Antenatal diagnosis of genetic disease / edited by A.E.H. Emery.

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Elements of Medical Genetics

ALAN E. H. EMERY MD PhD DSc FRCPE FRSE
Professor of Human Genetics, University of Edinburgh

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In many countries, including the United Kingdom, with liberalization of the abortion laws it is legal to terminate a pregnancy if there is a substantial risk that a fetus might have some serious physical or mental handicap. The ability to detect such disorders in the fetus in early pregnancy so that selective abortion may be carried out is the province of antenatal diagnosis. Interest in this subject has grown considerably in the last few years, and the object of this book is to review developments and indicate future possibilities in this field. It is hoped that it may prove useful to paediatricians, obstetricians, pathologists, geneticists and other hospital and scientific personnel who may be confronted with the problem of a couple at high risk of having a child with a serious genetic disorder. In the appendix are outlined practical details of laboratory methods of use in antenatal diagnosis.

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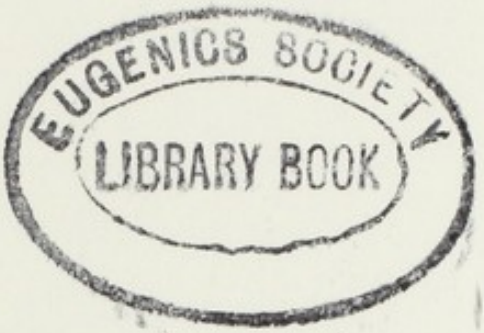

ANTENATAL DIAGNOSIS OF GENETIC DISEASE

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Edited by

A. E. H. EMERY

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Preface

In many countries, including the United Kingdom, with liberalization of the abortion laws it is legal to terminate a pregnancy if there is a substantial risk that a fetus might have some serious physical or mental handicap. The ability to detect such disorders in the fetus in early pregnancy so that selective abortion may be carried out is the province of antenatal diagnosis. Interest in this subject has grown considerably in the last few years, and the object of this book is to review developments and indicate future possibilities in this field. It is hoped that it may prove useful to paediatricians, obstetricians, pathologists, geneticists and other hospital and scientific personnel who may be confronted with the problem of a couple at high risk of having a child with a serious genetic disorder. In the appendix are outlined practical details of laboratory methods of use in antenatal diagnosis.

I should like to express my sincere thanks to my colleagues for their enthusiasm and willingness to collaborate in the production of this book, to Dr Rosalind Skinner for her editorial help, and to Miss Brenda King, B.Sc., for preparing the index.

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1973

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Contents

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Introduction

A. E. H. Emery

Aristotle, in his *History of Animals*, refers to the possibility of being able to predict the sex of the unborn child by such criteria as the side on which fetal movements are felt, or even the general condition of the mother. In fact only in comparatively recent times have precise techniques been developed for the study of the human fetus *in utero*. In the mid-1950s several laboratories reported almost simultaneously that fetal sex could be determined by examination of sex chromatin in amniotic fluid cells (see review, Emery, 1970). Some ten years later several groups reported that it was possible not only to determine the sex of the fetus from the study of amniotic fluid cells, but that these cells could be grown and their chromosomes studied (Klinger, 1965; Steele and Breg, 1966; Jacobson and Barter, 1967). In this way the chromosome constitution of the fetus could be determined. Later, Nadler (1968) showed that cultured amniotic fluid cells could also be used for the antenatal diagnosis of certain biochemical disorders. These reports paved the way for subsequent investigators and in the last three to four years there have been considerable advances in this field. It is the purpose of this book to review some of these developments, discuss techniques and indicate some of the problems and future possibilities.

Techniques which are, or may become, valuable in the antenatal diagnosis of genetic disease may be divided into those which study the fetus directly and those which study the fetus indirectly from changes in the mother's blood or urine (Table 1). Thus it has been found that a small proportion of the lymphocytes in the mother's circulation have an XY sex chromosome constitution if she is carrying a male fetus (Walknowska, Conte and Grumbach, 1969). Whether or not this finding has any relevance and might be expoited in antenatal diagnosis is not yet known. The study of metabolites in maternal urine is already being investigated from the point of view of its possible value in antenatal diagnosis (see pp. 118 & 120).

Table 1. *Techniques for antenatal diagnosis*

A. *Direct* (*fetal*)
1. Radiography
   (a) skeletal
   (b) soft tissue (amniography, fetography)
2. Sonography
3. Electrocardiography
4. Fetoscopy
5. Biopsy
   (a) membranes
   (b) placenta
   (c) fetus
6. Amniocentesis

B. *Indirect* (*maternal*)
1. Blood, e.g. fetal lymphocytes
2. Urine, e.g. oestriol excretion

Techniques for studying the fetus directly include radiography for skeletal abnormalities (Russell, 1969), amniography, whereby radio-opaque material is injected into the amniotic cavity and outlines the placenta and fetal soft tissues (Queenan and Gadow, 1970). and fetography whereby contrast medium with an affinity for the vernix caseosa is used and outlines the fetal soft tissues (Agüero and Zighelboim, 1970). These techniques, as with sonography which may also detect certain congenital abnormalities (Donald, 1971) and fetal electrocardiography which has been used to diagnose congenital heart block *in utero* (Järvinen and Österlund, 1963), are only of proven value in later pregnancy. Fetoscopy is a new development which may well prove the most valuable technique for the early detection of congenital abnormalities (see p. 49). The biopsy of fetal membranes (Mohr, 1968) or the placenta (Alvarez, 1966; Aladjem, 1968) are still very much at the experimental stage.

The approach most widely adopted is the study of amniotic fluid and its contained cells obtained by amniocentesis usually through the anterior abdominal wall. This book will be concerned almost exclusively with the results of such studies, directed at the antenatal diagnosis of genetic disease.

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2

Indications for Amniocentesis

K. Fried

The object of this chapter is to discuss in what situations amniocentesis is likely to be indicated. This is not an easy subject to define, but there are several broad clinical, statistical and moral considerations to be considered. Some of these considerations were discussed in 'Amniocentesis; *ad hoc* meeting of experts' (M.R.C., 1970) and by Emery (1970) and Nadler (1971).

Minimum Requirement and Parental Agreement

Amniocentesis is indicated for diagnostic purposes only if parents agree to act on the results. If for any reason the parents will not consider interruption of pregnancy under any circumstances, then there can hardly be any justification for amniocentesis. Obviously, apart from research, there is no indication for amniocentesis late in pregnancy when therapeutic abortion is no longer possible.

In every case, the possible risks of amniocentesis should be explained to the parents. Any diagnostic limitation of the procedure should be clearly stated. For example, amniocentesis with only chromosomal investigation can only exclude the possibility of a chromosomal aberration, but there is always the possibility of other genetic diseases, mental retardation or serious congenital abnormalities. To ensure that all the risks and limitations of the procedure have been explained to the parents, a form of consent is used in this Department (Fig. 1).

Low Procedure Risk

For the procedure to be justified, the risks of amniocentesis naturally have to be much lower than the genetic risk or probability that the fetus will be affected. While the risk of the child being affected can be calculated more or less exactly, the order of magnitude of the risks of amniocentesis to the mother and fetus are as yet unknown. It is very hard to estimate these risks because

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CONSENT FOR AMNIOCENTESIS

We, the undersigned have requested that amniocentesis should be carried out. The risks and limitations of this procedure have been explained to us by ..... and we appreciate that the procedure may have to be repeated. We understand that the birth of a normal child cannot be guaranteed from the results of studies on amniotic fluid and its contained cells.

Signed.....

.....

Date.....

Hospital.....

Case No.....

Figure 1. Form of consent for amniocentesis.

they depend on many factors such as the stage in pregnancy when the procedure is performed, the proportion and amount of amniotic fluid removed, the actual technique used, and the skill and experience of the obstetrician (see pp. 19 & 30). Nadler and Gerbie (1970) claim that reported maternal and combined fetal morbid-

ity and mortality from amniocentesis is less than 1 per cent. It should be noted, however, that in the *International Directory of Genetic Services* (Lynch, 1971), there were only eight genetic centres in the world which claimed to have performed more than 100 amniocenteses for genetic indications during 1970. Specific maternal mortality risks of the order of 1/100,000 cannot therefore be excluded. Few long-term maternal complications. except perhaps the possibility of initiating or aggravating blood-group sensitization, can be expected. Long-term effects on the unborn child have not yet been assessed. The risks involved in artificially terminating a pregnancy should also be borne in mind, including the possibility of future sterility.

In view of the uncertainty of the risks of amniocentesis it seems likely that many geneticists might therefore hesitate to recommend the procedure if the genetic risk of an abnormal child is less than, say, 5 per cent. However, there are those who recommend amniocentesis when the genetic risk is of the order of 1 per cent (Nadler and Gerbie, 1970).

Amniocentesis, Genetic Risks and Prevention of Birth of an Affected Child

Genetic disorders may be conveniently divided into those which are due to a single gene defect (*unifactorial*) and may be X-linked recessive, autosomal recessive or autosomal dominant, those due to many genes plus the effects of environment (*multifactorial*), and those due to a recognizable chromosomal abnormality.

X-linked recessive disorders

In those X-linked recessive disorders where the hemizygous affected male cannot yet be diagnosed *in utero* (e.g. haemophilia and Duchenne muscular dystrophy) it is possible to sex the fetus of a known carrier mother. If the fetus proves to be a male then selective abortion can be offered, since there is a 1 in 2 chance it will be affected. Of course, half the daughters of a known carrier will also be carriers and therefore the procedure may have to be repeated in the next generation if in the meantime no method has been found for diagnosing the affected male *in utero*. A more efficient means of dealing with X-linked disorders would be the selective abortion of female fetuses of fathers with X-linked disorders, since all his daughters must be carriers but all his sons normal. In this way, except for new mutations, the incidence of such disorders as haemophilia and Becker muscular dystrophy

would be reduced considerably.

If the mother is not known to be a carrier and there is no accurate test for the carrier state, the probability of her being a carrier may have to be calculated. It would seem that only if the probability of a mother being a carrier is high (10 per cent or more) should amniocentesis be offered. In a female with a 10 per cent probability of being a carrier the chance of a male fetus being affected is 5 per cent.

Autosomal recessive disorders

Most families with autosomal recessive disorders are ascertained after the birth of an affected child (retrospective prevention —see p. 140), and the mother has a risk of 25 per cent to any further children by the same father. In the future it may be possible to screen all couples and identify those where both parents are heterozygous for a recessive disorder, and therefore at high risk of having an affected child, in time to perform an amniocentesis. Already, the detection of heterozygotes for Tay-Sachs disease and sickle-cell anaemia by population screening is practised in some countries, although only the antenatal diagnosis of the former disease is possible at present.

Some serious congenital malformation syndromes are inherited as simple recessive traits which could be diagnosed *in utero* by fetoscopy.

Autosomal dominant disorders

The risk for an affected child in a fully penetrant autosomal dominant disorder, if one parent is affected, is 50 per cent. When penetrance is not complete, the risk is the product of the penetrance and 50 per cent. As no enzymatic defect has yet been identified in any dominant disorder, amniocentesis is at present of no use in the antenatal diagnosis of autosomal dominant diseases. However, some rare, serious congenital malformations are inherited as autosomal dominant traits (e.g. lobster claw), and could be diagnosed *in utero* by fetoscopy. Furthermore, genetic linkage with marker traits demonstrable in amniotic fluid or its contained cells is another possible approach to the problem of detecting dominant disorders *in utero* (pp. 125 & 148).

Multifactorial disorders

Many of the commoner congenital malformations are believed to be due to the effects of many genes plus the effects of environment (multifactorial). The risks of recurrence of such disorders

are usually less than 1 in 20 unless there is more than one affected individual in the family when the risks may exceed 1 in 10 (see p. 148).

Chromosomal disorders

The average risk of any woman in the population having a child with a serious chromosomal abnormality is about 0.2 per cent. This risk increases with maternal age. The risk if one of the parents is a translocation carrier, or a mosaic, may be much greater.

Down's syndrome is perhaps the most common cause of anxiety and indication for amniocentesis. The incidence increases with maternal age and is of the order of 15 per 1000 in mothers over the age of 40 (Wahrman and Fried, 1970). In the case of Down's syndrome due to an inherited chromosome translocation (D/G or 21/22) the recurrence risk may be as high as 1 in 10.

Amniocentesis for Maternal Anxiety

Amniocentesis is sometimes performed because of maternal anxiety consequent on the fear of having an abnormal child. The mother may have anxiety even though her risk of having an affected child is no more than that of any woman in the population. Maternal anxiety may justly be thought important in considering the indications for amniocentesis. For example, women over the age of 40 have a risk between 1 and 2 per cent of having a child with Down's syndrome. This might be considered a 'small risk' yet many women would be unwilling to take this risk and would wish the pregnancy terminated unless it could be shown that the fetus was normal.

Sex Prediction

Parents may wish to know the sex of the fetus for personal reasons, with interruption of pregnancy if the fetus is not of the desired sex. They may even state that only if amniocentesis is performed is there a possibility of their allowing the pregnancy to go to term. The ethical considerations of such a request are beyond the scope of this chapter, and they are closely related to those of abortion. In the present climate of opinion it is unlikely that any obstetrician would in fact agree to carry out amniocentesis for this reason.

Amniocentesis and Research

Criteria for doing amniocentesis are most important, for only when they are known is it possible to evaluate the procedure properly. Amniocentesis was frequently performed in the past for research purposes, and was carried out when it had been decided in advance to interrupt the pregnancy anyway. Indications for amniocentesis for diagnostic purposes in 155 pregnancies were tabulated by Nadler and Gerbie (1970) (Table 2). The reader of the most up-to-date tabulation of criteria for doing amniocentesis (Lynch, 1971) will find among the criteria, 'Viral infection in the first trimester' and 'History of exposure to mutagens'. The possibility that the above criteria were those for interruption of pregnancy and that the amniocentesis was done as a research investigation, cannot be ruled out. Doing amniocentesis for research can be justified if informed consent is obtained.

Table 2. *Indications for amniocentesis in 155 pregnancies.* (*From Nadler and Gerbie,* 1970, *and published with permission*)

Indication	No. of pregnancies	Outcome of pregnancy
Chromosomal	132	
Translocation carrier	22	7 with Down's syndrome (therapeutic abortion); 15 normal
Maternal age > 40 yr	82	2 with Down's syndrome (therapeutic abortion); 80 normal
Previous trisomic Down's syndrome	28	1 with Down's syndrome; 27 normal
Familial-metabolic	23	
Carrier of X-linked recessive disorder	7	2 males (therapeutic abortion); 5 females (normal)
Pompe's disease	8	1 with Pompe's disease (therapeutic abortion); 6 normal 1 normal (spontaneous abortion)
Lysosomal acid phosphatase deficiency	2	1 affected (therapeutic abortion) 1 normal
Metachromatic leukodystrophy	1	1 affected (therapeutic abortion)
Mucopolysaccharidosis	2	2 normal
Generalized gangliosidosis	1	1 normal
Maple-syrup urine disease	2	2 normal

Conclusions

The immediate and long-term risks to mother and fetus of amniocentesis carried out in early pregnancy have yet to be assessed. It might therefore be argued that amniocentesis and the possibility of selective abortion might only be offered to parents when the risks of an abnormal child exceed 5 per cent, or in exceptional circumstances where maternal anxiety is an overriding consideration. At present, parents most likely to be offered antenatal diagnosis are in cases of (1) unifactorial disorders where the affected fetus can be diagnosed *in utero*, or sex prediction in X-linked recessive disorders where it is not yet possible to detect the hemizygous affected male *in utero*; (2) congenital malformations where the risks of recurrence exceed 1 in 20 and where fetoscopy is available; (3) chromosomal disorders, particularly in Down's syndrome where the mother is over 40 years of age, or where one of the parents is a translocation carrier.

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3

Amniocentesis: Technique and Complications

J. B. SCRIMGEOUR

Amniocentesis is the technique by which a sample of amniotic fluid is aspirated from the amniotic sac. Research in relatively recent years has shown that from the study of amniotic fluid and its contained cells fetal wellbeing may be assessed. Such studies have inevitably led to an increase in the number of indications for amniocentesis. Depending on the indication and diagnosis expected from the amniotic fluid, amniocentesis may be required at virtually any stage in pregnancy from ten weeks gestation to beyond term. This must therefore be regarded as a direct challenge to the obstetrician to perfect the technique of amniocentesis as far as possible, so that minimal complications occur in mother and fetus, immaterial of what stage of gestation the procedure is carried out. An equal, but related challenge is to ensure that the specimen of liquor reaches the laboratory uncontaminated by skin, bacteria or blood of maternal or fetal origin.

Emphasis must be placed on the normal variation of the amniotic fluid content at any specific period of gestation and the variation which so often occurs in the course of normal pregnancy. These variables make it imperative that amniocentesis be carried out both at a gestation when normal values are already known and that the investigation can be carried out reliably by the laboratory concerned, using a technique familiar to that laboratory and on which the normal range was based. In any specific case a broad explanation of the procedure should be made to the patient, underlining the advantage that the examination of the liquor would have in her particular case, and a consent form for amniocentesis should then be signed by the patient (see Fig. 1). It follows that the result obtained from the study of the amniotic fluid should be acted on. Should no action be contemplated, whatever the result, it is doubtful whether amniocentesis is justified in the first instance.

The Development of Amniocentesis

While amniotic fluid has fascinated obstetricians over the centuries, a reliable and safe technique to obtain it before labour has only recently been perfected. In a monograph entitled *Contributions to the Theory of Liquor Amnii and Its Origin,* Prochownick (1877) gave a detailed analysis, both qualitative and quantitative, of amniotic fluid obtained from intact sacs or by using Kluge's 'egg-membrane piercer' to puncture the membranes and then aspirate amniotic fluid into a syringe. Actual amniocentesis was suggested by Schatz (1882), but not until 1919 does there appear a report of it having been carried out, at first in a case of polyhydramnios (Henkel, 1919), and a year later in a case where no polyhydramnios was present (Wormser, 1920).

The introduction of X-rays allowed the first real practical use of amniocentesis in placental localization by amniography (Menees, Miller and Holly, 1930). Soon after, an account was published not only of the technique of amniocentesis in amniography, but also the problems of interpretation of the films and possible complications of the procedure (Munro Kerr and Mackay, 1932). These complications have since been attributed to the irritant effect of the radio-opaque substances used at the time. In one instance a twin pregnancy was noted on the X-ray, but the amniogram showed only the lower amniotic sac with a filling defect, interpreted as the placenta, extending down to the cervix. At delivery, however, no placenta praevia was present but the misinterpretaion was due to *both* amniotic sacs extending to the level of the internal cervical os. Such reports, where multiple pregnancies have caused problems in interpretation of results, recur throughout the literature on amniocentesis. The moral in this early report must therefore be heeded, and care taken to avoid this pitfall. Rivett (1933) described the use of amniocentesis in ten cases of hydramnios and pointed out that in these cases the liquor did not appear to be under pressure.

Further impetus to the development of the technique of amniocentesis came in 1950 and 1952 when Bevis reported analyses of liquor in cases of rhesus isoimmunization. These observations were subsequently confirmed and a practical policy for the management of such patients, based largely on the results of amniotic fluid analyses, was pioneered by Liley (1961).

The majority of amniocenteses until recently have been carried out between 30 weeks gestation and term, but since 1960 it has been extended into the second trimester and even earlier.

Technique of Amniocentesis in General

General principles

The needle is usually inserted through the maternal anterior abdominal wall, through two layers of peritoneum, myometrium and decidua or placenta, into the amniotic sac. Structures which are particularly liable to be damaged during this procedure are the placenta, umbilical cord or even the fetus itself. The technique must therefore aim to minimize the chance of any such complication.

Asepsis

The procedure should be carried out under full antiseptic conditions. The hazard of amnionitis and subsequent fetal death was emphasized by Liley (1960) when he reported two perinatal deaths due to this cause—the *only* deaths attributable to amniocentesis is his series of 151 patients and in 216 amniocenteses. A technique which the author has adopted is the generous application of a reliable skin antiseptic, such as thiomersal 0.1 per cent (Merthiolate) in 50 per cent ethyl alcohol. A non-touch technique is used, each swab soaked in thiomersal being discarded after use and another two applications made. In obese patients or those with generalized skin infection where the risk is higher, a further precaution against skin contamination may be taken by inserting a larger bore needle through the skin and then inserting the amniocentesis needle down the lumen of the larger needle (Mandelbaum, 1970).

Placental localization

In principle, the placenta should be avoided if possible. Whether a technique is used to actually locate the placenta or whether clinical palpation of the abdomen alone is relied upon, depends on a number of factors: (1) the equipment available and the experience of the radiologist or obstetrician who interprets the results, (2) the gestation at which the amniocentesis is carried out, and (3) whether or not it is vital to obtain a blood-free specimen of liquor.

Of the 21,000 amniocenteses reviewed in the literature before compiling this chapter, the majority were undertaken in the management of patients with rhesus isoimmunization. Penetration of the placenta is liable not only to give a blood-stained specimen but also to precipitate fetomaternal haemorrhage or fetal exsanguination if a fetal vessel on the surface of the placenta is pierced. It is doubtful if penetration would precipitate abruption of the

placenta if the pregnancy were not complicated by rhesus isoimmunization (Zilliacus and Eriksson, 1958).

A subsequent rise in antibody titre is equally difficult to interpret but in only a few instances can amniocentesis *per se* be blamed. The complications of placental trauma are detailed on page 25.

Route—transabdominal or transvaginal?

The only advantage a transvaginal route has over the transabdominal method is that it is possible to obtain liquor by this technique before 12 weeks gestation. Therefore this technique would only be indicated in those genetic disorders where study of the liquor or its cells would give a diagnosis of normality or otherwise in fluid obtained before the thirteenth week. In such instances, if affected, the pregnancy could be terminated most safely by suction aspiration of the uterus (Scrimgeour *et al.*, 1972). Aspiration of fluid before 12 weeks would, of course, allow more time for cell culture within the period when termination per vaginum is still possible. Technical difficulties and the greater risk of infection are largely the reasons why this procedure has not been widely adopted and why the transabdominal route is preferred.

Site of amniocentesis

The actual point where the needle is inserted depends on whether the placental site is known and whether the exact location of the fetus at that time is known. Before 20 weeks gestation, the placental site may be found by ultrasonography or radioisotope scan, but the fetus is so mobile and small that it cannot be held out of the way of the approaching needle. After 20 weeks gestation, however, it is possible to ascertain both factors, i.e. the placental site and the position of the fetus, and the needle can be inserted accordingly.

Transvaginal amniocentesis has been done through the anterior fornix, the cervical canal or the posterior fornix. The actual technique depends entirely on the experience and success of each individual operator.

Bladder preparation

If amniocentesis is carried out when the patient is beyond 20 weeks gestation, the bladder should be emptied immediately prior to the procedure. When a transabdominal approach is planned prior to 20 weeks gestation, however, the author has found it helpful to have the bladder full for two reasons: (1) if placental localization is carried out by ultrasonography the full bladder serves as a useful landmark, and during the insertion of the needle

the bladder helps to hold the uterus out of the pelvis allowing easier access to the amniotic sac; and (2) it also stabilizes the uterus and prevents excess dextro rotation, which would expose the left uterine vessels to possible puncture. No urinary infections, symptoms or haematuria have resulted from this technique. If a transvaginal approach is used, the bladder should be empty.

Outpatient or inpatient?

Virtually all amniocenteses are carried out on an outpatient basis unless the patient lives a long distance away or unless any complication arises either during or immediately after amniocentesis. Patients should be observed for at least one hour following the procedure, particularly if carried out before 20 weeks gestation.

Premedication

No premedication is used by the author, but local anaesthetic, 1 per cent plain lignocaine, is infiltrated into the site of insertion. Should no such local anaesthetic be used, then a sedative such as promazine may be given.

Fetal heart monitoring

Using a standard Pinard fetal stethoscope it is simple to check the fetal heart rate both before and after amniocentesis if the gestation is more than 28 weeks. At an earlier gestation, however, an ultrasonic aid may be used, such as the Sonicaid or Doptone. Such a precaution ensures that the pregnancy is continuing before amniocentesis is carried out and that no fetal distress has been caused by the procedure when checked subsequently.

Fetomaternal haemorrhage

This complication of amniocentesis is of particular importance in patients who are known to be rhesus-negative. This implies that the patient's blood group and rhesus type must be determined before amniocentesis is carried out. Should a fetomaternal haemorrhage occur then, unless the father of the child is known to be rhesus-negative, IgG may be given.

