosinophilic or cyanophilic were also seen (Figures 13 and 15). These too showed nuclear 'shadows'. They were thought to be degenerate forms of the large nucleate squamous cells which are described later. A third type of anucleate cell occurred. These were small and round or oval, most frequently cyanophilic but eosinophilic forms were also seen. Their association with the 'parabasal' type cells and their resemblance in size and shape, suggested that these were degeneration products of the 'parabasal' cells (Figures 14, 15 and 18).

3.3.2 Nucleate Squamous Cells

Large nucleate squamous cells were seen in all the samples examined from 25 weeks gestation onwards (Figures 13, 14 and 15). these were either cyanophilic or eosinophilic, but the cyanophilic ones were seen more frequently in the specimens from female foetuses. The nuclei of these squamous cells were usually vesicular, as in intermediate cells, but some were pyknotic. In specimens from male

foetuses the eosinophilic cells showed a higher proportion of degenerate nuclei than the cyanophilic cells in the samples from female foetuses. The cyanophilic, nucleate, squamous cells were sometimes in 'navicular' form, i.e. their edges were thickened, the nucleus was often eccentric and their cytoplasm contained granules but clumping was not a conspicuous feature (Figure 13). Buccal smears, scrapings from the vaginal introitus of females and cells from the urine of newborn infants all contained a preponderence of nucleate, squamous cells, which suggested that these three sites provide the origin of the nucleate, squamous cells in the amniotic fluid. Urine from newborn female infants yielded fairly numerous, cyanophilic, intermediate type squamous cells, some of which were in navicular form (Figure 30). The female infants' urine also contained occasional very small cells with pyknotic nuclei and a small amount of cytoplasm. Urine from newborn male infants contained a few nucleate squamous cells which were cyanophilic, with vesicular nuclei, and a few very small dark cells with little cytoplasm (Figure 29). These were also seen occasionally in amniotic fluid specimens (Figure 20) as well as in male and female infants' urine and are thought to originate from the urinary tract.

Buccal smears from male and female newborn infants contained both cyanophilic and eosinophilic, nucleate, squamous cells (Figures 25, 26, 27 and 28). The nuclei were mostly vesicular but a few were pyknotic. There were no navicular forms. Keratohyaline granules occurred commonly in these cells. The karyopyknotic index was between

0 and 4. The proportion of cyanophilic to eosinophilic cells varied greatly from one specimen to another. There was no significant difference between those from male and female infants (Table XI).

Smears taken from the vaginal introitus of newborn female infants contained an abundance of cells. The appearance of the Papanicolaou stained preparation resembled a 'clean' vaginal smear from a pregnant woman (Figures 23 and 24). The cells were indistinguishable from intermediate cells from the adult vagina, and many of them were in navicular form. An occasional parabasal cell was present and an occasional superficial squamous cell. The difference between these smears and the adult vaginal smear of pregnancy was the absence of leucocytes, bacteria and cytolysis, and of cell clumping. The karyopyknotic index was between 0 and 5. The cells were nearly all cyanophilic, counts varying between 92 and 99 per cent.

3.3.3 'Parabasal' Type Cells

The third type of cell in amniotic fluid is small and round or oval, with fairly dense, blue-staining cytoplasm, which is often vacuolated. Their nuclei were well defined, vesicular and usually central (Figures 17 and 18). These cells frequently occurred in groups, sometimes joined by intercellular bridges. The proportion of these cells has been shown by several authors to decrease with the advance of gestation beyond the 32nd week, (Van Leeuwen, Jacoby and Charles (1965), Hoyes (1968), Votta, Bobrow de Gagneten, Parada and Giulietti (1968), Floyd, Goodman and Wilson (1969), Lind, Parkin and Cheyne (1969), Mandelbaum and Evans (1969), Bishop and Pollock (1970),

Stenback and Ojala (1970) and Husain and Sinclair (1971)). These cells were not counted in this study, but they could always be seen in preparations from male foetuses, at any time beyond the 32nd week, with much greater frequency than in preparations from female foetuses where they are outnumbered by the abundance of cyanophilic, nucleate, squamous cells. Mention has already been made of small anucleate cells, similar in size and shape to the small 'parabasal' type cells. These were seen most frequently in preparations showing numerous nucleate 'parabasal' type cells, and often closely associated with those cells. These small anucleate cells were usually cyanophilic but eosinophilic forms were seen as well. It was deduced that these were degeneration products of the nucleate, 'parabasal' type cells. Additional evidence of the degenerative process was seen in the variation of the state of preservation of the nuclei of these cells, from well preserved with clear nuclear detail to small pyknotic nuclei, both types occurring in the same preparation.