Fetal cells, identified by the Kleihauer-Betke technique, occur in varying amounts in normal adults (Jensen and Sorensen, 1965; Cassady, Cailleteau, Lockhard and Milstead, 1967). It is therefore important that a specimen be taken before amniocentesis is carried out. It is also noteworthy that fetal cells disappear quickly from the maternal circulation. A fetal cell count should therefore

be taken immediately after amniocentesis and 30 minutes later (Woowang, McCutcheon and Desforges, 1967).

Documentation

It is essential that *any* amniocentesis, even an unsuccessful attempt, should be clearly indicated on the antenatal record card. A rubber stamp inked with a distinctive colour will particularly draw the attention of the paediatrician to this point. Should the baby develop any peculiar behaviour, then a more thorough check for injury, such as pneumothorax (Creasman, Lawrence and Thiede, 1968) would be made. A register of amniocenteses, as suggested by Kihara (1970), would help to establish the true incidence of complications following this procedure.

Equipment

Apart from the equipment necessary for placental localization, that required for the actual amniocentesis is simple and easily autoclaved on a preset tray (Plate 1).

On tray:

- 4 squares (100 × 100 cm)
- 1 gown
- 1 paper towel
- 2 gallipots
- 1 pair sponge-holding forceps
- Cotton-wool balls.

Added to tray once opened:

- 1 pair surgeon's gloves (disposable)
- 1 21 g gauge needle
- 1 2 ml syringe } for local anaesthetic
- 1 20 ml syringe (disposable)
- 1 20 gauge 4 in needle with stylus.

Also to hand:

- Skin antiseptic (thiomersal 0.1 per cent)
- Local anaesthetic (plain lignocaine 1 per cent)
- Sterile plastic container for specimen.

The initial amniocentesis may be done in the X-ray department or in the ultrasonogram room as soon as the placenta has been localised. Should a further amniocentesis be necessary on

the same patient in whom the placenta has already been localized, this may be done in a separate room set aside for the purpose.

Technique of Amniocentesis after 20 Weeks Gestation

Placentography, as a preliminary, may be done using ultrasonography, radioisotopes such as Cr^{51} (Bowman and Pollock, 1965), radioiodinated serum albumen (Hibbard, 1966) or soft tissue placentography (Free and McDonnell, 1970). Freda (1965, 1966, 1967) and Crystle and Rigsby (1970) have used a combination of techniques involving radioisotopes and thermography. Placentography is particularly indicated in obese patients. These techniques will at least give a guide as to where the bulk of the placenta lies and on some occasions the actual margin of the placenta may be delineated. Amniocentesis can then be done in an area where the placenta is absent.

The patient should empty her bladder before lying as comfortably as possible on a firm couch. A brief explanation of the procedure is again made, and the lower abdomen then exposed. The height of the uterine fundus is noted, and palpation of the fetus carried out to find in particular the area in which the limbs are lying or whether the presenting part can be eased out of the brim of the pelvis to allow access to this region (Fig. 2 A and B). Queenan (1967) advocates insertion of the needle in the area of the fetal neck posteriorly where there is usually a good-sized pool of liquor. Haworth, Milic and Adamsons (1968) found that by using the suprapubic region in more than 6000 consecutive punctures no complications were attributable to the direct effect of amniocentesis, and they felt it unnecessary, using this technique, to perform placentography. Wiklund (1969) also advocated the suprapubic approach.

In the final analysis, the site chosen will depend on the experience of the operator and on where he feels he can confidently obtain a clear specimen of liquor with minimal trauma to mother and fetus. The fetal heart should then be checked, maternal venous blood taken for fetal cell count and the aseptic ritual adhered to.

Using a fine needle the skin is infiltrated with local anaesthetic and with a 21 gauge needle the infiltration is continued down to the level of the peritoneum. Freda (1965) points out that if the uterus contracts either *in toto* or at the point where the needle is being inserted, then the sensation on penetration of the uterine wall is more difficult to assess; for this reason, it is better not to

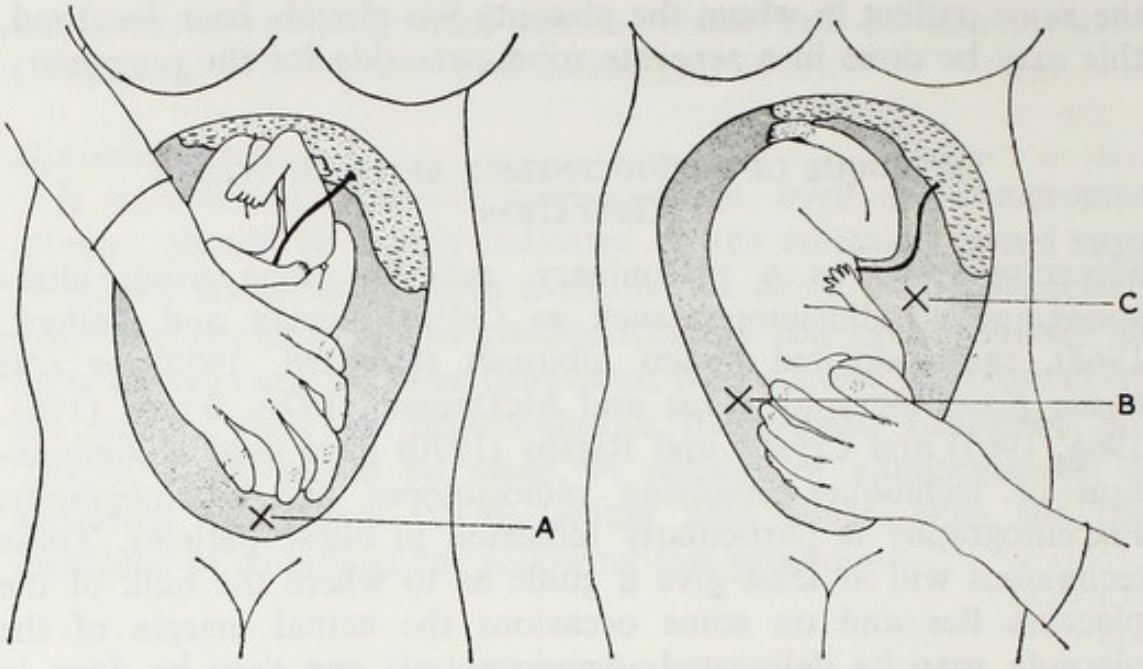


Figure 2. Sites for amniocentesis: A, suprapubic. B, nuchal. C, over fetal limbs.

infiltrate local anaesthetic into the myometrium. The disposable spinal needle should then be inserted at the same point, the fetus being confirmed by palpation to be out of the way of the needle, and the final insertion into the amniotic sac made with a sharp thrust rather than a tentative prod (Scrimgeour, 1971). With experience a definite sensation can be recognized as the needle penetrates rectus sheath, myometrium and finally amnion. Should the needle then swing about violently it is most likely that the fetus itself has been penetrated and the needle should therefore be withdrawn slightly until fetal movement settles. At this juncture the stylus is withdrawn and usually the liquor will well up in the hub of the needle if the operator is patient and the needle is in the correct place. If the liquor does not well up, it is often worthwhile rotating the needle through 180 degrees as this would appear to clear any obstruction at the tip, be it amnion or fetal parts. Do not swing the needle from side to side on its axis as separation of the choriodecidual space with resultant bleeding is almost certain to take place (Fort, 1971).

A syringe may now be attached to the needle and gentle suction applied, 10 ml of liquor may be aspirated with ease, and if it is to be used for bilirubin estimations in the management of rhesus immunization it is then put into a dark container, otherwise a sterile plastic container is sufficient.

If blood is aspirated with some difficulty into the syringe it is likely that the tip of the needle is still in the myometrium, the stylus is therefore reinserted and the needle inserted further into

the wall. If blood is aspirated easily, or wells into the hub on removing the stylus, it is probable that the tip on this occasion is in the placental bed. The aspirated blood should be kept for analysis to determine whether it is maternal or fetal in origin, and if the latter the fetal blood group and rhesus type may be determined. A reassessment of the original placental localization in relation to the current fetal position should be made, and if it is felt that it is unlikely that liquor will be obtained in any other site then the needle, with stylus *in situ*, should be inserted further and aspiration again attempted. If, however, in these circumstances, another site or route (Stenchever and Cibils, 1968) is possible, then this should be chosen in preference.

Having removed sufficient liquor for the investigation concerned, the needle is swiftly withdrawn. No dressing is generally required. The fetal heart is again checked and further maternal specimens of blood taken to check for fetomaternal haemorrhage.

Complications

Amniocentesis as a technique would appear on superficial examination to be a potentially hazardous procedure both for the mother and the fetus. Inevitably some authors have reported these complications in full, while others have minimized them. In an effort to put amniocentesis into perspective, a review of some 21,000 amniocenteses after 20 weeks has been carried out, and as far as possible those done for hydramnios, amniography and in the course of intrauterine transfusions have been excluded (Tables 3 and 4). Results for amniocentesis before 20 weeks gestation are detailed separately (page 29 and Tables 5 and 6).

Maternal death

Among the 21,000 amniocenteses reviewed only one maternal death has been reported (Hesseltine, 1962). The hazard of infection was underlined in the following brief report: 'The prospect of contamination of the amniotic cavity is inescapable. It is unlikely with proper care that a lethal complication will occur. Even so, some years back, a mother and her child died from infection' (Hesseltine, 1962). In fact, even when amniocentesis was done for hydramnios, amniography and intrauterine transfusion only one other report of a maternal death could be found. In a patient with hydramnios, Williams and Stallworthy (1952) stated briefly that 'one patient had died of complications following the formation of a uterine haematoma at the puncture site'. No further details in either case were given.

Table 3. *Transabdominal amniocenteses after 20 weeks gestation without placental localization (amniograms and intrauterine transfusions excluded)*

Author	Year	Number of amniocenteses	Placental trauma	Failed or bloody tap	Liquor with fetal blood	Fetal trauma	Comment
Alpern *et al.*	1966	101	N	11	N	N	3 patients liquor never obtained; 1 case amnionitis
Asensio & Pelegrina	1968	500	N	20	N	N	2 premature rupture of membranes a few minutes after tap through lower uterine segment
Beecham *et al.*	1962	13	N	4	N	N	
Bevis	1952	158	N	N	N	None	
Burnett & Anderson	1968	N	1	1	N	N	Definite fetal vein rupture by needle causing fetal death (two other deaths but after taps for polyhydramnios)
Cary	1960	89	N	18	N	None	One patient had 12 attempts but no liquor obtained
Cassady *et al.*	1967	54	N	28	N	N	10 with fetomaternal haemorrhage
Creasman *et al.*	1968	1	N	1	N	1	Tension pneumothorax of fetus; skin healed quickly
Fairweather & Walker	1964	178	N	16	4	1	10 specimens pure blood; 1 fetus injured but hydropic; no more premature labours than in 'control' group
Haworth *et al.*	1968	> 6000	N	N	N	N	No complications; suprapubic approach—no longer does placentography
Hesseltine	1962	N	N	N	N	N	MATERNAL DEATH DUE TO INFECTION
Hinselmann *et al.*	1970	73	N	15	8	N	Compare with results when placenta localized (Table 4)
Jensen & Sorensen	1965	55	1	22	4	N	Definite fetal vein rupture causing fetal anaemia (Hb 53%); 5 with fetomaternal haemorrhage
Liley	1960	216	41	17	11	No direct trauma	49 other possible placental punctures; 2 fetal deaths attributed to amnionitis

Table 3 (*continued*)

Author	Year	Number of amniocenteses	Placental trauma	Failed or bloody tap	Liquor with fetal blood	Fetal trauma	Comment
Macbeth & Robertson	1961	46	N	3	N	N	
Mackay	1961	223	N	Occasionally	N	N	Maternal abdominal wall emphysema
Mandelbaum	1970	3000	N	69	N	1	2 cases amnionitis; 1 case premature labour; 2 intrauterine deaths after bloody tap but more probably due to rhesus than amniocentesis
Misenhimer	1966	4	N	3	N	N	1 case delivered 30 h after tap; haematocrit 34%; died after 1 h
Parrish *et al.*	1958	50	None	9	N	None	All non-rhesus affected patients; no premature labours
Peddle	1968	410	N	N	N	N	46 with fetomaternal haemorrhage
Queenan	1967	604	N	289	59	N	1 case of premature labour
Robertson	1964	252	N	'A number'	N	N	One fetal death attributed to blood-stained tap
Stenchever & Cibils	1968	>200	1	'frequent'	N	N	1 case major fetal vessel lacerated causing fetal death. Suprapubic approach—still enters intervillous space 'frequently'
Walker & Jennison	1962	500	N	50	N	N	5% failed tap; 5% blood-stained
Westberg & Margolis	1965	166	N	'several'	N	N	1 case premature labour with amnionitis
Woowang *et al.*	1967	54	N	14	N	N	2 intrauterine deaths attributed to difficult amniocenteses
Zipursky *et al.*	1963	13	N	8	2	N	Fetomaternal haemorrhage of 10, 20, 30 and 50 ml
	*Total	12,960	43/521 (8·2%)	597/5,551 (10·8%)	88/1139 (7·7%)	3	1 maternal death; 9 fetal deaths

* Total refers only to reports where the relevant information was given.
N=no details.

Table 4. *Transabdominal amniocentesies after 20 weeks gestation with placental localization (amniograms and intrauterine transfusions excluded)*

Author	Year	Method of Localization	Number of amniocenteses	Placental trauma	Failed or bloody tap	Liquor with fetal blood	Fetal trauma	Comment
Bowman & Pollock	1965	Cr^{51}	402	N	20	N	N	5 to 50 ml fetomaternal haemorrhage
Crystle & Rigsby	1970	Radioisotope and thermogram	416	N	4	1	N	case fetal anaemia (Hb 4 g%) but no evidence of fetal or placental trauma; baby rhesus-negative —survived
Freda	1967	Radioisotope and thermogram	5000	N	29 (Freda, 1965)	N	N	
Free & McDonnell	1970	Soft-tissue placentography	185	N	58	13/39	N	1 fetal death attributed to fetomaternal haemorrhage (60 ml)
Hibbard	1966	$RISA^{132}$	48	N	1	N	N	
Hinselmann *et al.*	1970	Ultrasound	107	N	15	1	N	Good comparison of results when no placental localization (Table 3); 1 with fetomaternal haemorrhage
Mayer *et al.*	1961	Soft-tissue placentography	253	1	N	N	N	1 fetal death due to laceration of succenturiate lobe
Walker	1970	'Recommended'	2000	N	N	N	N	1 fetal death attributed to fetomaternal haemorrhage
		*Total	8411	1/253 (0·4%)	127/6158 (2%)	15/562 (2·7%)	–	3 fetal deaths

* Total refers only to reports where the relevant information was given.
N = no details.

Fetal death

Twelve fetal deaths were attributed to amniocentesis itself rather than to any concomitant complication of pregnancy which may have indicated the need for amniocentesis. The fetal mortality rate for this procedure is therefore less than 0.6 per 1000 amniocenteses (Tables 3 and 4). Infection, in the form of amnionitis, caused two fetal deaths (Liley, 1960), and one died with the mother of generalized infection (Hesseltine, 1962). In two cases a major fetal placental vessel was shown to have been punctured (Burnett and Anderson, 1968; Stenchever and Cibils, 1968), and in one case a succenturiate lobe was lacerated (Mayer, Gueritat, Ducat and Lewi, 1961). Fetomaternal haemorrhage was the cause of death in two cases (Free and McDonnell, 1970; Walker, 1970). One death was attributed to a heavily blood-stained tap (Robertson, 1964) and two to difficult amniocenteses (Woowang, McCutcheon and Desforges, 1967). One delivered 30 hours after amniocentesis with a haematocrit of 34 per cent and died after one hour (Misenhimer, 1966).

By stringent antiseptic technique the three deaths due to infection may have been avoided. Both Walker (1970) and Free (1970) carry out preliminary placentography, but in spite of this two fetal deaths occurred. Placentography also failed to reveal the presence of the succenturiate lobe.

Infection

Amnionitis causing fetal death was mentioned in only three cases out of 21,000 amniocenteses, including one death in which the mother also died of generalized infection.

The very low incidence of amnionitis may be explained by the fact that amniotic fluid in itself appears to be unfavourable to bacterial growth. Sjostedt, Rooth and Caligara (1958) in 44 cases found a very low pO_2 of 11 mmHg comparable with the fetal venous pO_2 of 10 mmHg. This is much lower than the mean maternal venous pO_2 of 30 to 50 mmHg and a similar range was found intra-abdominally and subcutaneously. For most organisms such a low pO_2 range in the amniotic fluid would inhibit or at least retard growth, but such a medium would be ideal for clostridia. However, no instances of clostridial infection have been reported following simple diagnostic amniocentesis, but there have been reports of such infection following amniocentesis for termination of pregnancy (MacDonald, O'Driscoll and Georghegan, 1965). Florman and Tuebner (1969) found that until meconium is added, amniotic fluid will only poorly support bacterial growth, and amniocentesis in the presence of fetal death should therefore

be avoided. Galask and Snyder (1968) found amniotic fluid to be bacteriostatic, even when enriched with casein hydrolysate, to both Gram-positive and Gram-negative bacteria as well as to *Candida albicans.*

In spite of such an inimical environment, one case has been documented in which infection was responsible for maternal death (Hesseltine, 1962). Amnionitis occurred in two cases out of 216 amniocenteses by Liley (1960) and both resulted in perinatal death. Westberg and Margolis (1965) reported one case out of 166 amniocenteses in which premature labour occurred two days after the tap, and large numbers of *Staphylococcus albus* were cultured. Alpern, Charles and Friedman (1966) reported a similar case, where premature rupture of membranes occurred and delivery did not take place until four weeks after the amniocentesis.

Transient pyrexia was first noted by Fairweather and Walker (1964). Whether this is attributable solely to infection or perhaps to micro amniotic fluid emboli or to fetomaternal haemorrhage is debatable. Fairweather and Walker (1964) found three patients with temperatures of more than 102°F within $\frac{1}{2}$ to 2 hours after amniocentesis; two of the amniocenteses had been successful, one a failure. Walker (1970) subsequently found six to seven patients with a similar reaction. Mandelbaum (1970) also reported two patients who developed chills, fever and uterine irritability 7 days after amniocentesis. Treatment consisted of antibiotics and immediate delivery. Both infants survived. It may be that actual amnionitis is more common than these statistics indicate, but the infection clears by the time of delivery in the vast majority of cases.

Fetal trauma

This complication has most commonly been reported in association with amniography or intrauterine transfusion, but it may also happen in a standard amniocentesis. In amniography the dye may be seen on X-ray to have penetrated the fetus, and in intrauterine transfusion the fetus is often delivered shortly after the transfusion. The fetus appears to have excellent healing powers and seldom is a scratch mark found, but Creasman *et al.* (1968) reported a case of tension pneumothorax which was attributed to amniocentesis. However, Crystle and Rigsby (1970) found no evidence of puncture, laceration or trauma of baby or placenta, in a case in which the haemoglobin was 4 g per cent. The baby was unaffected by rhesus isoimmunization and a Kleihauer check on a blood-stained tap showed no fetal cells. No logical explana-

tion for this anaemia could be made, but occult transplacental bleeding may have occurred (Kristofferson, Gert Jensen and Felbo, 1962a). They also cited two other cases with blood-stained liquor and concluded that it is advisable to have blood ready for the baby on delivery if the liquor contains erythrocytes of fetal origin. Misenhimer (1966) also reported one case in which three amniocenteses had yielded only blood and, on delivery, the haematocrit was 34 per cent and the baby died after one hour. Should the fetus be lethargic, as is likely in developing hydrops fetalis, it is less likely to avoid the needle point and therefore more likely to be impaled (Fairweather and Walker, 1964).

Placental trauma and failed or blood-stained taps

When amniocentesis is carried out without preliminary placental localization (Table 3), fetal deaths, placental trauma and failed or bloody taps are more common than when the placenta is first localized (Table 4). Clearly it is therefore important to localize the placenta before carrying out amniocentesis.

Success in obtaining amniotic fluid undoubtedly improves with experience. Fairweather, Murray, Parkin and Walker (1963) reported 16 amniocenteses out of 220 in which no fluid at all was obtained. Liley (1960) also abandoned amniocentesis in 16 cases out of 216: six of the failures were attributed to an interior placenta which could not be penetrated by a $3\frac{1}{2}$ inch needle, and the remaining 10 were all associated with an anterior position of the fetus. He rightly points out that where the fetal back is anterior and closely applied to the uterine wall, particularly if the fetal head is well flexed, then there may only be a very thin layer of liquor, perhaps only 2 to 3 mm deep, which is technically extremely difficult to locate accurately. In such circumstances it is obviously better to aim to get fluid by pushing the fetal head out of the pelvis—even if a rectal examination is necessary to do this (Strand, 1967)—and inserting the needle suprapubically if the placenta is known to be absent from that area (Fig. 2, p. 18).

Related to the failure rate, but often not specified in the literature, is the actual number of attempts at varying sites which may have been used before amniocentesis was finally abandoned. Free and McDonnell (1970) failed completely on four occasions out of 43, but admitted that to obtain the remaining 39 specimens no less than 61 abdominal punctures were necessary, i.e. a failure rate of 36 per cent of needle insertions. Grobbelaar and Trott (1968) also found that if a suprapubic approach was used and the number of 'needling attempts' were limited to two instead of four or more, as in their earlier work, the number of blood-

stained fluids fell from 52 per cent to 19 per cent. In one patient, 12 attempts to obtain liquor were made by Cary (1960) but all failed and at delivery very little liquor was present. Luckily, in spite of these attempts, no damage to fetus or mother was noted. Rather surprisingly, failure to obtain liquor after 20 weeks gestation is not related to the size of the uterus.

The amount of blood contaminating the specimen also varies from series to series. Both Fairweather *et al.* (1963) and Queenan (1967) found a high incidence of macroscopic bloodstaining, 52.4 per cent and 47 per cent respectively. Free and MacDonnell (1970) in a prospective series of 39 specimens examined microscopically found only 11 had less than 100 erythrocytes per ml, 19 could be microscopically judged to be blood-stained, while the remaining nine required microscopic confirmation. Among the blood-stained specimens not all authors state whether the blood was of maternal or fetal origin. Of the 39 in Free's series, 13 (33 per cent) contained fetal cells, three of these contained more than 10,000 cells per mm^3 and one fetus (rhesus-negative) died as a result of haemorrhage. Liley (1960) reported 11 (5 per cent) cases of aspiration of fetal blood out of 216, and Fairweather and Walker (1964) reported four (2 per cent) cases out of 178 amniocenteses. In a study of 55 specimens, Jensen and Sorensen (1965) found slight blood-staining in 18 (33 per cent) and a further four (7 per cent) heavily blood-stained. Four of the 22 blood-stained specimens contained fetal haemoglobin. Such varying incidences of fetal blood in the specimens must emphasize the point made previously by Crystle and Rigsby (1970): that the source of the blood should be checked and, if fetal, blood should be ready for transfusion after delivery.

Fetomaternal haemorrhage

It is important to realize that there is no correlation between the amount of blood, fetal or maternal, found in the liquor and the amount of fetal blood found subsequently in the maternal circulation. Free and McDonnell (1970) reported four cases in which fetal cells were found in the liquor but none in the maternal venous blood, and conversely they found seven cases with fetal cells in the maternal blood but none in the liquor. It follows, therefore, that *both* liquor and maternal venous blood should be investigated to exclude fetal blood loss.

In assessing whether fetomaternal haemorrhage has taken place a baseline estimation of the number of fetal cells in the circulation must be made. Cassady *et al.* (1967) found that 1 in 31 males, 1 in 21 non-pregnant females and no less than 10 out of 34 ante-

partum patients had fetal blood in their circulation. Cohen, Zuelzer, Gustafson and Evans (1964) found that 0.05 to 0.10 cm^3 of fetal cells are found in the maternal circulation during normal pregnancy. This confirms the work of Kristofferson *et al.* (1962b) and similar figures are given by Jensen and Sorensen (1965). Woowang *et al.* (1967) pointed out that fetal cells disappear quickly from the maternal circulation so that checks for feto-maternal harmorrhage should be done immediately after amnio-centesis and again after about 30 minutes. The incidence of fetomaternal haemorrhage varies not only in terms of volume, but is related to the difficulty in obtaining liquor. Woowang *et al.* (1967) recorded eight cases out of 74, and all of these occurred in patients where difficulty had been encountered in the amnio-centesis. Cassady *et al.* (1967) reported a higher incidence (10 out of 54) but found no significant difference in fetal cell counts when amniocentesis was easy or difficult. Free and McDonnell (1970) also recorded a high incidence of haemorrhage (16 out of 39) in spite of placental localization by soft tissue placentography, and concluded that if multiple punctures were required then feto-maternal haemorrhage is more likely. Hinselmann *et al.* (1970) in a control group without placental localization found 7 per cent (5 out of 73) to have fetomaternal haemorrhage, but when placental localization was carried out less than 1 per cent (1 out of 107) had fetomaternal haemorrhage.