Scrapings were taken from the amnion of a freshly delivered full-term placenta. The amnion had to be scraped hard with a metal spatula to obtain cells, which suggested that, near-term anyway, they do not desquamate readily. The cells tended to come off in sheets, especially from the umbilical cord and from that part of the amnion free from the placenta. The sheets of cells from the free amnion and from the cord had round or oval nuclei, usually centrally placed, showing more chromatin clumping than in intermediate squamous cells. Their

cytoplasm was thick, cyanophilic and sometimes vacuolated (Figures 33 and 35). When seen in histological sections these cells appeared cuboidal or flattened (Figures 34 and 36). Their resemblance to the 'parabasal' type cells in the amniotic fluid suggests that the amnion cells may be released into the amniotic fluid during pregnancy in small numbers, possibly in plaques which later become separated into individual cells. Scrapings from the amniotic surface over the placenta yielded cells and sheets of cells with nuclei indistinguishable from those from the free amnion and cord. However, these cells when seen laterally were columnar with nuclei placed at either end or in the centre of the cell (Figure 31). The columnar shape of these cells and the variable position of their nuclei was confirmed by histological sections (Figure 32). Desquamation of these cells has been suggested by Wachtel, Gordon and Olsen (1969) as the source of the fourth type of cell found in the amniotic fluid, the tall flask-shaped cells, which appear some time after the 30th week. These cells were seen only rarely in this study.

3.4 Histochemistry

Smears of cells from female foetus amniotic fluid and vulval smears from newborn infants, stained with methyl green and pyronin, showed a weak positive reaction for ribonucleic acid in the cytoplasm of the intermediate cells. This reaction was absent after treatment of the slides with ribonuclease. Papanicolaou staining after ribonuclease treatment did not show any change in the percentage of cyanophilic cells.

P.A.S. positive substances were present in amniotic fluid cells from male and female foetuses before and after salivary digestion. Papanicolaou staining after salivary digestion showed no change in cytoplasmic cyanophilia when compared with a control slide.

CHAPTER 4

DISCUSSION OF RESULTS OF SPECIAL CASES AND ABNORMAL CASES

4.1 Number 71. Abortion by Hysterectomy at 11 Weeks

Amniotic fluid oestrogen concentration 0.4 μg/100 mls.

There were plenty of cells in the sample. They were mostly of the 'parabasal' type and occurred often in groups and sometimes in sheets which were similar to those scraped from the free amnion and cord at term (Figures 21 and 22). Their cytoplasm was dense, sometimes vacuolated, and contained yellow-brown granules. Some cells showed degenerative changes in their nuclei from chromatin clumping to pyknosis. These cells corresponded to the Type 2 cells of Hoyes (1968), and are thought to be derived from the surface of the amnion.

A few cells were indistinguishable from nucleate superficial squamous cells. As the surface of the foetus at 11 weeks consists of periderm and does not differentiate into squamous epithelium until after 20 weeks these 'squamous-like' cells probably originate from the foetal periderm, and correspond to the Type 1 cells of Hoyes (1968).

The cytology of this case did not provide any new information. It confirmed the findings of Hoyes (1968) and showed that cells may be present in quite large numbers in the amniotic fluid as early as 11 weeks gestation. Their viability was not tested so this information is of limited value with regard to culture for early diagnosis of hereditary chromosome abnormalities.

4.2 Number 74. Anencephalic Foetus at 36 Weeks Gestation

Amniotic fluid oestrogen concentration 3.0 µg/100 mls

Maternal urinary oestriol ..... 3.2 mg/24 hrs

Karyopyknotic index 25.8

The very low oestrogen level in the amniotic fluid confirmed the findings of Aleem, Pinkerton and Neill (1969) that in anencephaly, which is associated with progressive atrophy of the adrenal cortex from the fifth month of gestation, the amniotic fluid oestriol concentration is markedly lower than normal and becomes progressively lower as gestation continues. The low maternal urinary oestriol supports the amniotic fluid findings.

The karyopyknotic index of 25.8 is near to the mean of the series, for female foetuses at 36 weeks, which is 21.2. This suggests that the karyopyknotic index is not dependent on the oestriol concentration, but on the maturity of the foetus. One case is clearly of only limited significance.

4.3 Number 67. Achondroplasia

The mother was an achondroplasic dwarf with a normal husband. They already had one normal female child. The female child of this pregnancy, which was achondroplasic, was delivered by caesarean section and died on the second day after delivery from respiratory distress syndrome. The amniotic fluid was taken at caesarean section. The oestrogen concentration of the amniotic fluid and the maternal urinary oestriol were within normal limits. The karyopyknotic index of 9.4 was the lowest recorded at 38 weeks gestation, but in accord with the trend towards lower values near term.

4.4 Numbers 27, 47 and 52. Twin Pregnancies

Specimens 47 and 52 were from the same patient. Both mothers were rhesus negative with antibodies. The amniotic fluid results were unremarkable. Number 27 had two male infants and it is not known which foetal sac was tapped. The amniotic fluid of numbers 47 and 52 was thought to have come from the female foetus. The second foetus was male and died in utero.

4.5 Diabetes

There were six specimens from five cases.

Only one result showed a considerable difference from those of normal patients at the same period of gestation. Number 20 had an amniotic fluid oestrogen concentration of only 10.6 μg/100 mls at 39 weeks, as against a mean of 47.9 μg/100 mls for the normal cases at 39 weeks. She gave birth at 40 weeks to a female infant which weighed 2380 g which is below the fifth percentile for 40 weeks gestation.

4.6 Hydramnios

There were four cases. Only the one case with an anencephalic foetus showed amniotic fluid oestrogen and maternal urinary oestriol levels markedly lower than the normal pregnancies.

4.7 Essential Hypertension

Four mothers had blood pressure of 140/90 or more recorded on at least two occasions before 28 weeks. The amniotic fluid oestrogen concentration of Number 79 was much lower, at 8.4 μg/100 mls, than

those of the normal pregnancies at 40 weeks. She was delivered at 41 weeks of a female infant weighing 2370 g, which is below the fifth percentile for 41 weeks gestation.

4.8 Rhesus Iso-immunisation

Twenty-eight specimens of amniotic fluid were obtained from 17 patients with rhesus antibodies. The two cases of twins have been discussed under a separate heading.

Numbers 46 and 56 were from the same patient, who had a spontaneous delivery at 38 weeks of a male infant weighing 2090 g which is below the fifth percentile for 38 weeks gestation. The infant died on the second day from respiratory distress syndrome.

Numbers 99 and 106 were from one patient who did not know the date of her last menstrual period. She had a 2740 g male infant, by caesarean section, which was severely affected by erythroblastosis and required four exchange transfusions. The amniotic fluid samples were not taken within one week of caesarean section and therefore would not necessarily be expected to indicate the outcome.

The lowest amniotic fluid oestrogen concentration recorded within one week of delivery was number 38, at 5.8 μg/100 mls. The infant was not severely affected.

Only seven patients in this series had amniocentesis within one week of delivery. The evidence from these seven patients supports the findings of Michie and Robertson (1971) that urinary and amniotic fluid oestriol levels did not give a reliable guide to the state of the foetus in rhesus iso-immunisation.

Comparison of the mean values for karyopyknotic index, amniotic fluid oestrogen concentration and maternal urinary oestriol for rhesus and non rhesus patients, divided into those with male and those with female foetuses showed no significant difference between the mean values for rhesus and non rhesus allowing for the week of gestation. On this evidence it was decided to combine the results of rhesus and non rhesus patients for analysis.

4.9 Low Birth Weights (Table V)

Nine patients had infants whose birth weights were below the fifth percentile for the time of gestation at delivery according to the tables published by Thompson, Billewicz and Hytten (1968) of data from births in the City of Aberdeen between 1948 and 1964.

Number 20 was a diabetic, whose amniotic fluid oestrogen concentration eight days before delivery was 10.6 µg per 100 mls, the lowest recorded in this series at 39 weeks, and it was probably an indication of lack of foetal well being.

Numbers 46 and 56 were from a rhesus negative mother with antibodies. Her infant, delivered spontaneously at 38 weeks, died of respiratory distress syndrome within 48 hours.

Number 74 had an anencephalic foetus with correspondingly low amniotic fluid oestrogen concentration and urinary oestriol.

Number 79 was hypertensive throughout pregnancy. Amniotic fluid oestriol concentration at 40 weeks was 8.4 µg per 100 mls.