It must therefore be concluded that if it is possible to avoid the placenta then this should be done. Otherwise fetal blood may leak in varying volumes into the liquor or into the maternal circulation. Some authorities have found that by using the suprapubic approach (Haworth *et al.*, 1968) it is unnecessary to perform placentography, while others (Stenchever and Cibils, 1968) have found that by using this very technique the intervillous space is entered 'on frequent occasions'. For the majority of obstetricians, therefore, to obtain a blood-free specimen of amniotic fluid with-out traversing the placenta, placentography should be undertaken and the proposed site of amniocentesis modified accordingly.

Risk of increasing isoimmunization

In determining whether amniocentesis, with an associated feto-maternal haemorrhage, has increased the degree of isoimmuniza-tion the natural history of rhesus isoimmunization must be taken into account. In 409 patients, where no amniocentesis was per-formed, Kelsall, Vos and Kirk (1959) found that 55 per cent had a rise in antibody titre after 34 weeks gestation. With the use of amniocentesis, Peddle (1968) found a significant difference in

antibody titres two weeks after the procedure when compared with a control group who had not had amniocentesis. There was no relationship between the rise in antibody titre and any change in the perinatal mortality rate. The difficulty in interpreting varying antibody levels during pregnancy, with or without amniocentesis, was reported by Aickin (1971). He found that in patients with none or only mild rhesus isoimmunization in a previous pregnancy, but in the current pregnancy had a marked rise in antibody titre, this group had the lowest perinatal mortality rate. Those with a similar history but with a *lesser* rise in antibody formation had a higher perinatal mortality rate.

Abruptio placentae and premature labour

Zilliacus and Eriksson (1958) reported the complications which occur in rhesus isoimmunization and in particular found 20 cases out of 147 in which there was a varying degree premature placental separation. They therefore felt that such patients are more likely to have abruptio placenta even without amniocentesis being done. Mackay (1961) reported the deaths of two infants which may have been attributable to abruptio placentae, but in which there was considerable doubt.

The incidence of premature labour in rhesus isoimmunization is also increased. However, Liley (1960) described two cases in which amniocentesis had to be postponed for 24 hours, but in that time both patients started spontaneously in labour. Had the amniocenteses been done, then the procedure would certainly have been blamed for this.

Should the membranes rupture spontaneously but prematurely then premature labour is likely, particularly if amnionitis intervenes (Alpern *et al.*, 1966). Using a suprapubic approach for amniocentesis, the lower uterine segment will be entered. If entered too low then the puncture in the membranes may extend as far as or allow leakage of liquor into the cervical canal. This would appear to have occurred in two cases of Asensio and Pelegrina (1968) in which the membranes ruptured a few minutes after amniocentesis.

Abdominal pain

This may be attributed to haematoma formation either in the abdominal or uterine wall. A smaller number may be due to leakage of amniotic fluid or blood into the peritoneal cavity. Asensio and Pelegrina (1968) found pain at the site of amniocentesis to be the commonest complication, especially if the suprapubic area was used. In 50 per cent of cases, Parrish, Rountree, Lock and

Winston-Salem (1958) reported no particular discomfort, 40 per cent had some abdominal soreness the day after amniocentesis, 8 per cent had abdominal soreness for two to three days after amniocentesis and finally one patient out of 50 stayed in bed for one day. Abdominal emphysema was noted by Mackay (1961) in one case.

Of more serious nature, however, Sjostedt *et al.* (1958) reported one patient who complained of upper abdominal pain for half an hour after the amniocentesis, but this then subsided: this was attributed to a small intraperitoneal haemorrhage. The potential seriousness of this complication was reported by Williams and Stallworthy (1952) in a case of hydramnios which had been relieved by amniocentesis but resulted in maternal death.

Transabdominal Amniocentesis Before 20 Weeks Gestation

While the basic technique is the same as in later pregnancy, a number of additional precautions must be remembered. At 14 weeks gestation, the fundus of the uterus is just above the level of the symphysis pubis but in the obese this may be difficult to define. The fundus may also not be palpable because the pregnancy is not as far advanced as expected because of uncertainty over the date of the last menstrual period or because the uterus is retroverted and has not yet grown out of the pelvis.

It may be possible to push the uterus further out of the pelvis by a vaginal examination but further abdominal examination will almost certainly make it revert to its former position. To obviate this problem it has been found that by keeping the bladder full during the procedure it will hold the uterus out of the pelvis and also steady. If the table is at a slight Trendelenberg slope then the fundus becomes more easily palpable, and in so doing the maternal intestines move away from the uterine fundus, thus avoiding their possible perforation.

The procedure is carried out in the ultrasonography room. The principles of the procedure are again explained to the patient. She lies comfortably on the table with only a single pillow so that she is not tempted to sit up to see what is going on. Such a movement would tighten her abdominal muscles and force the intestines near the fundus of the uterus. The fetal heart may be checked using either the B scan or a Doptone machine.

Careful ultrasonography is now undertaken by an experienced operator; interpretation is helped by the presence of the full bladder and frequently a clearly defined placental 'line', or margin,

can be seen (Fig. 3). The fetal skull is outlined and a biparietal measurement made to check its actual size against the expected size. Ultrasonography at this period of gestation not only gives the above information but also will exclude a multiple pregnancy, hydatidiform mole, missed abortion or anencephaly (Donald, 1969).

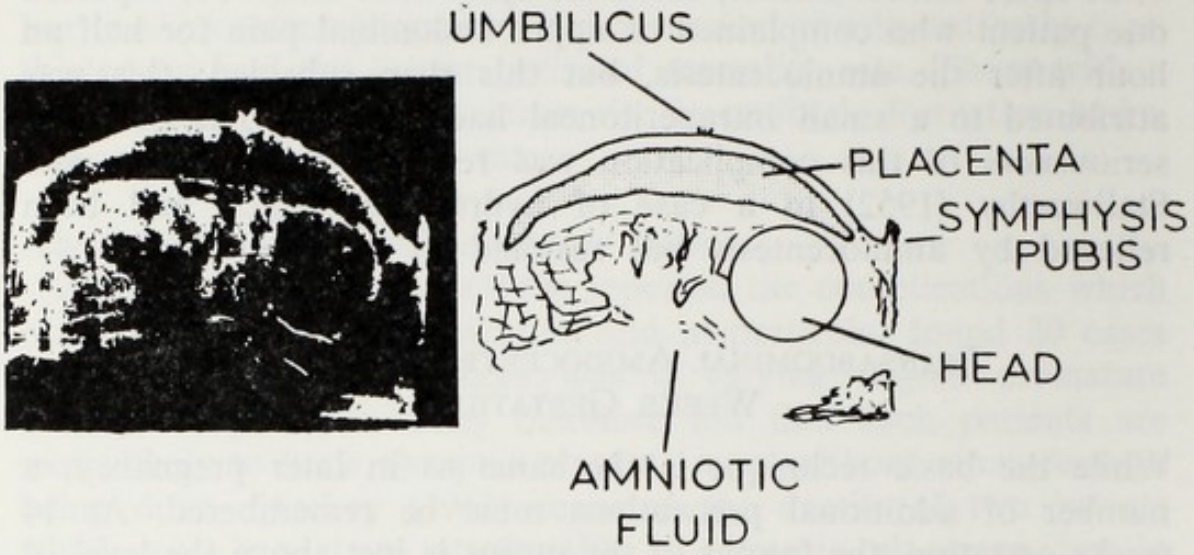


Figure 3. Ultrasonogram showing an anterior placenta.

Maternal venous blood is taken to check for the presence of fetal cells.

The site of puncture is chosen to avoid the placenta if possible and if necessary the bladder can be penetrated. No complication has arisen from this. If the amniocentesis is undertaken primarily to obtain amniotic fluid cells then it may be helpful to shake the uterus before inserting the needle, otherwise the cells will tend to sediment to the lower posterior parts of the amniotic sac. The fundus of the uterus is held steady by the left hand (Plate 2), pushed firmly against the anterior abdominal wall so that no loop of bowel may intervene. The needle is then inserted at the chosen site and if necessary at an angle. Any blood-staining of the fluid should be checked to determine whether it is of fetal or maternal origin and similarly maternal venous blood is taken to exclude any fetomaternal haemorrhage. The needle is then swiftly withdrawn, the fetal heart checked and the patient then allowed to micturate.

Complications

No maternal or fetal deaths have been reported following transabdominal amniocentesis before 20 weeks gestation (Table 5).

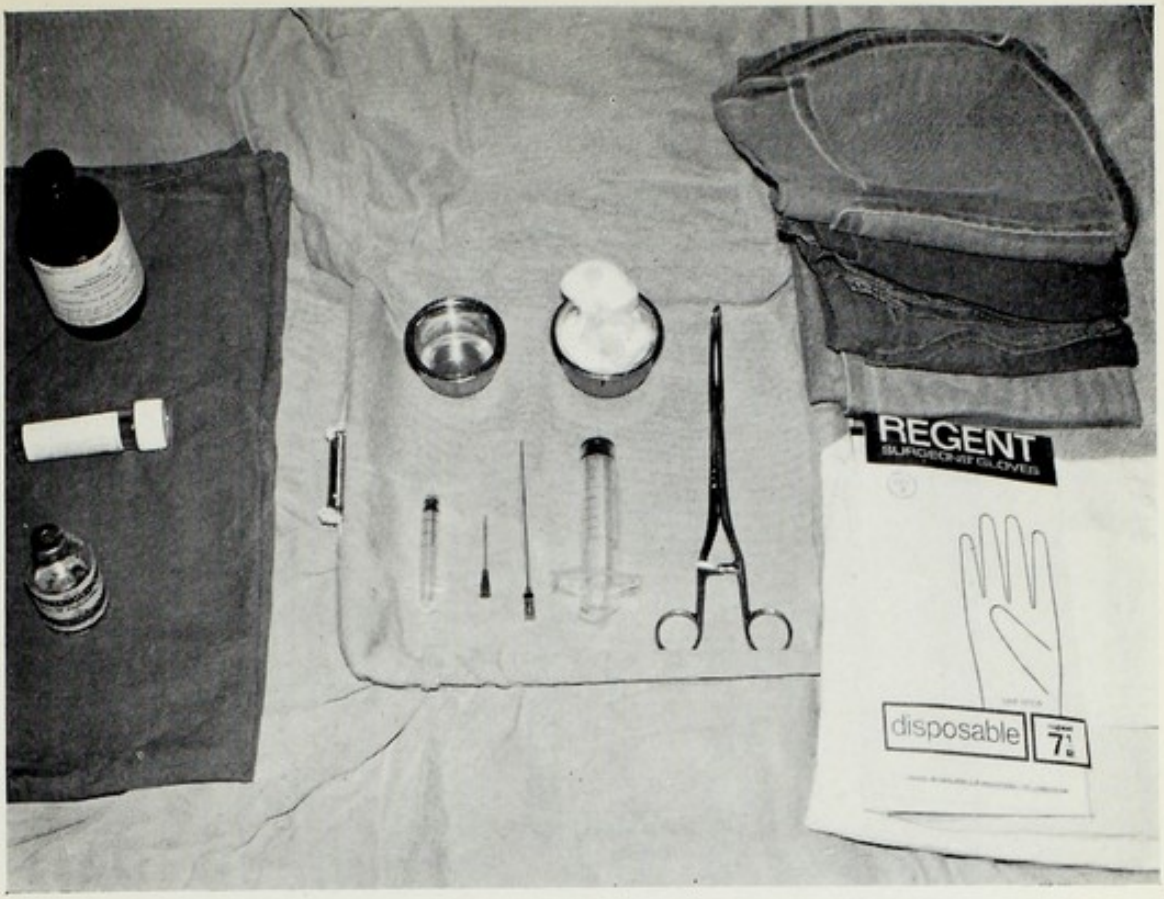


Plate 1. Equipment for amniocentesis.

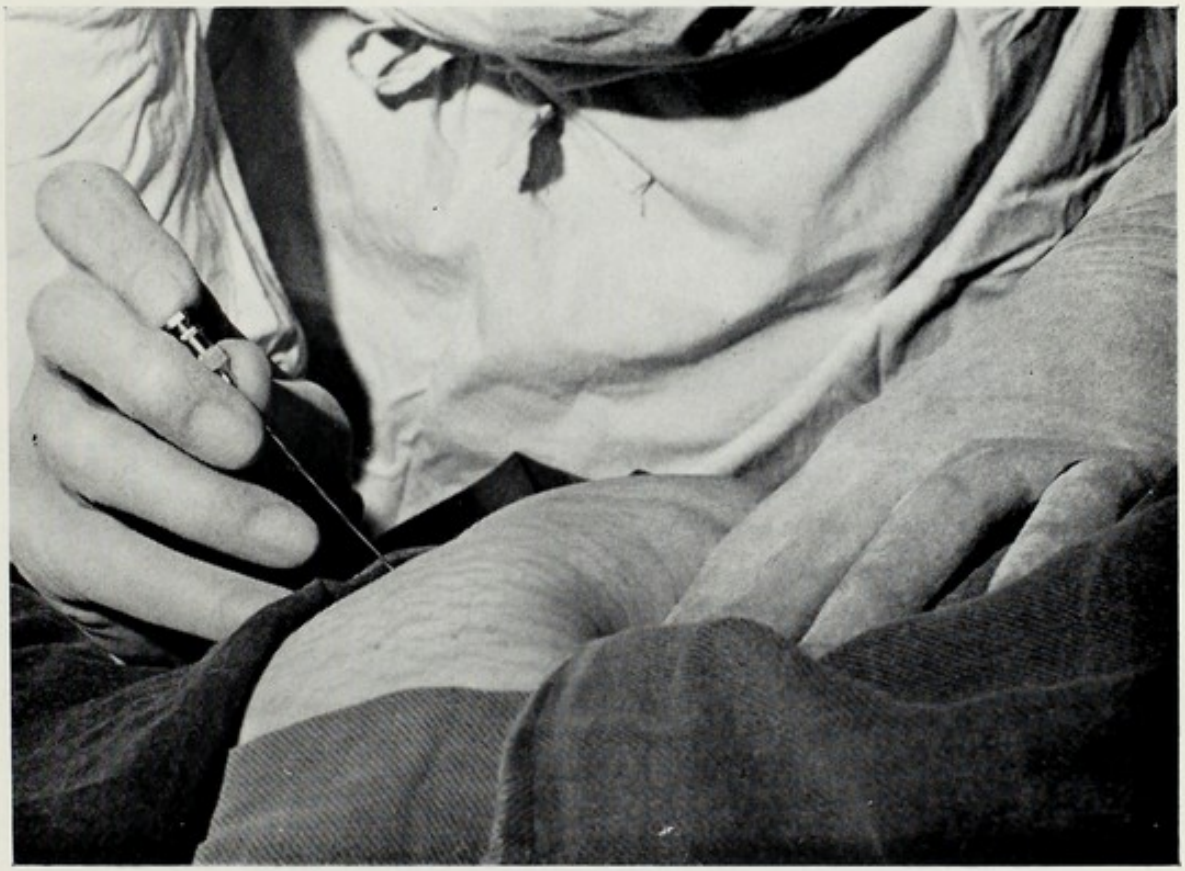


Plate 2. Amniocentesis before 20 weeks gestation.

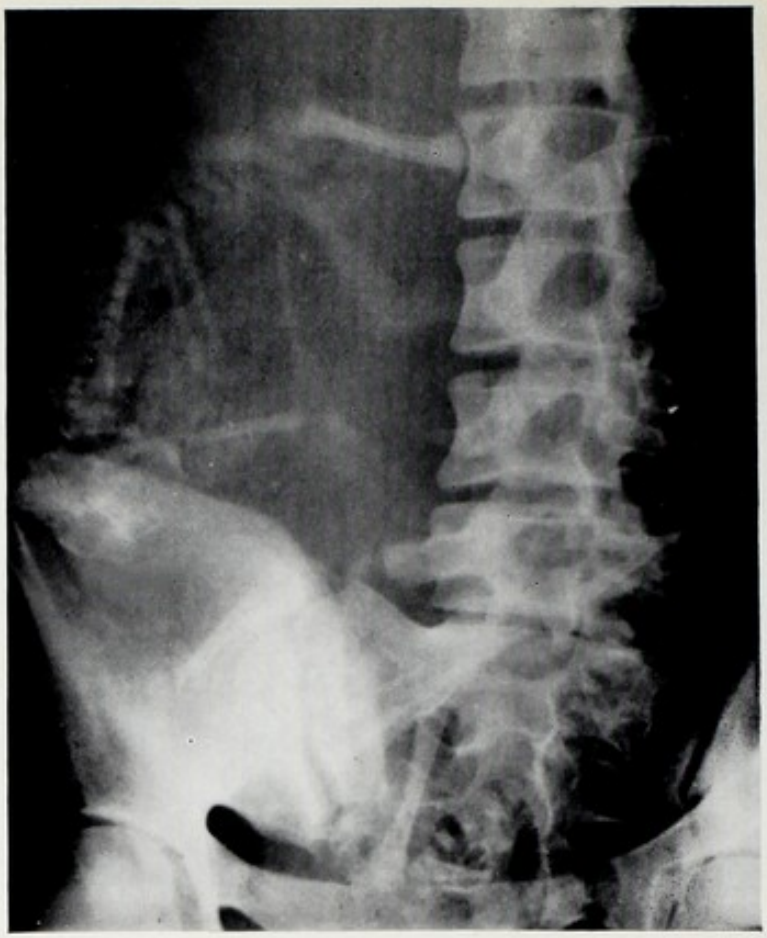


Plate 3. Twin pregnancy, anencephalic fetus on left.

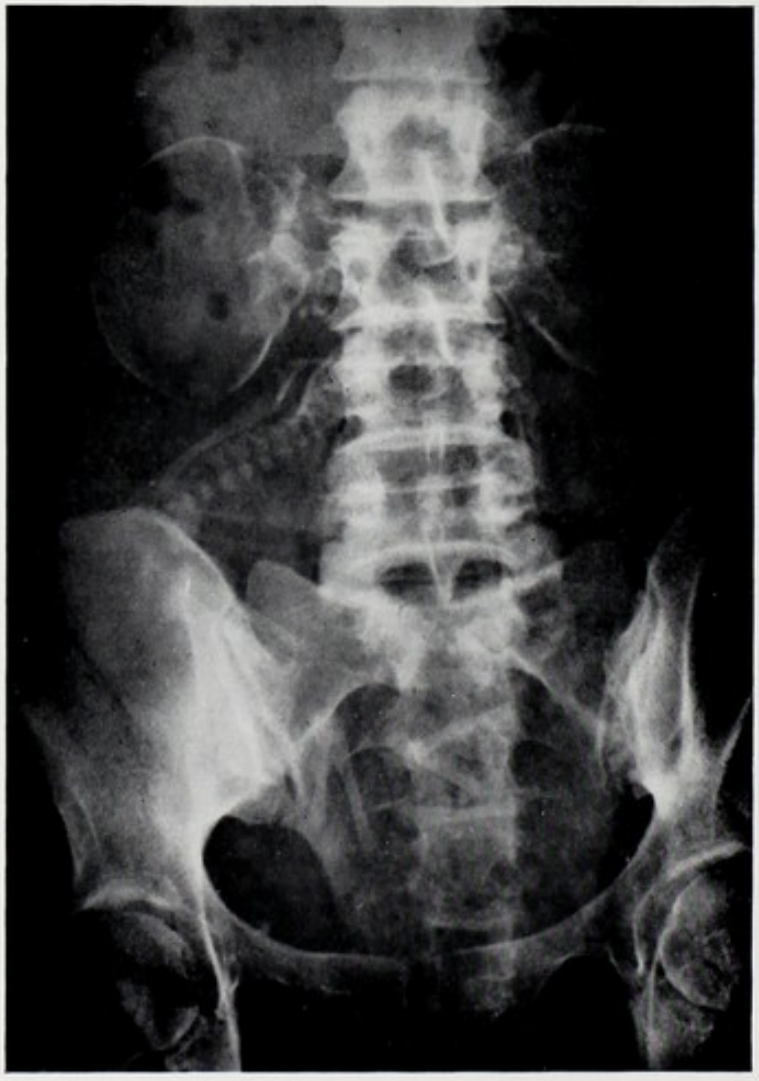


Plate 4. Twin pregnancy: thoracopagus.

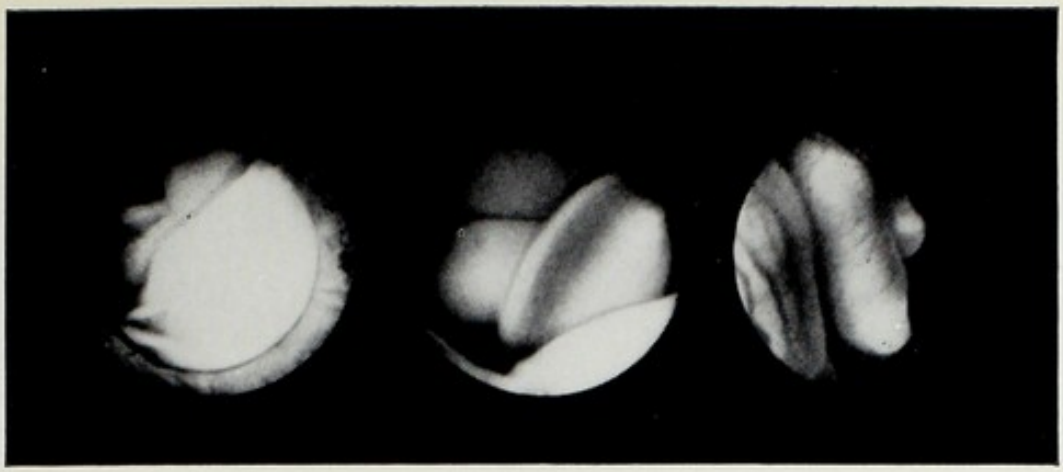
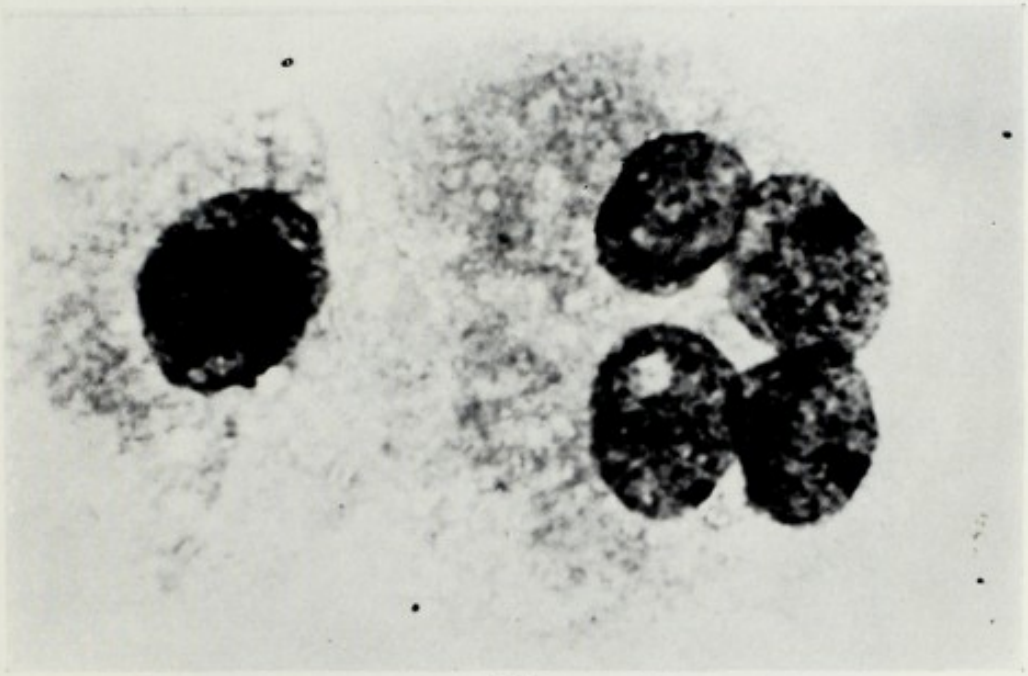
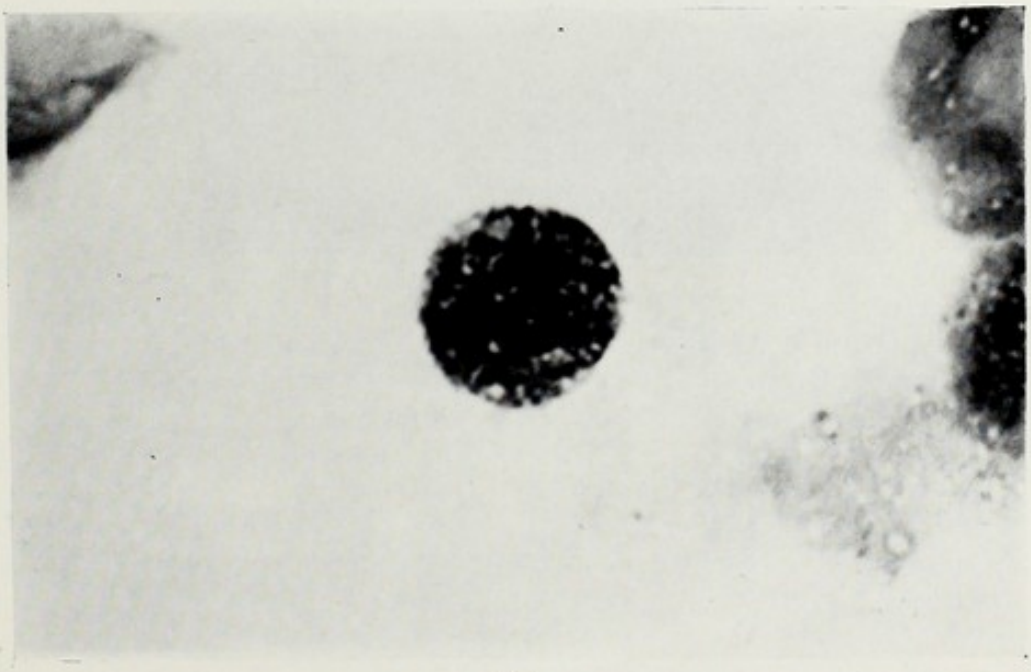


Plate 5. Fetoscopy at 18 weeks gestation. Left: View down 2 mm cannula, amnion and cord visible. Centre: Amnion, cord and fetal parts. Right: Fetal fingers.