Numbers 12, 63, 87, 102 and 104 were small for dates. Nile Blue Sulphate tests for foetal maturity at the time of amniocenteis

showed fat cell counts of 10 per cent or more in each case, which confirmed foetal maturity. Numbers 12 and 63 showed low amniotic fluid oestrogen concentrations of 9.3 μg per 100 mls at 42 weeks and of 10.1 μg per 100 mls at 37 weeks.

This evidence provided reasons for these low birth weights which made it unnecessary to doubt the foetal maturity as estimated from the date of the patient's last menstrual period, except in the case of number 102 whose dates were quite uncertain. Her results have been excluded from the graphs and statistics which relate to weeks of gestation.

CHAPTER 5

DISCUSSION

5.1 Karyopyknotic Index

The results show that there is no direct, positive, relationship between the amniotic fluid oestrogen concentration and the karyopyknotic index of the cells in the amniotic fluid contrary to the expectations when this investigation was begun. However, the karyopyknotic index has been shown to have a significant inverse relationship with weeks of gestation in the presence of both male and female foetuses, males $r = -0.43$, females $r = -0.42$, (Figure 4). The most striking result is that of the highly significant negative correlation between the karyopyknotic index in the presence of female foetuses and the amniotic fluid oestrogen concentration $r = -0.43$, whereas in the presence of male foetuses, $r = -0.13$, which is not significant (Figure 3).

With male foetuses, the partial correlation coefficient for karyopyknotic index and weeks of gestation calculated for a constant amniotic fluid oestrogen concentration, $r = -0.43$, is highly significant at 1 per cent, which suggests that the karyopyknotic index with male foetuses is related to the weeks of gestation rather than to the amniotic fluid oestrogen concentration. With female foetuses the partial correlation coefficient for karyopyknotic index and weeks of gestation at a constant amniotic fluid oestrogen concentration, $r = -0.29$, is significant at 5 per cent, but the partial correlation coefficient for karyopyknotic index with amniotic fluid oestrogen

concentration at the same weeks of gestation for female foetuses, $r = -0.31$, is also significant at 5 per cent, (for males, $r = -0.15$, which is not significant). These calculations from the results suggest that the karyopyknotic index with female foetuses is dependent on weeks of gestation ($r = -0.42$) and is significantly (5 per cent) related to the amniotic fluid oestrogen concentration even when considering specimens at the same week of gestation. With male foetuses the karyopyknotic index is dependent on the weeks of gestation and independent of the amniotic fluid oestrogen concentration at any one week of gestation.

The dependence of the karyopyknotic index on weeks of gestation with foetuses of either sex raises the question of whether this index could have a clinical application in the assessment of foetal maturity. The answer is that the variation of results at any one period of gestation is too high to allow a reliable assessment (Figure 4). Further studies of the karyopyknotic index of amniotic fluid cells in normal and abnormal pregnancies are necessary before the significance of the index can be finally evaluated.

It is suggested that the fall in the karyopyknotic index with male foetuses is due to the more rapid exfoliation of cells towards term, described by Votta, Bobrow de Gagneten, Parada and Giulietti (1968), principally from the buccal mucosa. The karyopyknotic index of buccal smears of newborn infants is between 0 and 5, which suggests that more rapid exfoliation near term produces a high per-

centage of cells with vesicular nuclei. Specimens of amniotic fluid obtained earlier in gestation when exfoliation is taking place more slowly will contain a higher percentage of degenerating cells, some of which will have pyknotic nuclei.

With female foetuses the same reasoning applies to cells desquamated from the buccal mucosa, but in addition there are the large number of cells exfoliated from the foetal vagina, which have a karyopyknotic index which falls towards term in a similar way to the maternal vaginal karyopyknotic index. This foetal vaginal epithelium is dependent on the circulating hormone levels which are reflected by the amniotic fluid oestrogen concentration. Hence the amniotic fluid karyopyknotic index with female foetuses shows a highly significant correlation with weeks of gestation and also a highly significant correlation with amniotic fluid oestrogen concentration, whereas the amniotic fluid karyopyknotic index for male foetuses only shows a highly significant correlation with weeks of gestation and not with amniotic fluid oestrogen concentration.

Evidence of the size of this exfoliation from the foetal vagina is provided by the higher mean total number of cells per cu.mm. in liquor from female foetuses (Figure 8) and also by the predominance of cyanophilic intermediate cells in amniotic fluid cell preparations from female foetuses.

The mean amniotic fluid karyopyknotic index at 40 weeks for female foetuses, 13.8, is higher than the karyopyknotic index of the cells scraped from the vulval area of the newborn infant, (0-5) and

higher than that of the maternal vaginal smear at term which in Leeton's series (1963) of ten normal pregnancies was always below 10. This cannot be accounted for by the presence of nucleate cells from the buccal mucosa or bladder because cells from these sources in newborn infants have a karyopyknotic index of 4 or less. The discrepancy is greater with male foetuses whose mean karyopyknotic index at 40 weeks is 19.8. It can be explained by the degeneration of cells after they have been shed into the amniotic fluid. Although liquor is continuously being ingested by the foetus, there is unlikely to be a complete change of cell population as a result of foetal swallowing. Nuclear degeneration results in a higher percentage of cells with pyknotic nuclei. Further evidence of cell degeneration is provided by the presence, in amniotic fluid, of large anucleate cells which often show nuclear 'shadows'. These are found only occasionally in scrapings from the surfaces of newborn infants. They may be eosinophilic or cyanophilic and can be distinguished from the smaller, anucleate cells derived from the vernix, which do not take up the Papanicolaou stain.

According to MacNaughton (1969), the foetal ovary, on present evidence, is rather inactive, possibly because it is inhibited by the large amount of circulating oestrogen and progesterone. Therefore it is not surprising that the foetal vaginal epithelium responds to maternal hormone levels in the same way as the maternal vaginal epithelium. The maturation of hormone-sensitive squamous epithelium in pregnancy is thought to be due to the combined action of

oestrogens and progesterone. Leeton (1963) suggested that "the paradoxical fall in the maternal vaginal pyknotic index in association with a progressive rise in oestriol level is possibly due to a correspondingly greater rise in progesterone". This is unlikely, as pregnanediol excretion levels off and may even fall slightly near term (Russell, Paine and Coyle (1957), Shearman (1959)) while oestriol excretion is still rising. This paradoxical association of a low karyopyknotic index with high oestriol excretion was also reported by Aubry and Nesbitt (1970) who found that a maternal vaginal karyopyknotic index above 10 in the last trimester of pregnancy was accompanied by a falling urinary oestriol. The converse was not always true, which suggested a poor sensitivity of the relationship. Stamm, Rawyler and Riotton (1959) reported a closer relationship of a 'poor' vaginal smear in pregnancy to low oestrogen excretion than to any other hormonal variant.

Two alternative explanations are offered for this paradoxical behaviour of vaginal epithelium, both maternal and foetal, in the later weeks of pregnancy. The first is that an increase in oestrogen in the presence of the high normal level of progesterone in pregnancy results in a lower karyopyknotic index. This is supported by the work of Nesbitt, Aubry, Goldberg and Jacobs (1965) who showed that administration of oestrogens in the presence of adequate progesterone tended to lower the maternal karyopyknotic index or to produce no effect if it is already low. An alternative explanation would be the action of a third, as yet unknown, hormone on the vaginal epithelium. The first

explanation is more easily acceptable as it involves hormones which are known to be present but there are still unknown mechanisms in pregnancy, in particular those responsible for the onset of labour, and it is possible that as yet unknown substances which influence the maturation of maternal and foetal vaginal epithelium in pregnancy will be discovered.

5.2 Amniotic Fluid Oestrogen Concentration

The results of this series showed a rise during the last trimester of pregnancy which was most marked after 35 weeks of gestation, before which only one result over 30 μg/100 mls was recorded, and a small fall after 40 weeks. Statistical analysis confirmed a positive correlation between the amniotic fluid oestrogen concentration and weeks of gestation, $r = +0.42$ with female foetuses and $r = +0.50$ for male foetuses, both of which are significant at 5 per cent (Figure 5). The values for amniotic fluid oestrogen concentration with male foetuses are higher than with female foetuses but the difference is not significant.

In addition to the pregnancy with an anencephalic foetus, five specimens of amniotic fluid taken between 36 and 41 weeks had an oestrogen concentration below 10 μg/100 mls. One of these, number 79, was from a patient with essential hypertension who gave birth to an infant of birth weight below the fifth percentile. One other case was of rhesus iso-immunisation but the infant was not severely affected. The other three were apparently normal. Three results were above 200 μg/100 mls. Two of these were at 39 and 40 weeks, but one was at

32 weeks. This was a specimen from a rhesus mother and was contaminated with blood, but the exceptionally high oestrogen value could not be accounted for. These three results were not included in the statistical analysis because they would have caused a distortion of the figures which would not have represented the general trend of the results. Figures for amniotic fluid oestriol concentration reported by Schindler and Herrmann (1966) go up to 300 μg/100 mls near term, but their spread of values is more even than in this series where all the other values are below 100 μg/100 mls, and the mean value at term is 54 μg/100 mls. Differences in absolute values between series probably reflect slight differences in the methods of estimation. Such differences between laboratories, in Aberdeen Edinburgh and Belfast have been analysed by Klopper, Michie and Aleem (1971), who concluded that although there was a great difference between the actual levels measured from the same specimens, either method would place any particular result in the same category in terms of their own criteria of normal values.