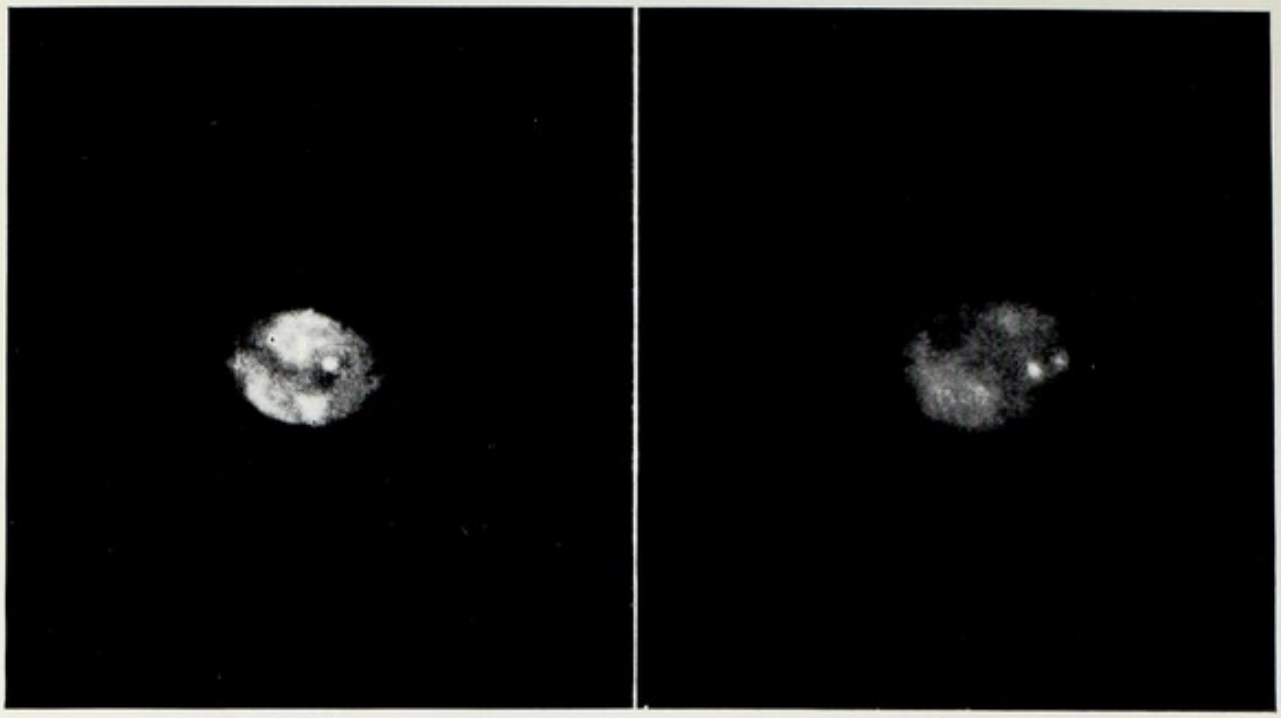


(a)



(b)

Plate 6. Amniotic fluid cell nuclei (sex chromatin).
(a) Female — sex chromatin positive. (b) Male — sex chromatin negative.



(a) (b)

(c)

Plate 7. Amniotic fluid cell nuclei (fluorescence).
(a) Male — single F body. (b) Male — duplex F body.
(c) Female — no F body.

Table 5. *Transabdominal amniocentesis before 20 weeks gestation*

Author	Year	Placental localization	Number of amniocenteses	Failed or bloody tap	Fetal trauma	Comment
Butler & Reiss	1970	None	25	4	None	If failed amniocentesis, repeat in two weeks
Emery *et al.*	1971	N	170	N	N	Risk estimated to be less than 1%
Ferguson-Smith *et al.*	1971	Ultrasound	26	1	None	Failed tap attributed to an anterior placenta
Gerbie *et al.*	1971	None	238	6	N	1 aborted one month after tap but attributed to cervical incompetence; 7 delivered prematurely—34 to 37 weeks gestation; 5 sets twins undiagnosed
Jacobson & Barter	1967	N	28	N	None	
Scrimgeour	1972	Ultrasound	42	1	None	1 failure at 14 weeks; 1 feto-maternal haemorrhage
		*Total	529	12/331 (3·6%)	None	No maternal or fetal deaths

* Total refers only to reports where the relevant information was given.
N=no details.

Gerbie, Nadler and Gerbie (1971) reported successful amniocenteses in 238 out of 244. Grossly bloody specimens represented 6.8 per cent of that total but no fetal blood was detected. Microscopic evidence of erythrocyte contamination was present in the vast majority. No improvement was in fact found when placental localization was used, but they admitted that their experience was too limited to determine the potential advantages of placentography. In a personal series of 42 amniocenteses, placental localization was carried out and the site of amniocentesis chosen accordingly. One attempt failed but was successful two weeks later. No cases of macroscopic bloodstaining of the liquor were found and in only one case was fetomaternal haemorrhage confirmed. Only 12 patients in this series continued with their pregnancies. None of these had evidence of fetal or placental trauma at delivery. The remaining 30 were terminated following the amniocentesis, by the instillation of hypertonic saline. Placental localization by ultrasonography has been found extremely useful in obtaining clear liquor. There were no multiple births in this series which confirmed the findings on sonography.

Transvaginal Amniocentesis Before 20 Weeks Gestation

Transvaginal amniocentesis is associated with a higher failure rate and a higher complication rate than the transabdominal route (Table 6). Among those cases in which the pregnancy was allowed to continue three spontaneous abortions and one premature stillbirth occurred (Riis and Fuchs, 1966).

If this approach is used before 12 weeks gestation, the patient must be reminded that at about this period of gestation she may abort spontaneously from natural causes irrespective of whether or not amniocentesis is carried out (Goodlin, 1964).

Various techniques have been tried, but most prefer to insert the needle through the anterior fornix. Initially Ruttner (1966) catheterized the patient and injected methylene blue into the bladder. He subsequently found that this precaution was unnecessary as the bladder was not traumatized nor was the needle inserted into the bladder yielding urine rather than the desired amniotic fluid.

If amniocentesis is attempted before 10 weeks gestation, the extraembryonic coelom (Fig. 4) forms a natural obstacle surrounding the amniotic sac. The extraembryonic coelom is filled with a clear mucinous material which, even if the needle does enter the coelom, will resist aspiration due to its viscosity. It is not until

Table 6. *Transvaginal amniocenteses before 20 weeks gestation*

Author	Year	Number of amniocenteses	Failed or bloody tap	Fetal trauma	Comment
Juhl & Fuchs	1956	'Small series'	N	None	Termination carried out a few days after amniocentesis; no evidence of infection or trauma found
Queenan & Adams	1964	4	1	N	All four attempted at 14 to 16 weeks gestation
Riis & Fuchs	1960	3	2	N	Two failed vaginally but successful transabdominally
Riis & Fuchs	1966	11	N	None	3 abortions and 1 premature stillbirth
Ruttner	1966	18	N	N	All pregnancies terminated
Scrimgeour	1972	17	8	None	All 10 to 13 weeks gestation. Amniotic sac seen in 10 cases, liquor obtained in 9 out of those 10
Scrimgeour	1972	14	14	None	All before 10 weeks. Failure attributed to extra-embryonic coelom and its mucinous contents. 1 mole; 1 missed abortion
Wagner *et al.*	1962	230	35	N	Failed in less than 13 weeks gestation; also in 1 case with a large cystocoele and in 1 with anterior cervical fibroid
	*Total	297	60/268 (22·4%)	None	1 fetal death

* Total refers only to reports where the relevant information was given.
N = no details.

at least 10 weeks gestation that the extraembryonic coelom disappears as an entity and the entire uterine cavity is filled by the conceptus. In some, the coelom may persist until 13 weeks gestation. The small size of the uterus before 10 weeks gestation and the presence of the extraembyronic coelom undoubtedly account for the poor results in obtaining liquor at this stage of gestation.

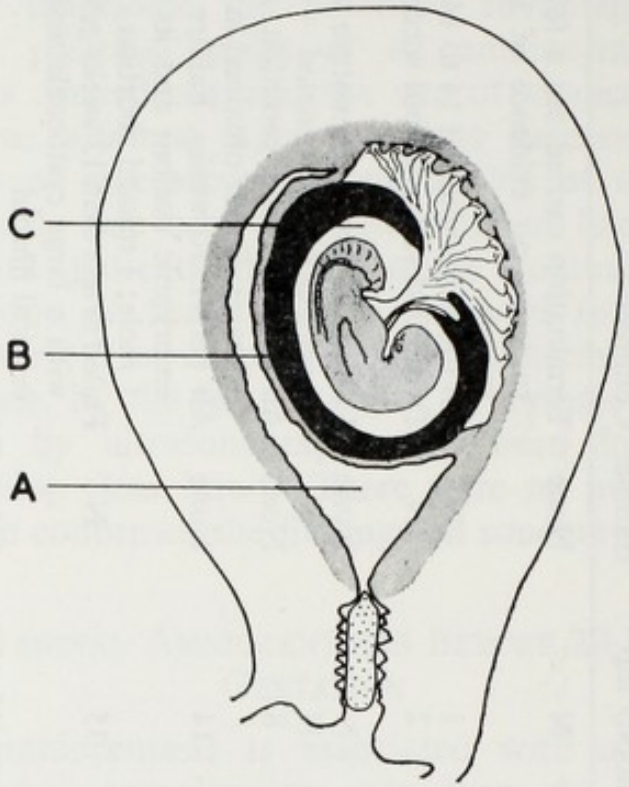


Figure 4. Pregnancy before 10 weeks gestation: A, uterine cavity; B, extraembryonic coelom; C, amniotic sac.

Another problem in this approach is that of sterilizing the rugose vaginal wall. Ruttner (1966) used iodine but cetavlon (1 per cent) gives equally good results without the hazard of local sensitivity. A preliminary vaginal examination is performed to check the exact size of the uterus, and whether it is anteverted or retroverted. In a series of 18 patients in whom termination of pregnancy was planned between 14 and 18 weeks gestation, Ruttner (1966) used a 16 cm long 2 mm bore needle inserted into the anterior lip of the cervix, along the line of the cervical canal into the amniotic sac. No problems were encountered in those in which the uterus was anteverted.

In a similar series of 230 patients for termination of pregnancy Wagner, Karker, Fuchs and Bengtsson (1962) were successful in obtaining fluid in 85 per cent of cases by inserting the needle at an angle of 15 degrees to the cervical axis. In the remaining 15 per cent, the majority were less than 13 weeks gestation, one had a large cystocoele and one an anterior cervical fibroid.

In a 'small series' of patients for whom termination of pregnancy was planned, Juhl and Fuchs (1956) allowed the pregnancy to continue for a few days after the transvaginal amniocentesis. No evidence of infection or trauma was found when the patients were terminated. Riis and Fuchs (1960) pointed out, however, that such a finding may not exclude a small lesion which would not give an immediate effect but may later cause fetal death.

Using a modified paediatric operating cystoscope introduced along the cervical canal, the author has tried to visualize the amniotic sac before inserting the needle. In 14 cases at less than 10 weeks gestation, a 'sac' was seen in only four but no liquor was aspirated. The 'sac' was undoubtedly the extraembryonic coelom. In 17 cases between 10 and 13 weeks gestation, the sac was seen on ten occasions and liquor aspirated in nine. The remaining one was a technical failure due to a blocked catheter. Of the seven in which no sac was seen, five were obscured by bleeding, one was a missed abortion and one a hydatidiform mole. All 31 cases were allowed to continue for a maximum of 14 days after the attempted amniocentesis and termination was then carried out as planned. In this series there was no evidence of infection or trauma.

Queenan and Adams (1964) allowed four pregnancies to continue after transvaginal amniocentesis at 14 to 16 weeks gestation. One attempt failed but the other three amniocenteses were successful. No complications were reported. In two patients, Riis and Fuchs (1960) reported one success while in the other two attempts to obtain liquor transvaginally failed. No complication transpired. Stenchever and Cibils (1968) found that in later pregnancy, if blood was aspirated at two different sites transabdominally, then a vaginal approach through the anterior or posterior fornix was a useful alternative. They used a Tuohy needle with trocar and reported no complications.

Conclusion

Transabdominal amniocentesis has an extremely low mortality and morbidity rate. The morbidity can be significantly reduced by preliminary placental localization. Transvaginal amniocentesis should only be attempted after 10 weeks gestation. The possible complications of this technique have still to be completely assessed.

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4

Other Techniques for Antenatal Diagnosis

J. B. Scrimgeour

That a fetus might have some serious genetic disorder may be suspected when there is a positive family history for the disorder in question, or if some associated complication of pregnancy arises such as polyhydramnios. Various techniques are now available, or are being developed, which allow the accurate diagnosis of such diseases at various stages of pregnancy. Amniocentesis is the method of choice in early pregnancy (Chapter 3), but other methods are also possible though usually only in later pregnancy.

Radiography

The use of X-rays is seldom indicated before thirty weeks gestation. Molegraphy (see p. 48) may prove an exception when other facilities are not available. The most frequent indication for a single antero-posterior film is polyhydramnios. When diabetes, multiple gestation and rhesus isoimmunization were excluded, Queenan and Gadow (1970) found that more than one-third of the patients with polyhydramnios delivered infants with congenital malformations. A straight film will allow a diagnosis of fetal skeletal abnormality in a majority of instances.

Only the fetal skeleton can be studied in detail on a straight film. Polyhydramnios frequently results in loss of definition, and sometimes the fetal spine or legs lie directly over the maternal vertebral column, making the delineation of fetal structure virtually impossible. Should this occur, an oblique or lateral film may prove helpful.

In Britain, anencephaly and other major central nervous system malformations form a major proportion of perinatal and infant mortality deaths. Anencephaly, if suspected, can easily be diagnosed in later pregnancy by a single film. All 88 patients referred for X-ray were diagnosed correctly by Russell (1969), but a further 25 patients who were not X-rayed and who presumably had no clinical features which might have raised the

suspicion of abnormality also delivered anencephalics. In the same series, a further 103 patients delivered infants with major neural tube defects, such as meningocoele, myelomeningocoele, hydrocephaly and microcephaly. In all 54 cases where antenatal X-ray was taken, craniolacunae were seen (one retrospectively).

The association between craniolacunae and such neural tube defects was first described by Hartley and Burnett (1943) but it is unfortunately not pathognomonic. Russell (1969) recorded one case with craniolacunae, but in a normal infant. Conversely, Lodge, Samuel and Heap (1967) found that in 100 patients with myelomeningocoele examined by X-ray before the age of 3 months, nine had no craniolacunae.

A standard straight X-ray will reveal major degrees of hydrocephaly, but other skeletal abnormalities may be diagnosed if a high index of suspicion prevails. In a seven year period in one hospital, Russell (1969) diagnosed antenatally three cases of achondroplasia, two cases of osteogenesis imperfecta, and one case each of symmelia and ectromelia. The last was not associated with thalidomide. Jonas (1959) detailed a prenatal diagnosis of osteogenesis imperfecta.

All patients in whom a multiple pregnancy is suspected should be X-rayed. This will not only confirm the diagnosis and the number of fetuses present, but also indicate whether or not there is any skeletal abnormality (Plate 3). When one twin is anencephalic, gross hydramnios is frequently present, and this may be relieved only by amniocentesis of the appropriate sac. Preliminary placental localization is essential in view of the large area of uterine wall covered by placenta. An amniogram is also indicated to locate the sac containing the anencephalic. It is helpful to inject 5 ml of methylene blue with the radio-opaque material, allowing time for mixing and then expose the film. If the anencephalic is in the sac into which the dye was injected, subsequent amniocentesis made at the same site will aspirate blue-stained liquor.

If, however, the anencephalic was not in the sac into which the dye was injected, another amniocentesis should be carried out at a different site and clear or only slightly blue-stained liquor aspiated. If more than two hours elapse between injection of the methylene blue and subsequent amniocentesis, then the liquor from *both* amniotic sacs will be stained blue, and therefore will not be helpful.

Monoamniotic twins may be separate or fused to varying degrees. In thoracopagus, the twins are joined from the upper sternum to the umbilicus (Plate 4). Characteristic features may

be recognized on X-ray as described by Gray, Nix and Wallace (1950): (1) heads at same level and in same place; (2) unusual extension of spines; (3) unusual proximity of spines; and (4) no change in their relative position after movement, manipulation and time.

Amniography

This technique is used to outline the amniotic sac. It was first described by Menees, Miller and Holly (1930) for placental localization, but other techniques are now available to localize the placenta without the need for amniocentesis. The advantage of amniography over a single straight film is that the former will allow diagnosis of major soft tissue abnormalities, some defects of the gastrointestinal tract and skeletal abnormalities, whereas the latter will only reveal skeletal abnormalities. Before performing an amniogram, each patient should be asked whether she is allergic to iodine, whether she has had any other similar X-ray such as a pyelogram, and whether she had any reaction to it. Patients with asthma must be monitored closely. If in any doubt, a sensitivity test should be carried out using a small dose of the proposed medium, injected intravenously. Unlike fetography, water-soluble contrast medium is usually used for amniography. Hypaque 75 per cent (sodium diatrizoate) is most popular, and 0.5 ml injected slowly intravenously over a period of 1 minute is recommended. Allergic reactions may not become manifest for 15 minutes, and this is therefore the minimum time which must elapse before proceeding further.

Technique

Following placental localization, the fetal heart is checked and the same technique as for a standard amniocentesis is followed (see p. 13). Before the injection of the medium it is essential that a free flow of liquor can be obtained. If this is not the case, the dye should not be injected, as the tip of the needle may be in a part of the fetus. Injection into the fetal biceps brachii, the pericardial sac, the urinary tract and even into cerebro spinal fluid have been recorded as a result of ignoring this precaution. The volume of medium injected is the same as the volume of amniotic fluid removed. Since the radio-opaque material is somewhat viscous, it is better warmed to body temperature before the injection is made. Berner (1967) found that Renovist 69 per cent (sodium diatrizoate 35 per cent, methylglucamine 34 per cent) had a lower viscosity than most, and can therefore be handled more easily. The volume of fluid removed varies with the gestation: 20 ml

between weeks 25 and 30, 30 ml between weeks 30 and 36, and 40 ml after week 36. In patients with hydramnios, 50 ml will be required. The needle is swiftly withdrawn after the injection, and the fetal heart checked.

The patient is now instructed to roll freely from side to side to allow mixing of the heavy radio-opaque medium throughout the amniotic fluid. If the fetus is particularly active, this will encourage the process. The X-ray is usually taken 15 to 30 min after injection, both postero-anterior and lateral views are necessary to obtain the optimal results.

The fetus actively swallows the opaque amniotic fluid and this outlines the fetal gastrointestinal tract. Some of the medium may be absorbed from the fetal gut and is then excreted into the maternal circulation via the placenta. It may also reach the maternal circulation by direct absorption through the amnion. The loss into the maternal circulation and thence into the maternal renal pelves and ureters, may be seen as early as 15 minutes after injection. In a later film, it may be seen in the maternal bladder.

Results

Amniography is currently most commonly used as a preliminary to intrauterine transfusion in the management of severe cases of rhesus isoimmunization. Advantage is taken of the fact that the fetus swallows the dye, and this is subsequently seen in the gut. This therefore serves as a target for the tranfusion needle.

Failure to see medium in the fetal gut when the fetus is known to be alive may be due to a number of fetal abnormalities. Oesophageal atresia in particular will give this result, but if the fetus has difficulty in swallowing as in cleft palate, or in anencephaly, a similar picture will result. Where the rate of swallowing is slow, as in hydrops, it may be 24 hours before the medium shows clearly in the fetal gut. A definite case of fetal diaphragmatic hernia was diagnosed and confirmed after delivery by Agüero and Zighelboim (1970b) and another case of intestinal obstruction by King and Wollschlaeger (1967) using amniography. Other abnormalities which may be diagnosed by amniography include external soft tissue swellings of the fetus or of the uterine cavity. Deformities of the fetal neural tube with associated tumour formation such as meningocoele, may be seen if the fetus is in profile on the film. If the fetus is not in profile, then the diagnosis may be missed. A fetal neck mass was diagnosed by amniography by Cash and Kornmesser (1968). A congenital abnormality of the uterus or a submucous fibroid would also show as a filling defect on amniography.

Fetography

An oil-soluble contrast medium with an affinity for vernix caseosa gives a much clearer outline of the soft tissues of the fetus than does amniography. Erbslöh (1942) suggested the name fetography for this procedure. Utzuki and Hashidzume (1941) appear to have been the first to describe the technique. In three cases of hydramnios with fetal death, they diagnosed correctly the fetal sex, and noted other details such as skin detachment, polydactyly, details of the face and subcutaneous oedema. Kräubig (1957) stated that fetography was indicated in cases of polyhydramnios, history of previous fetal malformation, diabetes, presumed fetal death and erythroblastosis. It is unlikely however, that it would now be justified in cases of presumed fetal death as other means are available for this diagnosis. In spite of the low risk attached to amniocentesis, infection is more likely to arise within the amniotic sac if fetal death exists. In erythroblastosis, Lennon (1967) found that fetography clearly outlined the anterior abdominal wall and was a valuable preliminary to intrauterine transfusion. Using Myodil (iophendylate) the outline was clear on a 48 hour film or sooner, depending on fetal activity. This medium had no harmful effect on mother or fetus.

In seven cases of hydramnios studied by fetography, Pauwen and Lammers (1948) found three cases of hydrocephalus, three of anencephaly and one with hydrops fetalis. A distended fetal abdomen due to hydrops or to intestinal obstruction would be outlined by this technique.

In patients with suspected conjoined twins, fetography can identify the site of fusion more accurately (Melin, 1967; Blunt, 1968).

Fetography initially did not prove practical because Erbslöh (1942) recommended that the amniotic fluid be drained off before the medium was injected. This has not in fact proved necessary. Agüero and Zighelboim (1970a) injected only 9 ml and found that doubling this volume did not give any clearer definition of the fetus. In some patients they used both Myodil and Hypaque to see if it would give some additional advantage, but the combination was only helpful when they wanted to outline the fetal gut. Patients with prolonged pregnancy, normal term pregnancy, twin pregnancy and transverse lie with placenta praevia were studied in the first instance. In all cases good fetal outlines were seen and fetal sex noted in one, external ear in two cases, detail of the fetal face in one instance and the fetal intestine in one instance. In the patient with a twin pregnancy, the second amniotic sac was

injected and a good fetal outline obtained. Presumably the position of the second sac could have been determined from that film and a similar procedure carried out for the first twin. In the patient with a known placenta praevia, the fetogram was satisfactory, but the placental site was not visualized.