5.3 Maternal Urinary Oestriol

The 59 results in this series are mostly within normal limits, but their mean value at term, 14.7 mg/24 hrs, is below the mean for normal pregnancies (Figure 6). It is possible that the low mean values for maternal urinary oestriol and for amniotic fluid oestrogen concentration are due to the selection of patients in this series, all of whom had indications for hospital delivery, for amniocentesis or caesarean section, therefore, they were not an absolutely normal group.

The correlation of maternal urinary oestriol with amniotic fluid oestrogen concentration, r = +0.20 is not significant. This compares with significant positive correlations reported by Berman, Kalchman, Chattoraj and Scommegna (1968), r = 0.69, and by Klopper (1972), r = 0.72, and a generally positive correlation found by Michie and Livingstone (1969) and by Bolognese, Corson, Touchstone and Lakoff (1971) but no statistically significant relationship. The absence of a significant correlation (r = +0.04) between maternal urinary oestriol and weeks of gestation in this series is probably due to the fact that 49 of the 56 urine specimens were collected between 36 and 42 weeks gestation, and the remaining seven specimens, between 29 and 34 weeks. These do not provide a representative spread of the results throughout the weeks of gestation. The significant correlation between amniotic fluid oestrogen concentration and weeks of gestation supports the suggestion that amniotic fluid oestrogen estimations may be a more reliable means of assessment of foetal well-being.

Further data on maternal urinary oestriol obtained from the Institute of Obstetrics and Gynaecology at Hammersmith Hospital established that there is no significant difference between mean values for male and female foetuses at the same period of gestation. This observation implies that oestriol levels, as reflected by the maternal urinary excretion, do not account for the sex difference in the karyopyknotic index.

5.4 Cytology of Amniotic Fluid

The cell morphology described agrees with the findings previously reported by Van Leeuwen, Jacoby and Charles (1965), Huisjes (1968a, 1968b), Wachtel, Gordon and Olsen (1969), Votta, Bobrow de Gagneten, Parada and Giulietti (1968) and Bishop and Pollock (1970). The decrease in karyopyknotic index with advancing gestation conflicts with the statement by Lind and Billewicz (1971) that 'cornified' cells appear at 36 weeks and come to equal the number of 'pre-cornified' cells at 37-38 weeks gestation, which implies a rise in karyopyknotic index to about 50 per cent. The examination of cells from foetal and amniotic surfaces for comparison with the cells in the amniotic fluid confirms the findings of most previous workers. Appearances suggest that the 'parabasal' type cells originate from the amnion, as suggested by Van Leeuwen, Jacoby and Charles (1965) and Wachtel, Gordon and Olsen (1969), not from the urinary tract as suggested by Huisjes (1970). Sections of amnion from full term placentae showed columnar, cuboidal and flattened epithelium, but columner epithelium was the predominant type in the placental amnion, flattened epithelium on the cord and cuboidal cells in the reflected amnion. These observations support those of Bourne (1962). The difference in cell content between amniotic fluid from male and female foetuses which is emphasised by this study is accounted for by the abundant, cyanophilic, intermediate cells from the female genital tract found in the vulval smears, which have a karyopyknotic index between 0 and 5 and a cyanophilic index between 92 and 100. Similar

cells are found in female infants' urine and a very few are found in male infants' urine, but the buccal mucosa from both male and female infants yields a mixture of eosinophilic and cyanophilic squamous cells which vary in proportions between individuals but do not show a significant difference between males and females.

5.5 Histochemistry

The presence of ribonucleic acid in cyanophilic squamous cells is not responsible for their basophilic reaction to the EA 50 stage of the Papanicolaou staining method. The chemistry of this stain still requires elucidation.

5.6 Total Cell Counts

The significantly higher mean total cells per cu.mm. with female than with male foetuses (Figure 8) confirms the abundant exfoliation from the female vagina which provides the basis for a cytological differentiation between sexes in the last three months of pregnancy. It also supports the statement of Rosa and Fanard (1949) that "the amount of centrifuged deposit is generally much more in the case of girls than in the case of boys", a point which had also been observed in this series. Two exceptionally high total cell counts were made, both showing more than 1500 cells per cu.mm. One was from a male which was stillborn two days later and one from a female whose Nile Blue Sulphate test for foetal maturity showed 75 per cent of fatty cells. These could be manifestations of post maturity but total cell counts on more specimens with follow up are necessary to prove this. Votta, Bobrow de Gagneten, Parada and Giulietti (1968) showed that the

total cells per cu.mm. increase with weeks of gestation. Out of 44 specimens between 38 and 42 weeks gestation their highest total cell count was 640 cells per cu.mm. at 42 weeks gestation. This suggests that 1500 cells per cu.mm. is exceptionally high. The next highest count in this series was 920 per cu.mm. for a female foetus at 40 weeks gestation. The high fatty cell count indicates that these very high counts are mainly due to exfoliation of anucleate cells of vernix type.

5.7 Pre-natal Determination of Sex

The suggestion that the sex of the foetus can be determined antenatally by differential cytoplasmic staining of amniotic fluid cells, first made by Rosa and Fanard (1949), has been confirmed and the deductions of Huisjes (1970), that the source of the increase in cyanophilic cells in the fluid from female foetuses is the infant's genital tract is supported. The acceptable percentage of cyanophilic cells indicating a female foetus varies as more quantitative results are published. (See Table XIII on page 117).

TABLE XIII

Prenatal Determination of Sex from Differential Cytoplasmic Staining of Amniotic Fluid Cells.

Comparison of Series

Authors	Number of Patients	Number Correctly Diagnosed	Percentage of Cyanophilic Cells	
			Highest Male Count	Lowest Female Count
Rosa & Fanard (1949)	40	40	-	-
Arendzen & Huisjes (1971)	67	66	20	22
Bennett, Morris & Davey (1972)	31	30	20	24
Nelson (1973)	29	28	42	50
Hudson	48	48	20	37

There is agreement between three out of four authors that 20 per cent of cyanophilic cells is the highest figure with a male foetus. The gap between the highest figure for a male foetus and the lowest for a female foetus in published series is too small to be diagnostic in every patient. The larger interval in this series, 20 to 37 per cent, (Figure 7) is thought to be due to the exclusion of the small 'parabasal' type amnion cells from the counts. When cyanophilic cell counts fall between 20 and 37 per cent it is suggested that the diagnosis of foetal sex should be made with caution, but the presence of navicular cells is a strong indication of a female infant. Alternatively Barr bodies may be looked for, but experience of this series has shown that the cyanophilic cell count is more conclusive. The choice of Papanicolaou or Harris-Shorr stain is a matter of personal preference. The reliability of this method of determining foetal sex is low before 30 weeks gestation, according to the findings of Nelson (1973). The clinical application of knowledge of foetal sex after 30 weeks is very limited at the moment. However, this marked cytological difference should be taken into account in all observations of amniotic fluid cytology and it has practical implications with regard to the estimation of foetal maturity by cytological methods.

5.8 Determination of Foetal Maturity

The Nile Blue Sulphate staining method of Brosens and Gordon (1966), picks out anucleate cells of vernix type with adherent fat. These would be expected to occur in similar numbers regardless

of the sex of the foetus but when the fatty cells are very few in number the percentage could be lower with female foetuses than male foetuses because of the larger number of cells exfoliated from the female genital tract. This is unlikely to upset the important implication of the test which depends on whether or not there are 10 per cent or more fatty cells present. If there are, the foetus is said to have reached 38 weeks maturity. The fatty cell count is known to give false negative results in at least 10 per cent of cases when the foetus is in fact mature. It would be of interest to know if these cases are equally distributed between male and female foetuses.

The method of Huisjes (1970), which consists of counting the percentage of anucleate, eosinophilic, 'polygonal' (vernix type) cells will be only minimally affected by the cytological differences between male and female foetuses. Attempted assessment of foetal maturity by this method from preparations used in this study was unsuccessful. The identification of 'polygonal' cells was too difficult to be useful, except in the obvious cases when the polygonal cells were in plaques. If this method is used it is suggested that the number of 'polygonal' cells be expressed as a percentage of the eosinophilic squamous cells rather than as a percentage of all the cells, to avoid lower assessment of foetal maturity with female foetuses.