In eight patients in whom fetal malformation was suspected or known to exist, good fetograms were obtained in all. Three fetuses were anencephalic, and in the remainder no external malformation was detectable. This was confirmed at delivery. The authors did not find that fetography was associated with premature onset of labour in this small series (Agüero and Zighelboim, 1970a).

The use of fetography is naturally governed by the presence of vernix caseosa on the fetal skin. Brosens, Gordon and Baert (1969), using a fat-soluble medium, Ethiodan (ethyliodophenyl undecylate), injected only 6 ml. Between 28 weeks and 38 weeks gestation, the fetus was almost completely outlined. Between 38 weeks and term, the outline of the fetal limbs and abdomen becomes patchy and in the majority of cases a clear outline was not seen. Confusion may arise if fetal movement occurs when the film is exposed, but this will also show as blurring of the long bones and if necessary another film can be taken. In the post-mature infant, the outline disappears from the limbs and abdomen, but may persist on the back and on the head of the fetus. Fetography may therefore be used as a rough guide to fetal maturity.

Placental and Amnion Biopsy

When fetal tissue is required for examination or culture it is theoretically possible to obtain it from the placental bed. If a diagnosis of fetal normality or abnormality is to be based on such tissue, it must obviously be of fetal origin rather than maternal. If the latter is inadvertently obtained, the result will be completely misleading.

The technique of placental biopsy was pioneered by Alvarez (1961), who was studying the morphopathology of the placenta. A similar technique has been used to study pressures in the inter-villous space during prelabour or the early first stage of labour (Schwarcz, Althabe, Fisch and Pinto, 1967).

Successful placental biopsy depends on an accurate knowledge of the placental site. It is only possible when the bulk of the placenta lies on the anterior abdominal wall (Fig. 5). In early pregnancy, the placenta can be localized using ultrasonography (see p. 52) or in later pregnancy by soft tissue placentography.

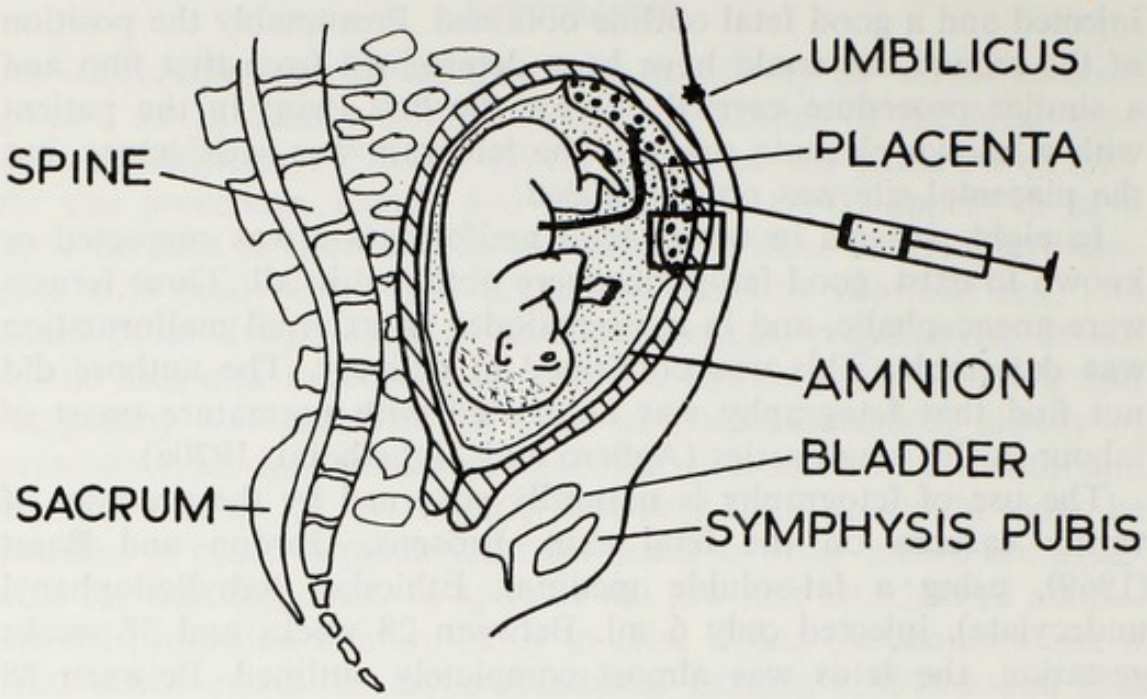

Figure 5. Placental biopsy.

Technique

The procedure is carried out when the patient's bladder is empty. The same antiseptic routine is used as for amniocentesis. The chosen site is infiltrated with local anaesthetic as far as the peritoneum. A Tuohy needle no. 16 is then inserted through the skin, rectus sheath and muscle. At this point the index finger is moved to almost 2 cm above the level of the skin, and the needle then advanced until the fingertip again touches the skin. This precaution allows penetration of the myometrium to this depth and avoids the needle tip going too far with possible entry into the amniotic sac. The stylus is then withdrawn and blood may well up into the hub of the needle. A 20 ml syringe containing 5 ml of heparinized saline solution is then attached and aspiration attempted. If only a little blood is obtained with moderate suction, then the tip of the needle is still in myometrium and must therefore be inserted further once the stylus is replaced. Conversely, if amniotic fluid is obtained it must be withdrawn, preferably completely, and another site chosen.

Once in the intervillous space the needle is rotated through 45 degrees at a time and repeated aspirations made through a complete circle. The needle is then swiftly withdrawn. Maternal venous blood should be taken both before and after the procedure to check for fetomaternal haemorrhage.

The aspirated material may then be examined using phase contrast microscopy and the chorionic villi selected for study.

Definite chorionic tissue can only be obtained by entering the

intervillous space (Fig. 6). The needle tip is then adjacent to the villi and aspiration easily detaches pieces of the villi. If aspiration is too strong a large area of chorionic tissue may be detached and fetal vessels torn. This would lead to loss of fetal blood into the intervillous space and consequently into the maternal circulation where it can be detected.

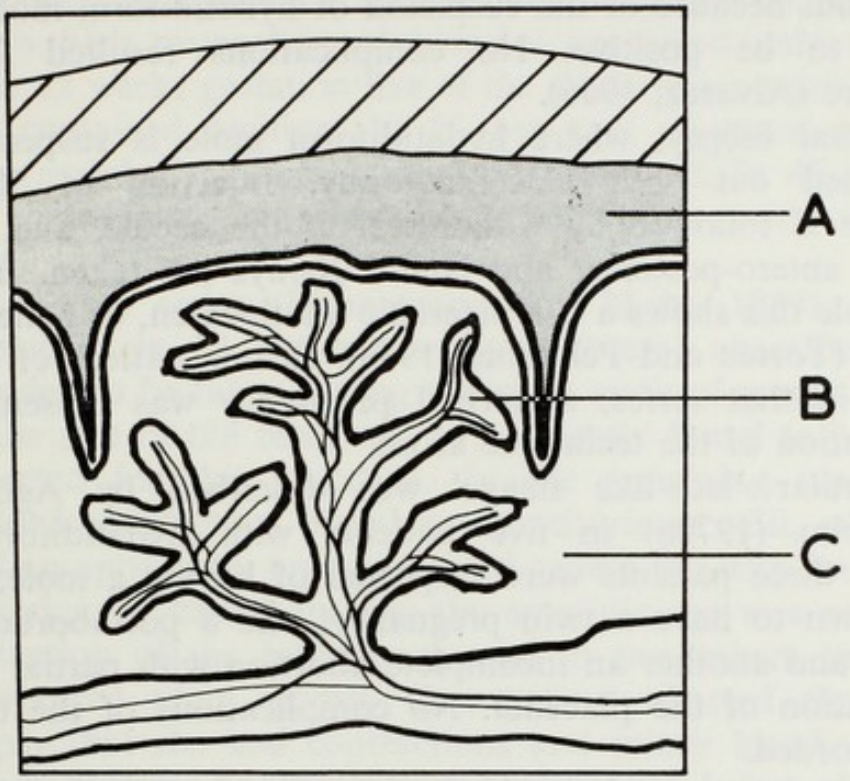

Figure 6. Placenta: enlarged area of Figure 5. A, Decidua (maternal). B, Chorionic villus (fetal). C, Intervillous space (maternal blood).

Aladjem (1968) carried out transabdominal placental biopsy in 92 patients. In this series all were more than 28 weeks gestation, but he quoted Alvarez, who had carried out biopsies as early as 14 weeks with no complications. In one patient no specimen was obtained and in the remaining 91 patients no less than 215 attempts were required in order to obtain a satisfactory specimen from each. This means that each attempt had a less than 50 per cent chance of success. Success was not related to the gestation of the patient. There were no maternal or fetal complications. Aspiration did not cause any histologic change in the villi. Using phase contrast microscopy, the chorionic villi may be readily recognized and any pathology noted (Aladjem, 1967, 1968a, b).

In earlier pregnancy, the transabdominal technique of placental biopsy may be used to confirm the diagnosis of hydatidiform mole (Alvarez, 1966). The technique should be modified, however, to include a syringe containing 15 ml of physiological saline

so that on aspiration the clear vesicles can be seen in the solution. The villi obtained are then mounted between a slide and cover slip and studied with the phase contrast microscope. The villi are characteristically oedematous and relatively avascular. Vim-Silverman's needle has been abandoned by Alvarez because of its traumatic action. In his series of 50 placental biopsies, three were carried out because of the suspicion of hydatidiform mole and all proved to be positive. No complications resulted from the procedure (Alvarez, 1966).

Placental biopsy, where hydatidiform mole is suspected, may be carried out with placentography. Injection of 50 ml of Hypaque is followed by withdrawal of the needle, and within 5 minutes antero-posterior and lateral X-rays are taken. In hydatiform mole this shows a characteristic 'moth-eaten, or honeycombed pattern' (Torres and Pelegrina, 1966). In one patient of the eight studied in that series, a normal pregnancy was present but no complication of the technique arose.

A similar 'lace-like image' was described by Agüero and Zighelboim (1970a) in five patients with hydatidiform mole. Another three patients were suspected of having a mole, but one was shown to have a twin pregnancy, one a postabortal haematometra and another an incomplete abortion with partial hydropic degeneration of the placenta. No complications of the technique were recorded.

This technique of 'molegraphy' (Agüero and Zighelboim, 1970a) may prove useful where quantitative urinary gonadotraphin estimations give equivocal results, no fetal heart is heard and no ultrasonic equipment is available.

Transvaginal placental biopsy

Before 10 weeks gestation the cavity of the uterus is not completely filled by the products of conception (see p. 34). It is therefore technically possible to insert an instrument through the cervical canal and obtain a biopsy of the adjacent membranes. Such a biopsy, however, almost inevitably will contain maternal tissue from the decidua capsularis and only little chorionic tissue. The membranes lining the extraembryonic coelom appears to be of fetal origin, and a biopsy of this would be satisfactory for culture. A more penetrating biopsy would reach the amnion itself, and a large biopsy from this source would be likely to precipitate abortion.

An instrument designed for transvaginal biopsy was described by Mohr (1968). The problems with the technique and subsequent results were recorded (Hahnemann and Mohr, 1968, 1969),

Initially, all patients underwent termination of pregnancy immediately after biopsy. In all 144 such cases were reported, but only after 82 was the technique sufficiently uniform to allow evaluation. In the remaining 63 cases, 62 were successful. In the unsuccessful case, rupture of the amniotic sac occurred when the instrument was introduced. It was found that the tenth week of pregnancy was the optimal stage for biopsy by this route. Earlier than this a high proportion of biopsies contained only decidua, while after 12 weeks gestation five of the six biopsies carried out at this time contained amnion. In 55 per cent of cases, a biopsy containing mainly fetal membranes was obtained. In 45 per cent a 'poor' result was obtained, including those with amniotic membrane.

In the second series (Hahnemann and Mohr, 1969), biopsies were attempted in 12 patients without general anaesthesia. All were intended to be taken during the tenth week of pregnancy, but in three the size of the fetus was subsequently found to be bigger than expected for this gestation. In the remaining nine cases, three yielded satisfactory biopsies with chorionic villi, while one other had an apparently uncomplicated course but with fetal death from an unknown cause. In one, the amniotic sac was punctured on introduction of the instrument, and in two others the membranes ruptured after biopsy. One patient aborted three days after biopsy, but she had contractions and minor bleeding a few days before biopsy. Another aborted within 24 hours of biopsy. Thus in five out of nine cases, complications arose which were attributable to the biopsy. Perhaps the use of a smaller instrument would minimize this if biopsy at this stage is essential.

Fetoscopy

In gynaecological practice, hysteroscopy has been used occasionally in Britain, in late pregnancy amnioscopy has recently found its place, but the technique of actually looking at the fetus *in utero* in early pregnancy has yet to be perfected.

Westin (1954) described a technique of hysterophotography performed between 16 and 20 weeks gestation. The instrument had an outside diameter of 10 mm and was introduced through the cervical canal. He observed fetal limb movements and swallowing when local anaesthesia was used, but neither if a general anaesthetic was used. All three of his patients subsequently underwent termination of pregnancy. In another report (Westin, 1957), the fetus was photographed and oxygen tensions in the umbilical vessels assessed, but the pregnancy was immediately terminated.

For antenatal diagnosis of fetal abnormality, a technique of fetoscopy must be developed which allows full inspection of the external surface of the fetus without excess hazard to the placenta or fetus.

Fetoscopy might be indicated when there is a high risk that the fetus might have one of the common congenital malformations (e.g. spina bifida or anencephaly) or in rare unifactorial (Mendelian) disorders associated with a well defined morphological abnormality (e.g. polydactyly in the Ellis–van Creveld syndrome).

In fetoscopy, accurate placental localization is essential. Should the trocar pierce the placenta, there is a high risk of fetal blood draining into the amniotic sac, the maternal circulation or both. As with a standard amniocentesis, ultrasonography appears promising in this field, but considerable experience is required to determine the margin of the placental site. The ultrasonogram will also confirm the size of the fetus in relation to its estimated gestation. The biparietal diameter at 18 weeks gestation is the most accurate measurement available at this time. A multiple pregnancy would also be excluded, and this finding would rule out the possibility of straightforward fetoscopy; the procedure should not be undertaken in these circumstances.

Equipment

To date, the author has used a fibre optic telescope with continuous cable from light source to the tip of the telescope. The outside diameter is 2.2 mm and the length is 20 cm. The lens has an angle of 110 degrees giving a good field of vision. The telescope is introduced by a similar size of trocar and cannula. To avoid any possible hazard to the developing fetus, a filter has been incorporated in the light source to reduce the ultraviolet and infrared light. The instrument, light source and photographic flash generator have been built by Wolf of Germany and Down Bros., Mayer and Phelps, England.

Technique

The procedure is carried out under general anaesthesia, so that if the fetus is found to be affected, termination by hysterotomy can be performed immediately. The bladder is emptied prior to induction of anaesthesia. Strict aseptic precautions must be observed, and usually it is carried out in a general operating theatre.

The skin is painted with antiseptic and the area draped. A small transverse suprapubic incision is made, sufficient to allow two

fingers of the left hand into the abdominal cavity. The uterus is then gently palpated and fetal parts felt. A small incision in the uterine wall may be helpful in exposing the amniotic membrane. A mattress suture, held by small artery forceps, inserted at each end of the incision will serve not only as a haemostatic but also as a retractor when the cannula is inserted. The site of the incision must be distant from the placental site, and the fetus must be held out of the way by one finger as the trocar is inserted into the amniotic sac.

Once the amnion is felt to be pierced, the trocar is removed, leaving the cannula *in situ*. Sometimes amniotic fluid will flow out, but in many the amnion itself floats over the orifice and arrests any flow. It is therefore advisable to insert the telescope even if no liquor appears. The fibre optic light source should now be turned up to maximum, and inspection carried out. If the cannula is not in the sac then the trocar must be reinserted and another attempt made. The more attempts that are made. the greater is the hazard of damaging the placenta or the fetus.

During inspection, traction must be kept on the mattress sutures so that only a minimum of liquor escapes. If a lot of fluid does escape, this obviously diminishes the area available for fetal movement and therefore makes it much more difficult to turn the fetus with the tip of the telescope. Should a malformation be present, photography is possible before termination is carried out (Plate 5). If no malformation is seen, photography is not advised, as the increased intensity of light from the flash generator might possibly have a deleterious effect on the fetus, though there is no proof of this as yet.

The telescope is withdrawn with the cannula and the two mattress sutures tied. A further suture may be necessary between the two to attain complete haemostasis and prevent any loss of liquor. The abdominal wound is then closed in the standard fashion.

Following fetoscopy, sedation with diamorphine for 36 hours has been found adequate. No evidence of uterine irritability has been noted. The patient is gradually mobilized after two days, the fetal heart checked with Doptone, and the skin sutures removed on the fifth day.

Results

Fetoscopy has been carried out in 19 patients already scheduled for termination by hysterotomy. All were undertaken with the patient's full consent. In gestations less than 15 weeks, entry into the amniotic sac with the trocar proved difficult. By 21 weeks

gestation the fetus has grown to a size which precluded any easy movement of it within the sac. The uterus should therefore be the size of an 18 week gestation to have the optimal chance of success.

The amniotic fluid was found to conduct light easily except in the presence of bilirubin or meconium. If blood contaminates the liquor, then full inspection of the fetus is virtually impossible.

Having established that it was possible to inspect the fetus *in utero* at about 18 weeks gestation, it remained to determine the effect of the procedure in pregnancies which were planned to continue if the fetus were found to be normal. Six patients have so far been studied. All had had two children affected by severe central nervous system malformations. In one patient, the first, fetoscopy showed a normal-looking fetus, no complications arose and she had a spontaneous delivery at 38 weeks gestation. The baby was normal.

One patient had come from Wales only to find that the uterus was only just 15 weeks size. The placenta was posterior and it was felt that the sac could be entered without hazard. In fact this did not occur, blood covered the lens and the fetus was not visualized. Termination was therefore carried out.

In another case, the fetus was subsequently found to be 20 weeks gestation and was certainly difficult to manipulate *in utero*. No abnormality was seen in the area inspected so the pregnancy was allowed to continue. Labour ensued at 34 weeks gestation and the child had a small spina bifida and subsequently died.

In one other case, at 18 weeks gestation, the sac was not entered and the technique was then modified to incise the myometrium, expose the membranes and then insert the trocar. The remaining pregnancies are currently continuing without evidence of abortion, infection or premature labour. All are believed to be normal fetuses.

Ultrasonography

Early growth and development of the fetus may now be studied using ultrasonography. Hellman, Kobayashi, Fillisti, Levanhar and Cromb (1969) reported 103 patients in whom ultrasonography had been carried out. They concluded that an abnormal fetus may be suspected if there is failure of the gestational sac to form a clearly defined ring-like structure, failure of growth of the gestational sac and the uterus, or finally a low implantation of the gestation sac.

In a detailed study of various methods of ultrasound examination in early pregnancy, Jouppila (1971) reported the results of 267 pregnant patients and 15 non-pregnant control patients. A-,

B- and Doppler methods were all used on the pregnant patients between the fifth and sixteenth weeks of pregnancy. The incidence of prematurity (4.7 per cent), perinatal, mortality (0.5 per cent) and anomalies of the newborn (1.6 per cent) were no greater than in the general clinic population. He concluded that there was no evidence of any detrimental effect of ultrasound.

Using B-scan he found that at 12 weeks gestation there appeared a transitional period when it was difficult to obtain a distinct gestation sac and the fetal head was equally difficult to define clearly before the thirteenth week. In diagnosing an abnormal pregnancy using B-scan Jouppila (1971) emphasized the necessity for serial examination before coming to a definite conclusion.

In later pregnancy anencephaly may be diagnosed by ultrasound (Donald, 1969), but until finer definition of the echoes is achieved only gross abnormalities would be suspected (Fig. 7).

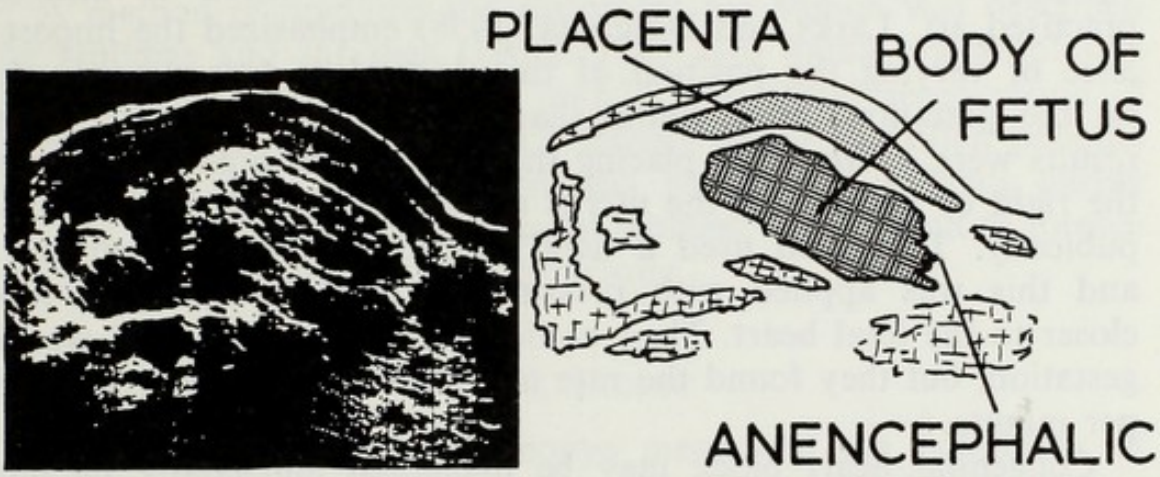

Figure 7. Longitudinal ultrasound scan: anencephalic fetus.

Serial measurements of the biparietal diameter by A-scan may give warning of developing hydrocephaly. Multiple pregnancy may also be diagnosed (Fig. 8).

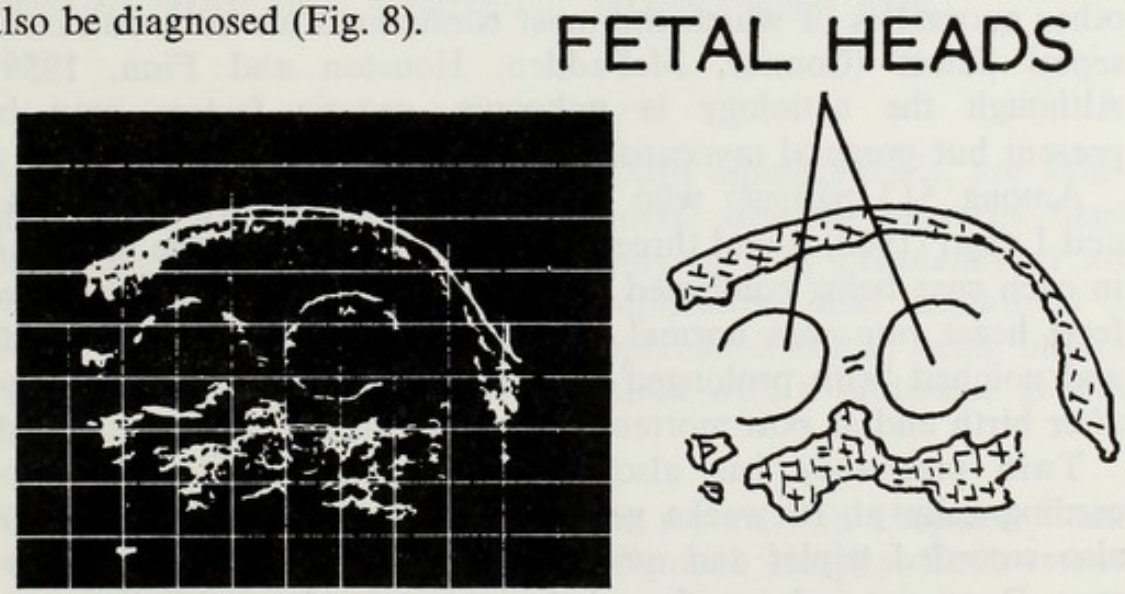

Figure 8. Transverse ultrasound scan: twin pregnancy.

Fetal Electrocardiography

Fetal heart rate monitoring is now an established part of labour ward intensive care. At that point in pregnancy, particular attention is paid to rate in relation to uterine contractions. Fetal cardiac anomalies may be suspected at a much earlier gestation and if diagnosed will avoid confusion at the time of labour.

Jouppila (1971), using a Doppler ultrasound method, found that the mean fetal heart rate at nine weeks gestation was 179 beats per minute but by 16 weeks gestation it had fallen to 149 beats per minute. In early pregnancy, where there is difficulty in detecting the fetal heartbeat, an intravaginal probe placed in the anterior or posterior fornix usually solves the problem.

The first actual recording of a fetal electrocardiogram was reported by Cremer in 1906. Since that time more sophisticated equipment has been developed but interpretation is still a practised art. Larks and Dasgupta (1958) emphasized the importance of varying the position of the electrodes, depending partly on the gestation. They found that in early pregnancy the best results were obtained by placing the longitudinal bipolar leads on the right or left side of the uterus and the transverse leads suprapubically. They also used a hand electrode in early pregnancy, and this was applied with pressure so that the electrode was closer to the fetal heart. Their earliest recording was at $11\frac{1}{2}$ weeks gestation, but they found the rate to be between 90 and 100 beats per minute.

Congenital heart block may be diagnosed antenatally by this method (Larks and Longo, 1960; Jarvinen and Osterlünd, 1963). Should such a diagnosis be made, the paediatrician must be warned when delivery is imminent. It is often associated with other anomalies of which the most common is an intraventricular septal defect (Connor, McFadden, Houston and Finn, 1959). Although the aetiology is unknown, genetic factors may be present but prenatal myocarditis or syphilis must be excluded.

Among 517 patients who had fetal electrocardiograms, Larks and Longo (1960) found three cases with heart block, the diagnosis in each case being confirmed after delivery. In one other case, the fetal heart rate was normal but the fetal complexes were wide and notched 'with prolonged overshoot'. The baby died five days after birth and at post-mortem coarctation of the aorta was found.

Twin pregnancy has also been diagnosed by fetal electrocardiography at 16 weeks gestation (Larks, 1959). Larks (1959) also recorded triplet and quadruplet pregnancies. In 1960, however, Dunn not only confirmed the presence of a twin pregnancy

at 34 weeks gestation but also found that one of the twins had a heart block.

Fetal Electoencephalography

The first antenatal electroencephalogram was made by Lindsley in 1942. Bernstine and Borkowski (1959) studied 75 women at varying gestations. Ninety-five varieties of frequencies of pattern were found. In 85 per cent, the waves were of low to moderate voltage at $\frac{1}{2}$ to 12 per second. In the remainder the waves were small and fast (15 to 30 waves per second). Maternal hyperventilation caused no change in activity in 73 per cent, but in 19 per cent there was increased voltage, and in 8 per cent increased frequency. After hyperventilation, return to normal was rapid. In one case there was a slow return after the appearance of abnormal activity. The baby was subsequently stillborn.

A method of applying the electrodes per vagina was described by Huhmar and Järvinen (1963). They also found low amplitude waves with low frequency. In anoxia they found accentuation of a high amplitude, low frequency activity which was often periodic and paroxysmal. Interpretation was particularly difficult during uterine contractions. At the present moment it is difficult to foresee a clinical application for this technique.

Fetal Biopsy

In a presentation in 1971, Inouye mentioned the possibility of fetal biopsy. This technique is currently being carried out in Japan but so far it is being done without ultrasonic guide and with such a technique it appears probable that any part of the fetus may be biopsied rather than a particular predetermined part.

Conclusion

Various techniques, other than standard amniocentesis, are available for antenatal diagnosis of genetic disease. Most of these techniques, however, are of value only in later pregnancy or are still experimental. The possible complications and limitations of these various techniques must be appreciated before being offered to a patient 'at risk' of having a child with a particular genetic disorder.

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5

Antenatal Sex Determination

M. M. Nelson

Most requests for antenatal diagnosis come from mothers who are known carriers of an X-linked disorder such as haemophilia or Duchenne muscular dystrophy which only affects males, or are at risk of having a child with Down's syndrome. For these reasons, sex prediction based on the study of uncultured amniotic fluid cells and cytogenetic studies on cultured amniotic fluid cells are important aspects of the problem of antenatal diagnosis of genetic disease. The culture of amniotic fluid cells and cytogenetic studies will be dealt with in the next chapter. Here we shall only be concerned with the antenatal determination of sex based on the study of uncultured amniotic fluid cells.

Amniotic Fluid Cells

It is accepted that cells present in the amniotic fluid are of fetal origin, irrespective of the exact site of derivation (Table 7). The majority probably come from fetal skin with smaller contributions from buccal mucosa and possibly upper gastrointestinal, genito-urinary and respiratory tracts, and from urine, umbilical cord and amnion.

In the past 15 years, study of these amniotic fluid cells has become a subject of increasing interest particularly since 1965. Potential use of uncultured cells for antenatal diagnosis is being explored now that amniotic fluid can be obtained in the second trimester with comparatively little risk.

Morphology

Basically four main types of cells may be distinguished: large irregularly shaped cells without a nucleus or with a small pyknotie nucleus, slightly smaller irregularly shaped cells with a larger vesicular nucleus, and small round cells with a large vesicular

Table 7. *Morphology of amniotic fluid cells*

Authors	Types of cells noted*	Postulated origin of cells		Changes in number as pregnancy advances	
Van Leeuwen *et al.* (1965)	a, d	a	—skin	a	—increase
		d	—amnion	d	—decrease
Votta *et al.* (1968)	a, b, c, d, e	a, b, c	—skin	a	—increase
			mouth	b	—increase
			vagina	e	—increase
			cord		
			urine		
		d	—amnion		
		e	—amnion		
Lind *et al.* (1969)	a, b, c, d, e	a, b, c, d	—skin	a, b, e	—increase
				d	—decrease
Floyd *et al.* (1969)	a, b, c, d, e	a	—skin	b, c	—increase
				a, d	—decrease
Huisjes (1970)	a, b, c, d, e	a	—skin	a	—increase
		a, b, c	—mouth		
		c	—vulva		
			urine		
		e	—amnion		
			cord		
Bishop & Pollock (1970)	a, b, c, d	—		a	—increase
				d	—decrease

*a = large polygonal or irregular anucleate cells.
b = large irregular cells, nucleus pyknotic.
c = large irregular cells, nucleus vesicular.
d = small round cells, nucleus vesicular.
e = others.

nucleus (Fig. 9). Most investigators agree that the number of large anucleate amniotic fluid cells increases during pregnancy, whereas the number of small round cells decreases (Table 7). However, Floyd, Goodman and Wilson (1969) noted a decrease in anucleate cells with advancing gestation. Only Wachtel, Gordon and Olsen (1969), Lind, Parkin and Cheyne (1969) and Bishop and Pollock (1970) included specimens of less than 20 weeks. Wachtel *et al.* (1969) make no comment on these early specimens except to say that the amniotic fluid 'was almost completely acellular'. Lind *et al.* (1969) stated that at 30 weeks or less 'cells are scanty' and mainly small, round and nucleated. Bishop and Pollock (1970) also noted that these small round nucleated cells are much more frequent, being 10 to 20 per cent of the total in the first 20 weeks. This is confirmed by our own unpublished observations.

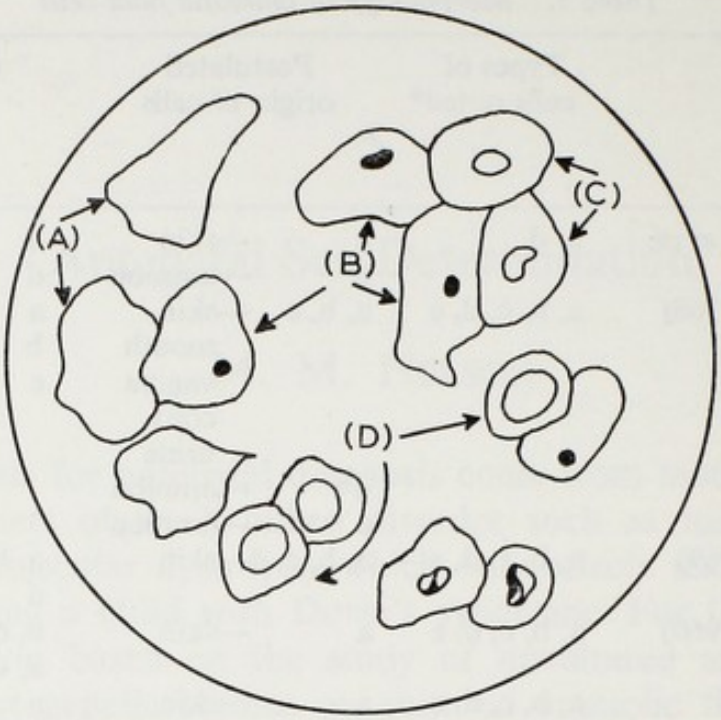

Figure 9. Drawing of four basic types of amniotic fluid cells: A, large irregular cells, anucleate; B, large irregular cells, nucleus pyknotic; C, large irregular cells, nucleus vesicular; D, small round cells, nucleus vesicular.

Cell count

The total cell content of the amniotic fluid rises during pregnancy. Actual figures obtained in different reports cannot be compared because of different counting methods. Votta, Bobrow de Gagneten, Parada and Giuletti (1968) showed a sharp rise in the mean count for weeks 28 to 36 (82.39 per ml) to that for weeks 38 to 42 and over (272.17 per ml). Fig. 10 shows the results in over 500 specimens studied in this laboratory by the method of Steele and Breg (1966) (see Appendix for method), whereby the cells are stained with trypan blue: the dead cells take up the stain but the viable cells remain unstained. The rapid rise in cell numbers after 13 to 16 weeks is probably correlated with changes in the fetal skin, and after 20 weeks the urine will contribute its share. The viable cell count rises only slightly as pregnancy advances.

Gordon and Brosens (1967) reported that staining with Nile blue sulphate (see Appendix) separated amniotic fluid cells into two distinct types, blue staining and orange staining, the latter containing lipid in the cytoplasm. Orange cells are very rarely seen before 30 weeks, but by 40 weeks, they may account for 50 per cent of the cells, and by 38 weeks appear in clusters. Many reports of the use of this stain have now been published, confirm-

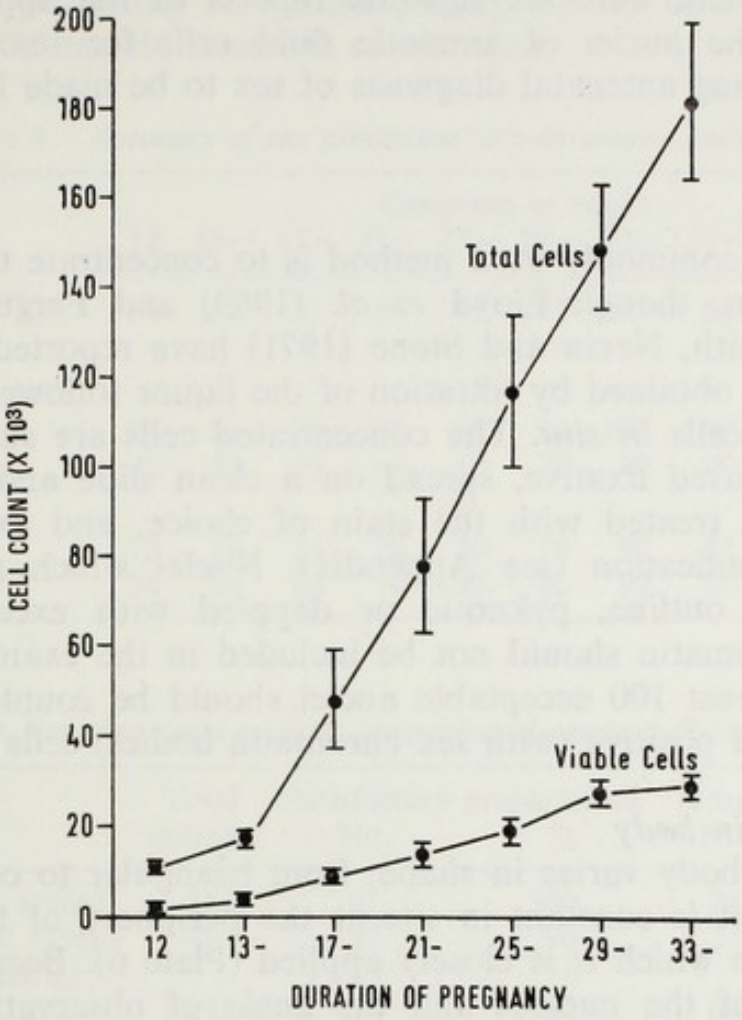

Figure 10. Total and viable cell counts in amniotic fluid.

ing the marked rise in orange cells towards the end of pregnancy. However, there is also agreement that in some fluids, even at term, there may only be 2 per cent of such cells present.

Attempts have been made to use total cell count or the percentage of orange cells as indicators of fetal maturity. However, neither is satisfactory: the standard error of the total cell count is too wide to give precise information, and a comparatively low proportion of orange cells (less than 10 per cent) may be found at term.

Sex Chromatin Sexing

In 1954, Moore and Barr reported finding in nuclei of cells from women a small, darkly staining particle which was not present in the nuclei of cells from normal males. This became known as 'the sex chromatin body' or 'Barr body'. It is an inactive X chromosome, so the number of these bodies is always one less than the number of X chromosomes present.

In 1956 there were six separate reports of the application of examining the nuclei of amniotic fluid cells for sex chromatin bodies allowing antenatal diagnosis of sex to be made for the first time.

Technique

The most commonly used method is to concentrate the cells by centrifugation, though Floyd *et al.* (1969) and Ferguson-Smith, Ferguson-Smith, Nevin and Stone (1971) have reported improved preparations obtained by filtration of the liquor followed by fixing the trapped cells *in situ*. The concentrated cells are suspended in an alcohol-based fixative, spread on a clean slide and air dried. The slide is treated with the stain of choice, and examined at X 100 magnification (see Appendix). Nuclei which are folded, irregular in outline, pyknotic or dappled with excess deeply-staining chromatin should not be included in the examination. A total of at least 100 acceptable nuclei should be counted and the percentage of positive (with sex chromatin bodies) cells calculated.

Sex chromatin body

The Barr body varies in shape, from triangular to convex, and in size, but it is constant in site at the periphery of the nuclear membrane to which it is closely applied (Plate 6). Because of the orientation of the nucleus and the angle of observation, it can only be identified with certainty in a proportion of cells. It can be seen in nuclei of 5 to 30 per cent of amniotic fluid cells derived from a female fetus. In some laboratories, counts of up to 3 per cent have been reported in cells from a male fetus. Standards must therefore be established for the normal counts in each laboratory before diagnostic examinations are undertaken.

Difficulties

One of the drawbacks to the simple and quick examination of the nuclei for sex chromatin bodies is the small total number of cells in early pregnancy. In addition, there are anucleate cells and cells with pyknotic nuclei where detail cannot be seen. When these are deducted and allowance made for distorted nuclei, the numbers remaining to be counted may be considerably reduced. For these reasons, preparations may therefore not be satisfactory (see Table 8). In satisfactory preparations, however, over 90 per cent accuracy in diagnosis of sex by this method may be achieved (Table 9). In early pregnancy, when this technique is most likely to be used, the diagnostic accuracy rises to over 90 per cent (Table 8). If this technique alone is used in sex prediction there

is always the possibility of misinterpreting the sex of a fetus with an XO or XXY sex chromosome constitution.

Table 8. *Accuracy of sex prediction (sex-chromatin body)*

	Gestation in weeks									
	12	13–	17–	21–	25–	29–	33–	37–	40+	Total
Total	34	66	51	59	91	117	125	55	22	620
Satisfactory preparations										
Number	16	33	28	38	59	93	100	49	18	434
%	47	50	55	64	65	79	80	89	82	70
Sexed correctly										
Number	15	31	24	28	50	76	92	42	15	373
%	94	94	86	74	85	82	92	86	83	86

Table 9. *Sex chromatin analysis in uncultured amniotic fluid cells*

	Total samples	Satisfactory preparations		Correctly sexed	
		No.	%	No.	%
Riss & Fuchs (1966)	21	21	100	20	95
Amarose *et al.* (1966)	41	37	90	37	100
Jacobson & Barter (1967)	85	68	80	64	94
Abbo & Zellweger (1970)	115	115	100	115	100
Ferguson-Smith *et al.* (1971)	66	66	100	66	100
Nelson (1972)	620	434	70	373	86
Total	948	741	78	675	91

Fluorescent Sexing

In 1969, Caspersson and his colleagues described the use of acridine derivatives in examining plant chromosomes. In the same year, Zech (1969) showed that the long arms of the human Y chromosome fluoresced very brightly when stained with chloroquin and examined under u.v. light. The following year, Caspersson, Zech, Johansson, Lindsten and Hulten (1970) and Pearson, Bobrow and Vosa (1970) described a bright fluorescent spot seen in the nuclei of cells obtained from males. This spot, the fluorescent F body, or the Y body, is the contracted Y chromosome in the interphase nucleus. Pearson (1970) first reported that it could be seen in amniotic fluid cells from male fetuses. Within 18 months, further reports of this technique applied to amniotic

fluid cells became available (Khudr and Benirschke, 1971; Polani and Mutton, 1971; Walker, Gregson and Hibbard, 1971).

Technique

The staining procedure is simple (see Appendix). Fixed cells prepared as for other studies are suitable. The optical equipment requires to be of a very high standard in order to examine for fluorescent bodies (F bodies) in the amniotic fluid cells. At least 50 nuclei should be counted.

The F body

The F body is small, at the limit of optical resolution, but of a characteristic very bright fluorescence against the duller greenish reaction of the nucleus (Plate 7). It can usually be clearly distinguished from other moderately bright but bigger spots scattered in the larger nuclei of certain of the amniotic fluid cells. It is easy to see the F body in nuclei which, by conventional methods of staining, are too pyknotic or too small to show sex chromatin. In this laboratory, in roughly 20 per cent of the satisfactory fluorescent preparations, examination for sex chromatin bodies has not been possible because of these difficulties (Nelson, 1972). The F body may be seen anywhere in the nucleus, the site not being as characteristic as in the case of the sex chromatin body. The size of the F body varies, but its brightness remains the same irrespective of size. The proportion of amniotic fluid cells in which the F body can be seen varies from 20 per cent to over 70 per cent in male fetuses, with an average in the 50 to 60 per cent range. A small proportion of cells in females have an F body so that normal laboratory ranges for male and female fetuses must be established.

Two F bodies (duplex) may be seen in a small proportion of the cells from normal 46,XY males. These are very close together. They must not be mistaken for the two F bodies in 47,XYY individuals which are separated by at least a quarter diameter of the nucleus and which represent two separate Y chromosomes.

The accuracy of sex prediction by F body counts is shown in Tables 10 and 11. The sex chromatin body is not usually seen in uncultured amniotic fluid cells examined under fluorescent light. Very occasionally, a faintly staining spot can be seen which resembles it in shape and site, but the number of nuclei with this spot is very low, and it is not possible to use this for diagnostic purposes. These cells may be ones which have come from the umbilical cord (Greensher, Gersh, Peakman and Robinson, 1971), or the buccal mucosa. However, if amniotic fluid cells are cultured,

then examined with the fluorescent technique, both sex chromatin body and F body can be clearly seen (Mukherjee, Blattner and Nitowsky, 1971).

Table 10. *Accuracy of sex prediction (fluorescent body)*

	Gestation in weeks									
	12	13–	17–	21–	25–	29–	33–	37–	40+	Total
Total	10	32	20	8	21	23	32	15	5	166
Satisfactory preparations										
Number	4	16	10	6	13	17	26	14	5	111*
%	40	50	50	75	62	74	81	93	100	67
Sexed correctly										
Number	3	15	10	6	12	15	24	13	5	103†
%	75	94	100	100	92	88	92	93	100	93

* 38/111 specimens examined at 3 weeks to 4 months after fixation.
† 3/8 Misdiagnoses examined at 3 weeks to 4 months after fixation.

Table 11. *Fluorescent body (Y) analysis in uncultured amniotic fluid cells*

	Total samples	Satisfactory preparations No.	%	Correctly sexed No.	%
Polani & Mutton (1971)	10	10	100	10	100
Walker *et al.* (1971)	50	50	100	50	100
Khudr & Benirschke (1971)	17	17	100	17	100
Ferguson-Smith *et al.* (1971)	13	13	100	13	100
Nelson (1972)	166	111	67	103	93
Total	256	201	79	193	96

Combined use of the sex chromatin and F body counts should give an accurate antenatal diagnosis of the sex chromosome constitution of the fetus. The two techniques can be completed, and an answer given with respect to the sex of the fetus within 24 hours as opposed to about two to three weeks for karyotyping. Most investigators, however, prefer to use all three techniques whenever possible.

Problems in the rapid sexing methods

These techniques of nuclear sexing will only give information on the sex chromosome constitution of the fetus. They give no

information at all about the autosomes nor about any other abnormality.

A negative F body and sex chromatin body count will be found in the 45,XO fetus, and this could be confused with a 46,XY when the Y chromosome is small. A small Y chromosome is found in less than 1 per cent of the male population (Court-Brown, 1966) and in such cases may not fluoresce brightly either in chromosome preparations or interphase nuclei (Borgaonkar and Hollander, 1971). A misdiagnosis of sex would be made in such a case even when using the complementary nuclear sexing methods. Both methods must be used to detect the 47,XXY fetuses, as in these the sex chromatin examination alone will predict a normal female and the F body alone a normal male.

In cases where discordant results using the sex chromatin body and F body counts are obtained, the problem of sex chromosome mosaicism must also be considered. Harris and Robinson (1971) found such mosaicism in 0.05 per cent in over 21,000 newborns, screening by Barr body counts in buccal smears and amnion and by physical examination of the infant. Confirmation of mosaicism was made by peripheral blood cultures. Examination of the amnion gave a more accurate identification of mosaicism than examination of buccal smears. Amnion cells are found among the amniotic fluid cells, their proportion being highest at the time when antenatal sex screening will be carried out, that is in the first half of pregnancy. Full karyotype analysis will have to be done to determine the correct chromosome complement in such cases.

Undiagnosed twins will lead to difficulties in antenatal sexing when the twins are discordant for sex. As most centres now use some method of placental localization before carrying out amniocentesis, at the same time it should be possible to delineate the fetus, and in this way the majority of twin pregnancies will be recognized.

Blood contamination of the specimen of amniotic fluid has not proved to be a problem as far as sexing is concerned. In fact, with the fluorescent technique, if any blood cells are from fetal sources, and show the F body, it is only further confirmation of the fetal sex, as determined from the positive F body count in the amniotic fluid cells.

Besides sex prediction, uncultured amniotic fluid cells can also be studied for determination of the fetal ABO blood groups (Scott, Coulson and Goulden, 1969). Enzymatic and biochemical investigations are discussed in Chapter 7.

Future Developments

The main use to date of uncultured amniotic fluid cells is in sexing the fetus. Antenatal sexing is already offered in cases of X-linked diseases such as Duchenne muscular dystrophy or haemophilia, where selective abortion of males may be considered, even though in these there is a 50 per cent chance of aborting a normal male. At present only in a few X-linked disorders is it possible to diagnose the hemizygous affected male *in utero* (see Chapter 7). The hope for the future is that it may become possible to predict the affected hemizygote in most X-linked conditions. In uncultured cells, many possibilities still lie unexplored by electron microscopy, histochemical, enzymatic and other biochemical studies. As the majority of the cells are, however, non-viable and may have undergone both structural and biochemical changes since they became detached from the living fetus, on the whole there is less chance of diagnostic advances resulting from their study than in the case of cultured amniotic fluid cells.

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6

Amniotic Fluid Cell Culture and Chromosome Studies

M. M. Nelson

As noted earlier (p. 58), the cells of the amniotic fluid are of fetal origin and information obtained from their culture is expected to reflect the state of the fetus.

In the mid 1960s the first successful attempts to culture amniotic fluid cells were reported (see p. 1). Since then it has become an accepted tool in the management of certain high-risk pregnancies.