The point-scoring method of estimation of foetal maturity suggested by Lind and Billewicz (1971) will be influenced by the cytological differences between amniotic fluid from male and female foetuses. They define three broad categories of maturity; namely,

before 32 weeks, where basal type cells equal squamous cells until 30 weeks, after which squamous cells predominate; 33-36 weeks, where the total number of cells increases and basal cells disappear; and 37 weeks or more, where anucleate squamous cells appear and signify the approach of term. The relatively high proportion of parabasal type cells with male foetuses may give a deceptively low score in weeks of gestation, between 30 and 37 weeks, as was found by Bennett, Morris and Davey (1972), with 14 per cent of the female foetuses in their series. In another paper, Lind (1970) recommends the use of haematoxylin and eosin stain instead of Papanicolaou. This method would not differentiate the male from the female foetuses. If a correction were to be made, according to the sex of the foetus, it would be necessary to use Papanicolaou or Harris-Shorr staining methods.

The observations in this thesis suggest that cytological assessment of foetal maturity should state the sex of the foetus as well as the percentage of fatty cells. A total cell count may also be useful if further work confirms that very high counts are consistent with post maturity because the Nile Blue Sulphate test is known to give false low results in some cases.

SUMMARY AND CONCLUSIONS

Estimations of karyopyknotic index and amniotic fluid oestrogen concentration were made on 108 specimens of amniotic fluid which were obtained by amniocentesis from women between 25 and 42 weeks pregnant. The results show that the karyopyknotic index falls with advancing gestation as the amniotic fluid oestrogen concentration rises. Attention was drawn to cytological differences between amniotic fluid from male and female foetuses by a greater fall in the karyopyknotic index towards term with female foetuses than with male foetuses and also by the correlation between the karyopyknotic index and amniotic fluid oestrogen concentration which was highly significant with female foetuses but not significant with male foetuses.

The cytological difference between amniotic fluid from male and female foetuses obtained during the last three months of pregnancy was demonstrated in two ways. First, counts of total cells per cu.mm. on 38 specimens showed that amniotic fluid from female foetuses contains on average significantly more cells than amniotic fluid from male foetuses. Second, differential cytoplasmic staining by Papanicolaou and Harris-Shorr methods showed that amniotic fluid from female foetuses contains a large number of cyanophilic squamous cells, whereas the cells in amniotic fluid from male foetuses are predominantly eosinophilic. Counts of the percentage of cyanophilic squamous cells in 48 specimens of amniotic fluid provided the correct diagnosis of the sex of the foetus in every case and established criteria for a reliable method of diagnosis of the sex of the foetus during the last three months of pregnancy.

Comparison of the cyanophilic squamous cells in female specimens of amniotic fluid with cells obtained from the surfaces of newborn infants and from amnion demonstrated that they are only found in large numbers in the vulval region of the female infant and it is reasonable to assume that they accumulate there after exfoliation from the vaginal epithelium. The large numbers of these cyanophilic intermediate squamous cells in the female specimens of amniotic fluid account for the lower karyopyknotic index with female foetuses.

The fall in the amniotic fluid karyopyknotic index towards term in pregnancies with a female foetus is comparable with the fall in maternal vaginal, karyopyknotic index during the last ten weeks of pregnancy, which suggests that the foetal vagina responds in the same way as the maternal vaginal epithelium to the circulating hormones, and not to the amniotic fluid oestrogen concentration. The higher karyopyknotic index with male foetuses is attributed to the relatively lower total number of cells which includes more pyknotic cells due to cell degeneration in the amniotic fluid after exfoliation.

Histochemical tests showed that ribonucleic acid and glycogen are present in the cytoplasm of the cyanophilic squamous cells but their removal did not affect the cyanophilia obtained with Papanicolaou stain.

Figures for maternal 24-hour urinary oestriol excretion in 82 pregnancies at 38 weeks gestation were analysed statistically but there was no significant difference between values for pregnancies with male as compared with female foetuses.

The significance of the cytological difference between amniotic fluid from male and female foetuses lies at present mainly with cytological tests for foetal maturity which depend on differential cell counts. This is because the large numbers of intermediate squamous cells from the genital tract may give female foetuses an advance in maturity over male foetuses.

The fall in the karyopyknotic index of the amniotic fluid cells towards term could have clinical implications if further studies were to show differences between values in normal and abnormal pregnancies, which could be used as a guide to foetal well being.

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