Methods

Culture

Each laboratory has its own variation of a basic culture method which has been found to give satisfactory results. The volume of fluid available from amniocentesis ranges from 5 to 40 ml and is usually about 10 to 20 ml. Two to 10 ml aliquots are used for each dish set up, depending on the age of the pregnancy and the culture dishes used in each laboratory. Most methods involve the concentration of cells by centrifugation, followed by re-suspension in a small volume of amniotic fluid, or serum or medium. The cells are then seeded, allowed to settle and attach, after which culture medium is added. The medium may be any of those suitable for tissue culture and is supplemented by the addition of up to 30 per cent fetal calf serum. Antibiotics (streptomycin and penicillin) and a fungicide may be added to minimize the risk of bacterial and fungal infection. The cells are incubated in an atmosphere of 5 per cent carbon dioxide at 36°C. After a period of three to seven days, the culture is examined for evidence of growth and the medium changed. This routine is repeated till sufficient cells are present either for chromosome preparations or for subculture. The method used in this laboratory at present is given in the Appendix.

Chromosome preparations

A cover slip can be included in the culture dish and the cells

growing on it examined directly or if no coverslip is used, the cells may be harvested and handled as a suspension. In either case, colchicine is added to the medium for the last few hours of culture. The cells are then subjected to hypotonic shock, fixed and stained. A number of metaphase plates are counted and a few photographed. From the photographs karyotypes are made in the conventional manner.

Histochemical tests

Histochemical tests can be carried out on the cover slips with primary growth or cells may be subcultured into fresh dishes containing coverslips, and then subsequently stained.

Biochemical studies

At the present time, biochemical studies require a far greater number of cells than any other tests. For these studies, the primary culture is harvested by detaching with trypsin and the cell suspension divided between two or more new dishes. This process is repeated as often as is needed. At present the number of cells required may be in the region of 2 million or over, and this number may only be reached at the fourth or fifth subculture.

Results of culture

The cells of the amniotic fluid are not easy to culture.

The plating efficiency is disappointingly low, in view of the number of viable cells present. These viable cells make up about 20 per cent of the total cell count at 16 weeks gestation, representing about 5000 to 10,000 cells per ml (see p. 61), which suggests that a moderately high initial colony count would be obtained. In practice, less than 10 colonies are seen in the primary culture. It seems that the viability of the cell as indicated by the exclusion of vital stains (e.g. trypan blue, eosin red or nigrosin) bears little relation to its growth potential (Nelson and Emery, 1970). Uhlendorf (1970) suggests that less than 10 'colony forming cells' per ml of amniotic fluid may be present. One point to be emphasized is that because of the scarcity in the inoculum of cells which grow, material must be handled with more than usual care to minimize trauma and loss. The latter may be minimized by using siliconized equipment whenever possible.

Culture success rate

Success in culture may be defined either as the presence of growing cells within two to three weeks of setting up a culture (Nelson and Emery, 1970) or as the persistence of growth after a

specified number of subcultures. However defined, it is now roughly 85 per cent. Karyotypes have been obtained in about the same proportion of the successful cultures (Table 12). Where growth has not been seen in the initial sample, a second sample of amniotic fluid has been satisfactorily cultured in about 80 per cent of cases. Most of the early reports on culture included specimens taken by amniocentesis at all stages of pregnancy, and a proportion of the specimens were from rhesus-immunized pregnancies. Later reports are almost all concerned with amniotic fluid cells obtained in the first 20 weeks of gestation and exclude specimens containing bilirubin. This difference may in part account for the higher success rate reported recently in most laboratories. Cells have been grown successfully from pregnancies where the bilirubin content of the amniotic fluid was high, but if repeated taps from severely affected Rhesus-incompatible cases are cultured, the cells take progressively longer to grow, and the success rate falls as pregnancy progresses (Nelson, 1972).

Table 12. *Culture success rate and length of time for chromosome studies*

Year	Author	Success in culture (%)	Time to process (days)	Success in karyotyping (%)
1966	Steele & Breg	19	—	17
1967	Jacobson & Barter	67	—	58
1968	Nadler	73	18 to 25	100
1969	Muntzer *et al.*	56	15 to 21	?100
	*Santesson †*et al.*	100	4 to 12	100
	Valenti & Kehaty	83	14 to 42 (26.6)	71
1970	Gregson	86	7 to 32 (13.0)	77
	*Lisgar et al.	90	3 to 26	100
	*Nadler & Gerbie	97	3 to 28 (14.2)	?100
	*Butler & Reiss	95	14 to 64 (27.0)	88
1971	*Barakat †*et al.*	100	17 to 27 (22.3)	100
	*Ferguson-Smith *et al.*	85	7 to 31 (18.4)	83

* Generally exclude Rh-pregnancies.
† Less than 20 cultures reported.

Storage of specimens

Growth has been obtained from amniotic fluid cells which have been stored for up to one week, provided they were kept at a low temperature (i.e. about 4°C). Cells have survived journeys by air, train and postal services without any special precautions being taken. When possible, however, the culture should be set up within hours of the amniocentesis. Viability of cells, as assessed by trypan blue staining, is known to decrease with storage (Steele and Breg, 1966) and may reach 50 per cent after 5 days (Wahlstrom, Brosset and Bartsch, 1970).

Duration of culture

The length of time taken to obtain sufficient growing cells for karyotype analysis varies from 3 to 64 days with most laboratories reporting means from 13 to 28 days (Table 12). Histochemical studies can be carried out after a similar period. Biochemical investigations can be considered after 28 days in culture when good growth has been obtained, but usually at least 35 days are required.

Types of cell growth

Growing amniotic fluid cells resemble epithelial cells or fibroblasts seen in cultures of other tissues. One or the other cell type or a mixture may be present. The epithelioid cells usually start as a small compact colony, while the fibroblasts are seen singly in the primary culture. The exact origin of either type is not known, but it is thought that the epithelioid cells may be mainly derived from amnion cells. They are not as 'robust' as the fibroblasts and, in mixed growth, will disappear after a few subcultures. Amniotic fluid fibroblasts may be repeatedly subcultured, but the culture does not survive as long as, for example, fibroblasts from skin.

Difficulties in Culture

Failure in culture

Culture failure rates in published reports are noticeably declining, especially when only diagnostic amniocenteses and aspirated liquor from therapeutic abortions are included. In these fluids the proportion of non-viable cells is less than in fluid obtained in later pregnancy, but why this should affect culture success is not clear.

As amniotic fluid cells will not grow in suspension, the greater the attachment of the cells to the dish, the greater the chance of successful culture. The attachment can be increased by methods such as re-suspending the cells in serum which is 'sticky', by plac-

ing a cover-slip over them to immobilize them or by leaving the concentrated cell suspension to stand in the dish for a while before dilution with the culture medium is carried out. The use of plastic containers rather than glass when the specimens are being transported is also advocated. Viable cells attach more readily to glass than plastic, and if left in contact with glass for any length of time before the culture is set up the numbers which will be available for transfer to the culture dishes will be correspondingly less.

The stage of pregnancy at which the amniocentesis is carried out is also of importance. Before 15 to 16 weeks, there are comparatively few cells present. After this period, the volume of liquor increases and therefore a larger volume of fluid with a greater number of cells can be removed.

There are, however, cell samples which will not grow in spite of all inducements. There is no obvious morphological difference in these specimens and they occur at any stage of pregnancy. no method is yet available for recognizing them nor for explaining the failure. They may for some reason lack colony-forming cells or these few cells may have been damaged in processing the cells for culture.

There is no specific growth stimulating agent for amniotic fluid cells. The addition of fetal calf serum to the medium supplies macroglobulins and glycoproteins, the active components used up in culture. However, increasing the proportion of serum added in culture above 30 per cent has no stimulating effect on initiation of growth. Higher concentrations may even be toxic. Individual batches of serum vary in their capacity to sustain growth and serum should only be obtained from reputable firms which test for toxicity.

Blood contamination

Blood contamination of samples of amniotic fluid is very common. Only about 20 per cent of specimens are completely free of erythrocytes on microscopic examination. However, the presence of blood in the culture has not proved to be a drawback to the successful culture of amniotic fluid cells. There may be a delay in observing growth, however, until the red cells have been removed by successive medium changes. The blood may be maternal or fetal in origin. If maternal, then mistakes in diagnosis may occur. However, long-term blood culture is not easy to sustain, especially with the techniques used for amniotic cell culture. In the majority of cases, when karyotypes are obtained at about 3 weeks, this complication can be disregarded, as

contaminating maternal leucocytes do not survive longer than a week.

Maternal tissues

Needle biopsies of maternal tissues, such as skin or muscle obtained during amniocentesis, were anticipated as a complication, but in practice they rarely occur. Some workers (Macintyre, 1971) do prefer to discard the first few millilitres of fluid obtained, in order to lessen the liklihood of contamination by such material, but most investigators do not consider this necessary.

Infection

Infection is a problem in the culture of amniotic fluid cells as in any other culture. The use of closed culture vessels (such as Leighton tubes, or Carrel or Falcon plastic flasks) rather than open ones (petri dishes) may be an advantage. Throughout the time of culture, close attention must be paid to the sterility of dishes, equipment and media. Technique must be impeccable. If possible, it is preferable to have an enclosed area set aside for such manipulations as are necessary. This area may have a mode of forced ventilation with ultraviolet light irradiation to keep environmental infection to a minimum. Bacterial infection is reasonably easy to combat with the above precautions and the use of antibiotics in the medium. Fungal infections are more difficult to control. A level of fungicide in the medium high enough to prevent fungal growth may be cytotoxic. A precaution generally taken is to set up as many separate culture dishes from the individual sample of amniotic fluid as possible to decrease the chance of complete loss of all cultures by infection. Viral infections may also occur, as well as may contamination with mycoplasma. Freezing down samples of cells for regrowth later is a useful precaution and insurance against loss of cultures.

Time in culture

The overall length of time required to produce a karyotype is about three weeks (Table 12). Probable failures can usually be predicted after about two weeks in culture. Therefore, diagnostic amniocentesis for chromosomal defect should be carried out about five to six weeks before the time limit for termination of pregnancy is reached. This allows three weeks for assessing whether or not the culture has been successful and, where failure is suspected, a margin for repeat amniocentesis and repeat culture. This means that with initial amniocentesis at about 16 weeks

gestation, termination may have to be carried out well into the second trimester. Recent reports of the hazards of termination at this time increases the pressure on laboratories to decrease the time taken to produce results. This is particularly true if the mother comes late to amniocentesis. Every effort is therefore being made to reduce diagnostic delay to a minimum.

There is always a risk of contamination with maternal cells as noted above. In some cultures growth of cells may be prolific at three days or so, but the majority of these cells die off by the end of the first week. This behaviour is similar to that of blood cells, especially macrophages which could be maternal in origin. Three cases have been described (Nadler, 1971) where contamination with maternal blood cells was considered to be responsible for 46,XX karyotypes obtained very early in culture from amniotic fluid where the fetuses later proved to be males. It is likely, therefore, that two weeks will prove to be the optimum time for karyotypic analysis, as, by then, growth of fetal cells will be well established and risk of contaminant cell growth will be minimal.

Results of Chromosome Studies

Good karyotypes can be obtained by either of the methods described above. Cover-slip preparations may not be easy to make because of loss into the medium or fixing fluid of cells in mitosis, a stage when they are known to be particularly liable to detach. A good, but not completely confluent culture has to be selected and handled with great care. Cover-slip preparations do have the advantage, however, that fewer cells are required than for a suspension preparation, and so karyotype analysis may be carried out about a week earlier on the average.

Techniques

The methods of preparing material for study include the staining of the chromosomes by conventional means, or by one of the newer techniques which reveal the banding patterns characteristic of individual chromosomes. These newer techniques include staining with chloroquin (Atebrin) and examination under a fluorescent light source (Polani and Mutton, 1971), or pretreatment of the chromosomes followed by staining with Giesma stain (Sumner, Evans and Buckland, 1971), or even more recently pretreatment of the chromosomes with trypsin and staining with Leishman's stain (Seabright, 1971). All of these methods have their own particular advantages, but for the last two, the chromosome preparations must be particularly good.

Indications for chromosome studies

There is unanimous agreement about the value of chromosome analysis when the mother or father is a translocation carrier or a known mosaic. Another indication is maternal age since there is an increased incidence of Down's syndrome, other autosomal trisomies and sex-chromosome abnormalities in children born to mothers of over 40 years of age (see p. 8). The rapid sexing methods described in Chapter 5 should also be supplemented whenever possible by cell culture and karyotype analysis in cases at risk from X-linked disorders to obtain confirmation of predicted sex

Long-term study of the newborn population may indicate where antenatal screening could be profitably applied. For example, should mothers be screened at certain times of the year or at the time of epidemics? Harris and Robinson (1971) recently published a report on a large series of newborns examined over a period of six years. They found, for example, a higher incidence of sex chromosomal anomalies among newborns conceived during a rubella outbreak, X-chromosome abnormalities for this group being nearly four times higher than in non-epidemic periods. They also found seasonal peaks of abnormality when the month of conception was studied, though other surveys have not shown this (Tunte and Niermann, 1968). Such seasonal fluctuation has also been noted in the autosomal trisomies (Taylor, 1968). Mothers exposed to radiation or chemical insults might also need to be considered. The feasability of such screening is discussed in Chapter 9.

Results of chromosome studies

So far, apart from the three exceptions quoted above the prediction of fetal sex on the basis of chromosome studies has been accurate in all cases. Nadler (1971) and Polani (1971) give summaries of published investigations and incorporate other cases studied in their own laboratories where amniocenteses had been carried out for specific diagnostic reasons. Nadler gives a diagnostic total of 16 cases of Down's syndrome, and five other unspecified anomalies in 255 high-risk cases studied. Polani quotes 159 high-risk pregnancies investigated, with 13 Down's syndrome and six other anomalies diagnosed antenatally. There is, however, some overlap in the series they report. A plea has been made for the establishment of some kind of central register where results would be pooled to give the maximum amount of information over as short a period of time as possible

(Kaback, 1971). It is to be hoped that such a centre will soon be set up in the United Kingdom as in the U.S.A.

Problems involved in chromosome studies

These problems are mainly those of establishing successful cultures and obtaining sufficient metaphase plates to allow a diagnosis to be made. The rate of cell replication varies, but a wave of mitoses usually occurs about 18 to 24 hours after medium changing. Advantage may be taken of this fact to determine the most suitable time to process the cells. Examination for the presence of cells in mitoses is mandatory, but the use of phase microscopy is helpful in confirming ordinary light microscopy findings for this phase of growth. Even so, occasionally only a very small number of mitotic figures may be found which can be analysed.

The interpretation of a translocation found on examination of amniotic fluid cell chromosomes may be difficult. If a similar translocation is present in the chromosomes of a phenotypically normal parent, it is tempting to assume that it is of no significance. However, minor differences in the amount of material involved in the translocation in the amniotic fluid cell chromosomes may not be detectable and the fetus could therefore be abnormal. Macintyre (1971) discusses these difficulties. Complete study of such chromosome anomalies in the parents and fetus may involve the use of many techniques and take a long time.

The question of mosaicism, though it must be rare as none has yet been reported in amniotic fluid cultures, has also to be considered. What degree of mosaicism is sufficient to indicate termination of pregnancy? In some cases, where a particular mosaic pattern is known to be associated with an abnormal phenotype (e.g. 47,XY,21+/47,XXY) there will be no doubt, and termination will be indicated. Mosaicism where abnormal autosomes are involved will probably always be severe in its effects and so be accepted as sufficient grounds for termination. If 46,XX/47,XXX is found, should the pregnancy be terminated? The higher the proportion of normal cells present, the less the mosaicism will manifest clinically. Can this be used as an indication of no obvious abnormality in the fetus and hence as an indication for not terminating pregnancy? As the incidence and effects of various degrees of mosaicism are not yet fully known, it is difficult to answer these question. Harris and Robinson (1971) found the incidence of mosaicism of the sex chromosomes in newborns to be 0.05 per cent. In the Edinburgh newborn survey carried out by the Medical Research Council Population and Clinical Cytogenetics Unit,

the incidence of mosaicism was 2 in 4901 live births (0.04 per cent) (Radcliffe, 1971). These are studies on buccal smears, amnions and peripheral blood cultures. Long-term follow-up will no doubt provide information on mosaicism and its effects on individuals. Until this information is available, finding such anomalies in amniotic fluid cell cultures will remain a diagnostic problem.

Abnormal chromosome results

There is a comparatively high incidence of polyploidy (tetraploidy) in amniotic fluid cell cultures (Table 13). So far, there is no evidence that this reflects an abnormality in the fetus, for in many such cases healthy babies with normal karyotypes have been subsequently delivered. Tetraploidy is not a common finding among studies of spontaneously aborted material, the frequency being less than 3 per cent in cultured embryonic cells (Sasaki *et al.*, 1971), 3.4 per cent in abortions up to 60 days of age (Carr, 1971), 2 per cent in abortions up to 154 days of age (Carr, 1965) and 11.7 per cent in abortions at all ages up to 17 weeks (Mikamo, 1970). The tissues examined in these reports included biopsies from the fetus, amnion and chorion. The membranes were more commonly used in very early abortions, as often no fetus is to

Table 13. *Polyploidy in cultured amniotic fluid cells*

Author	Number of amniotic fluids examined	Gestation of specimens (weeks)	Cells with polyploidy (%)	Comments
Kohn & Robinson, 1970	3	?	13 to 100	1 case with 100% and 1 case with 50% polyploidy; both had normal chromosomes in other tissues cultured
Milunsky *et al.*, 1970	20	14 to 16	0 to 43	10/20 cases delivered; infants normal phenotypically
Walker *et al.*, 1970	110	9 to 38	0 to 100	2 cases with 100% polyploidy gave fetuses with normal chromosomes in other tissues
Tadashi, 1971	1	15	39	No polyploidy in other tissues studied

be found in these grossly abnormal products of conception. In therapeutic abortions, polyploidy has been found in 11.5 per cent of cases (Sasaki *et al.*, 1971). These authors comment on the fact that in four of the six cases with tetraploidy, the cells examined were from cultures of amnion or chorion. Polyploidy is reported from other work (Petursson and Fogh, 1963; Böök, Kjessler and Santesson, 1968) to be common in the cells of normal amnions. As cells from the amnion are known to be shed into the liquor, their growth is likely to be responsible for the polyploidy in amniotic fluid cell cultures (Hoyes, 1971). A suggestion of an increased incidence with prolonged culture has been reported (Walker, Lee and Gregson, 1970).

Future Developments in Amniotic Fluid Cell Culture

The aim for the future is to achieve rapid and accurate diagnosis of abnormality in the fetus at high risk, to allow termination if necessary. To do this, the failure rate on the first attempted culture must be reduced to as low as possible. Improved methods of culture with perhaps a specific growth-stimulating factor would help achieve this. Improvement in culture success in reported studies has been noted to be occurring already. The time in culture should be reduced to the optimal and there are indications that this may be two weeks for chromosomal analysis. The time of carrying out amniocentesis should be fixed at the stage of gestation when the possibility of obtaining sufficient fluid with the maximum number of cells will be possible with the least risk to the pregnancy. It is likely that this has already been established and is about 16 weeks.

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7

Biochemical Studies on Amniotic Fluid Cells

D. J. H. Brock

Introduction

Recognition of a genetic defect *in utero* is the result of deliberate intrusion into the amniotic sac in search of abnormality. Usually amniocentesis is undertaken because a pregnancy is known to have an enhanced risk. The risk may be based on empirical observations, as in most chromosome abnormalities, congenital malformations and multifactorial disorders, or it may be derived from the modes of inheritance of mutant genes. In the latter case, where a disease is presumed to originate from a single genetic locus, the risk may be stated with precision and will be 25 per cent for recessives and 50 per cent for dominants. The difficulty lies in knowing what to look for as a marker of the disease state. In this sense antenatal detection of inborn errors of metabolism is closely tied to current understanding of the basic biochemical lesions in these disorders. But since it is also important to know whether an abnormality will be expressed in amniotic fluid cells, information on the relative tissue distribution of protein and enzyme deficiencies is also germane to consideration of future prospects for the *in utero* recognition of genetic disease.

Inborn Errors of Metabolism and Antenatal Diagnosis

Modern understanding of inborn errors of metabolism is based on the observations and ideas of Garrod (1908). He saw them as deriving from blocks in metabolism, which were the products of specific enzyme deficiencies, and he recognized that they behaved as typical Mendelian recessive characters. Since then there has been a gradual enlargement of Garrodian concepts to encompass defects in proteins which have no enzymatic capacity (structural, transport or regulator proteins), and to include disorders which are inherited as dominants. There is, at present, a tendency to regard all disorders which show Mendelian genetics as inborn

errors of metabolism and this has been reinforced by the one gene-one enzyme theory of Beadle and Tatum (1941), expressed in its modern form of one cistron-one polypeptide chain. It may be correct to call all dominantly and recessively inherited disorders inborn errors of metabolism; it is not correct to assume that all are based on mutant proteins. Some genes are transcribed but not translated (those for example which code for ribosomal RNA), while others, it would appear, may not even be transcribed. It is quite possible that some inborn errors will eventually be found not to be associated with any specific protein deficiency.

Nonetheless the gene-protein relationship has been enormously valuable in directing research in genetic disorders. A recent review lists more than 130 conditions in which the primary enzyme or protein defect has been defined with reasonable certainty (Brock, 1972). Most of these are recessively inherited, for with the exception of a few haemoglobinopathies, dominant conditions have so far defied analysis. These 'defined' or 'solved' recessives constitute the group of diseases which have yielded most readily to attempts at antenatal diagnosis and where future prospects are brightest, for the closer to the gene level at which a genetic disease can be monitored, the less the chance of being confused by the pleiotropic effects of mutation.

It is not, however, always convenient or possible to follow an inborn error at the protein level. In many unsolved disorders considerable experience has been gained about characteristic metabolites excreted in the urine which mark the disease. The discovery of these at unusual levels in amniotic fluid may be taken as a sign that the fetus is excreting them and so used to assess the possibility of abnormality (this is discussed in Chapter 8). In other disorders metabolites are stored in solid tissues and gradually accumulate until the cell is disintegrated by their presence. In these storage diseases, of which the glycogenoses, mucopolysaccharidoses and sphingolipidoses are the best-known examples, a characteristic histochemistry may often be found, based on the chemical properties of the stored compound. But because the relationship between the metabolic abnormality and the primary protein lesion is unknown, there is a greater chance of error in making a diagnosis. This is exemplified by experiences in antenatal diagnosis of the mucopolysaccharidoses (see pp. 98 & 120) and cystic fibrosis of the pancreas (*vide infra*).

It is difficult to know in advance whether an enzyme deficiency will be expressed in amniotic fluid cells and therefore amenable to antenatal diagnosis. If the enzyme is known to be comparatively tissue-specific this is unlikely; neither phenylalanine hydroxylase

(a liver enzyme responsible for phenylketonuria) nor glucose–6–phosphatase (an enzyme of liver, kidney and intestinal mucosa, responsible for Von Gierke's disease) are measurable in amniotic fluid cells. Enzymes of wide tissue distribution (e.g. those of the

Table 14. *Enzymes responsible for inborn errors of metabolism, which are expressed in cultured skin fibroblasts*

Disorder	Enzyme*
Glycogen storage disease type II (Pompe's)	α-1,4-Glucosidase
Glycogen storage disease type III (Forbes')	Amylo-1,6-glucosidase
Glycogen storage disease type IV (Andersen's)	Amylo-(1,4→1,6)-transglucosidase
Phosphohexose isomerase deficiency	Phosphohexose isomerase
Galactosaemia	Galactose-1-phosphate uridyl transferase
Galactokinase deficiency	Galactokinase
Glucose-6-phosphate dehydrogenase deficiency	Glucose-6-phosphate dehydrogenase
Acatalasia	Catalase
Pyruvate decarboxylase deficiency	Pyruvate decarboxylase
Maple syrup urine disease	α-Ketoacid decarboxylase
Propionic acidaemia	Propionyl-CoA carboxylase
Methylmalonic acidaemia	Methylmalonyl-CoA isomerase
Homocystinuria	Cystathionine synthetase
Cystathioninaemia	Cystathioninase
Citrullinaemia	Argininosuccinate synthetase
Argininosuccinic aciduria	Argininosuccinase
Hyperlysinaemia	Saccharopine synthetase
Hyperornithinaemia	Ornithine transaminase
Generalized gangliosidosis	Non-specific β-galactosidase
Tay-Sachs	Hexosaminidase A
Globoside storage disease	Hexosaminidase A and B
Fabry's	α-Galactosidase (ceramide trihexosidase)
Gaucher's	β-Glucosidase (glucocerebrosidase)
Niemann-Pick	Sphingomyelinase
Metachromatic leucodystrophy	Arylsulphatase A
Lactosyl ceramidosis	Lactosylceramide β-galactosidase
Krabbe's	Galactosylceramide β-galactosidase
Acid phosphatase deficiency	Acid phosphatase
Refsum's disease	Phytanate α-hydroxylase
Lesch-Nyhan	Hypoxanthine-guanine phosphoribosyl transferase
Orotic aciduria	Orotidylate pyrophosphorylase Orotidylate decarboxylase
Xeroderma pigmentosum	'Endonuclease'
Congenital erythropoietic porphyria	Uroporphyrinogen III cosynthetase

* References may be found in Brock (1972).

glycolytic pathway and those found in lysosomes) are more likely to be found. Probably the best rule-of-thumb which has emerged is that if an enzyme is expressed in cultured skin fibroblasts then it is more than likely to be part of amniotic fluid cell metabolism. Table 14 lists those enzymes, whose deficiencies cause inborn errors of metabolism. which are measurable in cultured fibroblasts derived from skin explants.

Methodology

Uncultured cells

Histochemical or biochemical attempts to monitor inborn errors of metabolism in amniotic fluid cells raise the question of whether or not the cells should be cultured. Uncultured cells have obvious advantages; assays can be performed in a matter of hours rather than after a delay of weeks and the vagaries of cellular growth in culture can be circumvented. But the use of such 'raw' material has even greater disadvantages in the high proportion of non-viable cells and the possibility of contamination by maternal blood cells. Nadler (1969) quotes 30 to 50 per cent non-viable cells in the early stages of pregnancy; Santesson, Akesson, Book and Brusset (1969) found 65 to 80 per cent non-viable cells at 15 to 20 weeks, and Nelson and Emery (1970) found 66 to 88 per cent non-viable cells between 17 and 20 weeks of gestation. Trypan blue uptake as a measure of non-viability, is not necessarily an indication of cellular death or the inactivation of intracellular enzymes. But it does suggest that quantitative enzymology will be imprecise and that values will fluctuate considerably.

Maternal blood cell contamination is less easy to control for even the most carefully performed amniocentesis may on occasion yield a grossly stained specimen. Erythrocytes can be seen under the microscope when the cellular button is examined and selectively removed by careful use of hypotonic shocks (Fig. 11). However, interference by erythrocyte enzymes with assays for enzyme deficiency conditions is not often a problem as only a small proportion of the solved inborn errors of metabolism are expressed in these cells (Brock, 1972). Contamination by white cells is more likely to be serious; Cox, Douglas, Hutzler, Lynfield and Dancis (1970) have pointed out that in the *in utero* detection of Pompe's disease by α-1,4-glucosidase assay there may be considerable distortion of results by neutrophils and lymphocytes among the amniotic fluid cells. These authors discuss the possibility of removing the neutrophils by incubating cells with iron filings and then subjecting them to a magnetic field, for the amniotic cells them-

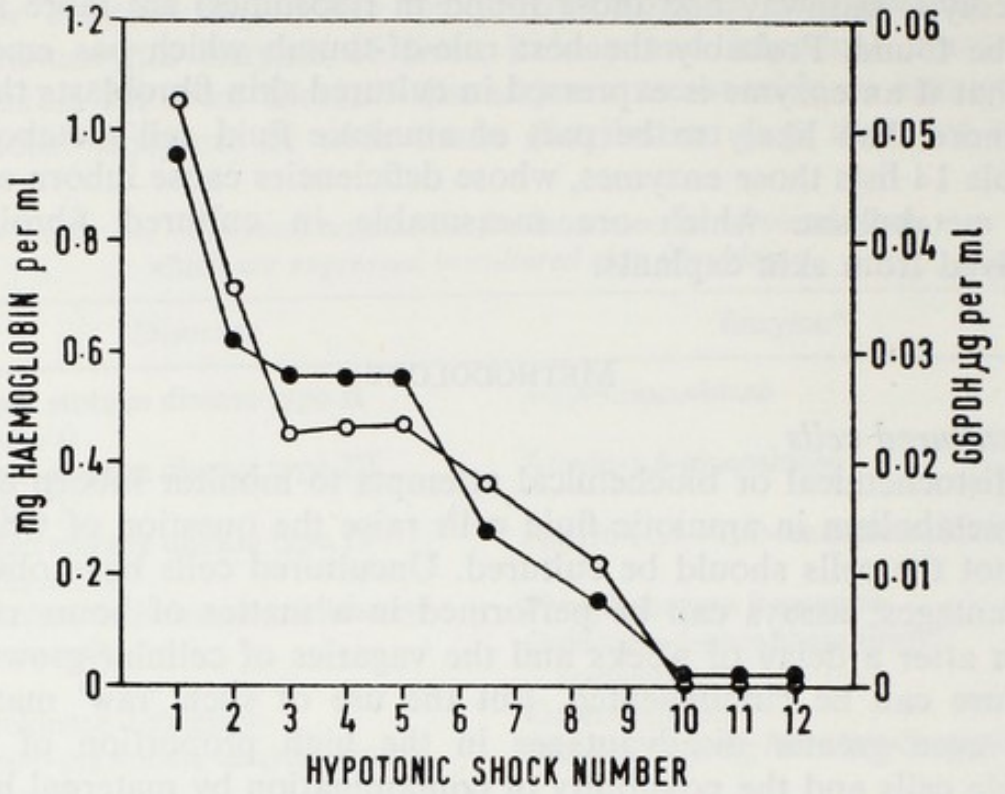

Figure 11. The removal of red cells from a grossly stained pellet of amniotic fluid cells. The pellet was suspended in water for 10 seconds and then made isotonic by addition of concentrated saline. Haemoglobin and G6PD were assayed in the supernatant. Each separate exposure to water represents one hypotonic shock. The simultaneous decrease of haemoglobin (●) and G6PD (○) in the extracts indicates that G6PD is largely derived from red cells. (Sutcliffe and Brock, unpublished observations.)

selves have no phagocytic activity. But recourse to such complicated manoeuvres is an argument against the convenience of uncultured cells. Even if the technique were completely successful, there still remains the possibility that enzymes of the amniotic fluid itself could absorb to the recovered cells and not easily be removed by conventional saline washing. For example, α-1,4-glucosidase activity in amniotic fluid (Fig. 12) reaches a peak at about 15 weeks of gestation (shortly before the time of most amniocenteses). Other enzymes have similar gestational profiles (Sutcliffe and Brock, 1972) and this may constitute a source of error in the enzymology of uncultured cells.

Despite these reservations, various attempts have been made to estimate the enzyme content of uncultured cells. Many of these studies have been qualitative, and have given no indication of the range or mean of the enzyme values (Dancis, 1968; Nadler, 1968b; Nadler and Gerbie, 1969), though they may be useful in indicating what enzymes may be anticipated in cultured cells.

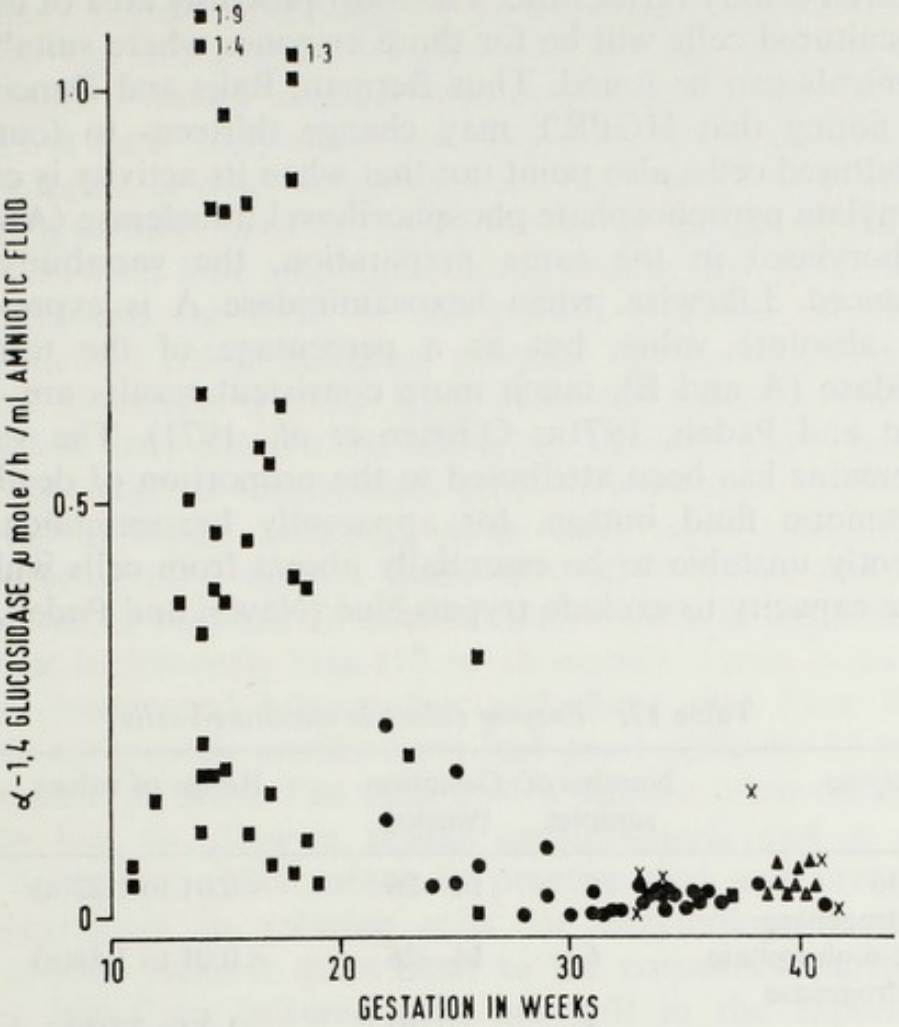

Figure 12. α-1,4-Glucosidase activity in amniotic fluid at different stages of gestation. ■, Fluids obtained at termination of normal pregnancies; ●, fluids from pregnancies at risk for Rh isoimmunization; ▲, fluids obtained by artificial rupture of membranes in normal pregnancies; X, fluids from anencephalic fetuses (Sutcliffe and Brock, unpublished observations.)

Where quantitative measurements have been undertaken, they have revealed great variability of values. Sutcliffe and Brock (1971) assayed isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase, phosphohexose isomerase and lactate dehydrogenase, and concluded that uncultured cells were poor material for the precise enzymology necessary in making diagnoses (Table 15). Similarly scattered values were observed for hypoxanthine-guanine phosphoribosyl transferase (HGPRT), β-D-N-acetylhexosaminidase (hexosaminidase), α-1,4-glucosidase, and arylsuphatase A (Table 15). However, Nadler and Messina (1969) have recorded more consistent levels of α-1,4-glucosidase in uncultered cells, while Nadler (1968b) has claimed that G6PD in cells 'in culture for 0 weeks' (presumably

uncultured cells?) varies little. The most probably area of usefulness for uncultured cells will be for those enzymes where suitable internal controls can be found. Thus Berman, Balis and Dancis (1969), while noting that HGPRT may change thirteen- to fourteenfold in uncultured cells, also point out that when its activity is compared to adenylate pyrophosphate phosphoribosyl transferase (AMP pyrophosphorylase) in the same preparation, the variability is less pronounced. Likewise, when hexosaminidase A is expressed, not as an absolute value, but as a percentage of the total hexosaminidase (A and B), much more consistent results are obtained (Navon and Padeh, 1971a; O'Brien *et al.*, 1971). The variability that remains has been attributed to the proportion of dead cells in the amniotic fluid button, for apparently hexosaminidase A is sufficiently unstable to be essentially absent from cells which have lost the capacity to exclude trypan blue (Navon and Padeh, 1971b).

Table 15. *Enzyme values in uncultured cells*

Enzyme	Number of samples	Gestation (weeks)	Range of values	Ref.
Isocitrate dehydrogenase	6	14—26	<0.01 to 1.22(a)	1
Glucose-6-phosphate dehydrogenase	6	14—26	<0.01 to 1.88(a)	1
	4	10	1.3 to 2.5(b)	2
6-Phosphogluconate dehydrogenase	6	14—26	0 to 0.01(a)	1
Phosphohexose isomerase	4	14—26	8 to 38(a)	1
Lactate dehydrogenase	5	14—26	<0.01 to 12.2(a)	1
HGPRT	8	12—20	13 to 180(c)	3
α-1,4-Glucosidase	7	16—20	14.4 to 119(c)	4
	7	14—16	2.4 to 5.1(d)	5
Hexosaminidase	21	15—20	141 to 826(e)	6
	7	17—21	50 to 770(e)	7
β-Galactosidase	7	17—21	10 to 30(e)	7
Arylsulphatase A	7	17—21	5 to 34(e)	7

(a) μmoles NADH or NADPH/min/g protein; (b) μmoles NADPH/h/ng protein; (c) arbitrary units; (d) μmoles maltose hydrolysed/min/g protein; (e) μmoles substrate hydrolysed/h/mg protein.

References: 1. Sutcliffe and Brock, 1971; 2. Nadler, 1968b; 3. Berman *et al.*, 1969; 4. Cox *et al.*, 1970; 5. Nadler and Messina, 1969; 6. O'Brien *et al.*, 1971; 7. Kaback and Leonard, 1970.

Cultured cells

The technique of amniotic fluid cell culture is discussed in detail elsewhere in this book (Chapter 6). What is often overlooked

in the many papers describing the methodology of culture is that success to the biochemist is more elusive than success to the cytogeneticist. Primary cultures will usually suffice for karyotype analysis, but until microassays for enzymes and proteins are more fully developed, several subcultures are necessary to produce the two million or more cells necessary for biochemical analyses. This will take between four and eight weeks, and in both slow and fast growing cultures the limit of the growth potential of the cells may be reached before sufficient material has accumulated. Each subculture introduces a fresh opportunity for infection while each trypsinization may shorten the life-cycle. Detailed data on success rates to the stage of biochemical analysis are scarce; Nadler and Gerbie (1970) report achieving more than three subcultures in 75 per cent of their sample while Uhlendorf (1970) records the accumulation of between 20 and 80 million cells in approximately 80 per cent of his early taps (13 to 18 weeks). There is no doubt that less experienced laboratories and those that have focused largely on cytogenetic studies have had great difficulty in growing large numbers of cells. The failure of a culture often occurs at a stage too late to allow a repeat amniocentesis and a second attempt at culture. This means in practice that any investigator whose experience in growing cells for enzymological assay is limited, should make it quite clear to the concerned parties that diagnoses based on cultured cells are still in the experimental stage. Extensive success with amniotic fluid cells grown for cytogenetic analysis is not an adequate safeguard.

Even when cells proliferate adequately and within an appropriate time-span, various problems remain. Littlefield (1971) has discussed a number of these. They include the effects on enzyme levels of : (1) contamination by maternal cells or by fungi and bacteria; (2) the apparent heterogeneity of cultured amniotic fluid cells; (3) differing culture media and the nutritional factors they contain; (4) the stage of the cell cycle at the time of harvesting and the possibility of inadvertent synchronization of the mitotic cycle; and (5) the method of extraction of proteins from the mature cells.

Of these, perhaps the most disconcerting is the possibility that the culture consists largely of maternal cells. Nadler and Gerbie (1970) have commented on a culture in which there was an early outgrowth of what appeared to be maternal macrophages, which subsequently died out leaving only fetal cells; Uhlendorf (1970) has described a similar experience, though in his case the maternal cells were subcultured several times. It is unlikely that this is a common phenomenon, but if it can happen once it can happen

again. The discovery of a characteristically adult or fetal marker isoenzyme, easily demonstrated by electrophoresis, would be reassuring to those confronted by this doubt (Kirkman, 1971).

The morphological heterogeneity of cultured amniotic fluid cells has been known for some time (Steele and Breg, 1966). The two commoner types are usually referred to as the shorter-lived epithelial-like cell and the longer-lived fibroblast-like cell. The comparative biochemistry of the two types has not been seriously investigated, and presumably most massive cultures are made up largely of fibroblasts. Recently, Melancon, Lee and Nadler (1971) have made an attack on the problem and have demonstrated that at least with respect to one enzyme, the two cellular types have quite distinct properties. Histidase, an enzyme present in skin and liver, and whose deficiency is responsible for histidinaemia, is

Table 16. *Enzymes in cultured amniotic fluid cells, for which values have been quoted*

Enzyme	Disorder	References
β-Galactosidase	Generalized gangliosidosis	Sloan *et al.*, 1969 Uhlendorf, 1970 Kaback and Leonard, 1970
Hexosaminidase A	Tay-Sachs	O'Brien *et al.*, 1971 . Navon and Padeh, 1971a Kaback and Leonard, 1970
α-Galactosidase	Fabry's	Brady *et al.*, 1971
Glucocerebrosidase	Gaucher's	Uhlendorf, 1970
β-Glucosidase	Gaucher's	Beutler *et al.*, 1970
Arysulphatase A	Metachromatic leucodystrophy	Kaback and Howell, 1970 Kaback and Leonard, 1970
Sphingomyelinase	Niemann-Pick	Uhlendorf, 1970
α-1,4-Glucosidase	Pompe's (GSD II)	Nadler, 1968a Nadler and Messina, 1969 Cox *et al.*, 1970
Amylo-1,6-glucosidase	Forbes' (GSD III)	Howell *et al.*, 1971
Amylo-(1,4→1,6)-transglucosidase	Andersen's (GSD IV)	Howell *et al.*, 1971
Ornithine transaminase	Hyperornithinaemia	Shih and Schulman, 1970
Argininosuccinase	Argininosuccinic aciduria	Shih and Littlefield, 1970
Cystathionine synthetase	Homocystinuria	Uhlendorf and Mudd, 1968 Uhlendorf, 1970
Histidase	Histidinaemia	Melancon *et al.*, 1971
Galactose-1-phosphate uridyltransferase	Galactosaemia	Nadler, 1968a
G6PD	G6PD deficiency	Nadler, 1968b
HGPRT	Lesch-Nyhan	Boyle *et al.*, 1970
'Endonuclease'	Xeroderma pigmentosum	Regan *et al.*, 1971

expressed primarily in epithelial cells and apparently not at all in pure fibroblast populations. Since epithelial cells are usually overgrown in amniotic fluid cell cultures by the more robust fibroblasts (Littlefield, 1971), this presents a particular problem for the antenatal detection of histidinaemia, and perhaps a more general problem is establishing normal levels for other enzymes.

The other problems in enzyme assay cited above—the effects of contamination by extraneous organisms, of culture medium variation, of cell-cycle stage and of method of harvesting—are in principle soluble by appropriate experience. This is now being acquired at a steady rate. The enzymes which have been assayed in cultured amniotic fluid cells and for which normal values have been quoted are listed in Table 16. Specific activities are not given since each laboratory (and perhaps each investigator) should establish a range of values for the particular assay conditions and should not depend on the results of others. Furthermore, laboratories with extensive experience of cultured skin fibroblasts should be cautious in using enzyme values from these as criteria for comparing enzyme values in amniotic fluid cells. The differences in range and mean for the few enzymes where there is adequate data are shown in Table 17. It has also been pointed out (Condon, Oski, DiMauro and Mellman, 1971) that the relative contribution of the Embden-Meyerhof and hexose monophosphate pathways to glucose utilization differs in skin fibroblasts cultured

Table 17. *Comparative enzyme values in amniotic fluid cells, adult skin fibroblasts and fetal skin fibroblasts**

Enzyme	Amniotic fluid cells	Adult fibroblasts	Fetal fibroblasts	Ref.
Cystathionine synthetase	170 to 500	100	N.M.†	1
β-Galactosidase	140 to 330	100	50 to 80	2
Hexosaminidase	75 to 100	100	25 to 90	2
Arylsuphatase A	50 to 90	100	20 to 80	2
Argininosuccinase	40 to 50	100	45	3
Ornithine ketoacid transaminase	~50	100	N.M.	4
α-1,4-Glucosidase	~100	100	N.M.	5
Amylo-1,6-glucosidase	~100	100	N.M.	5
Amylo-(1,4→1,6)-transglucosidase	~100	100	N.M.	5
Acid phosphatase	~100	100	100	6
Sphingomyelinase	~100	100	N.M.	7
Glucocerebrosidase	~50	100	N.M.	7

* Based on adult fibroblasts as 100%. † N.M. = not measured.

References: 1. Uhlendorf & Mudd, 1968; 2. Kaback and Leonard, 1970; 3. Shih and Littlefield, 1970; 4. Shih and Schulman, 1970; 5. Howell, Kaback and Brown, 1971; 6. Brock (unpublished); 7. Uhlendorf, 1970.

from adult and fetal explants, and though fetal skin fibroblasts are not the same as fibroblasts derived from amniotic fluid cells, this is another warning that adult skin fibroblasts are not necessarily good control material for making antenatal diagnoses.

There remains the question of whether the stage in gestation at which amniocentesis is performed has any influence on enzyme patterns in cultured cells. Nadler (1968b) observed changes in the isoenzyme bands of G6PD and lactate dehydrogenase, but not in a variety of other enzymes, when cells were obtained from pregnancies of different maturities. He also found that quantitative levels of G6PD (an X-linked enzyme) were approximately twice as high in cells derived from 10-week female fetuses as in equivalent-stage male fetuses. During six weeks of culture, the values in the female cells declined to the level of the male cells. This intriguing suggestion of a pre-Lyonization state in amniotic fluid cells, followed by *in vitro* differentiation and X-chromosome inactivation, has yet to be confirmed. The work of Steele (1970) on G6PD levels in early embryonic tissues, where approximately 70 per cent higher values were found in females, points in the same direction. But both studies may suffer from the difficulty of assessing the contribution of the autosomally-linked enzyme 6-phosphogluconate dehydrogenase to the apparent G6PD values.*

There do not appear to be any other studies which have assessed the effect of gestational stage on cultured cell enzymes. In eight of the nine enzymes assayed by Nadler (1968b) no significent differences in activity were demonstrable in cells obtained at 10, 16, 20, 24, 28, 32 and 36 weeks of gestation. Even the high G6PD values in early female cells tended to normality after six weeks in culture. This suggests, as Littlefield (1971) has pointed out, that cultured amniotic fluid cells are not to be regarded as representative of the state of differentiation of the fetus, but rather as adapted to the unnatural environment of tissue culture. and thus largely independent of the gestational age of the fetus.

Diagnoses which have been made

As yet only a few prenatal diagnoses of inborn errors of metabolism have been made using amniotic fluid cells as the marker material. A considerable range of metabolic disorders have been covered in these diagnoses, but experience in most individual diseases is still disturbingly small. Thus galactosaemia has been cited as being

* Steele (1970) found no difference in the activities af HGPRT (another X-linked enzyme) in the male and female embryonic tissues.

detectable antenatally (Milunsky *et al.*, 1970), but the only diagnosis reported was on a term fluid sample and was not confirmed (Nadler, 1968a). It is, of course, more than probable that galactosaemia will be easily monitored through assays of galactose-1-phosphate uridyl transferase in cultured amniotic fluid cells obtained early enough to allow termination of pregnancy. But until this is done, the experimental state of the science should be emphasized rather than suppressed. To date, the only genetic disorders in which antenatal diagnosis may be repeated with confidence are Tay-Sachs disease and Pompe's disease, while the only group of metabolic disorders in which reasonable progress has been made is the sphingolipidoses (Fig. 13 and Table 18). For this reason, individual disorders are discussed in some detail in the ensuing sections.

Table 18. *Antenatal diagnosis of sphingolipidoses*
(*See Fig. 13 for key number*)

Key no.	Disorder	Deficient enzyme	Antenatal diagnosis*
1	Generalized gangliosidosis	β-Galactosidase	+
2	Tay-Sachs	Hexosaminidase A	+++
3	Globoside storage disease	Hexosaminidase A and B	–
4	Fabry's	α-Galactosidase	+++
5	Lactosyl ceramidosis	Lactosyl ceramide β-galactosidase	–
6	Gaucher's	β-Glucosidase	+++
7	Niemann-Pick	Sphingomyelinase	+++
8	Krabbe's	Galactosyl ceramide β-galactosidase	+++
9	Metachromatic leucodystrophy	Arylsulphatase A	+

* +, Antenatal diagnosis made but inadequately reported; +++, antenatal diagnosis made and fully reported.

Tay-Sachs Disease

The primary defect in Tay-Sachs disease appears to be a deficiency of the A component of the lysosomal enzyme β-D-N-acetylhexosaminidase (Okada and O'Brien, 1969). Its virtual absence in practically all tissues studied leads to the massive accumulation of a specific lipid called GM_2, or 'Tay Sachs ganglioside' (Fig. 13). Two closely related disorders are Sandhoff's disease (or globoside storage disease), in which there is a deficiency of both A and B components of hexosaminidase (Sandhoff, 1969), and the juvenile

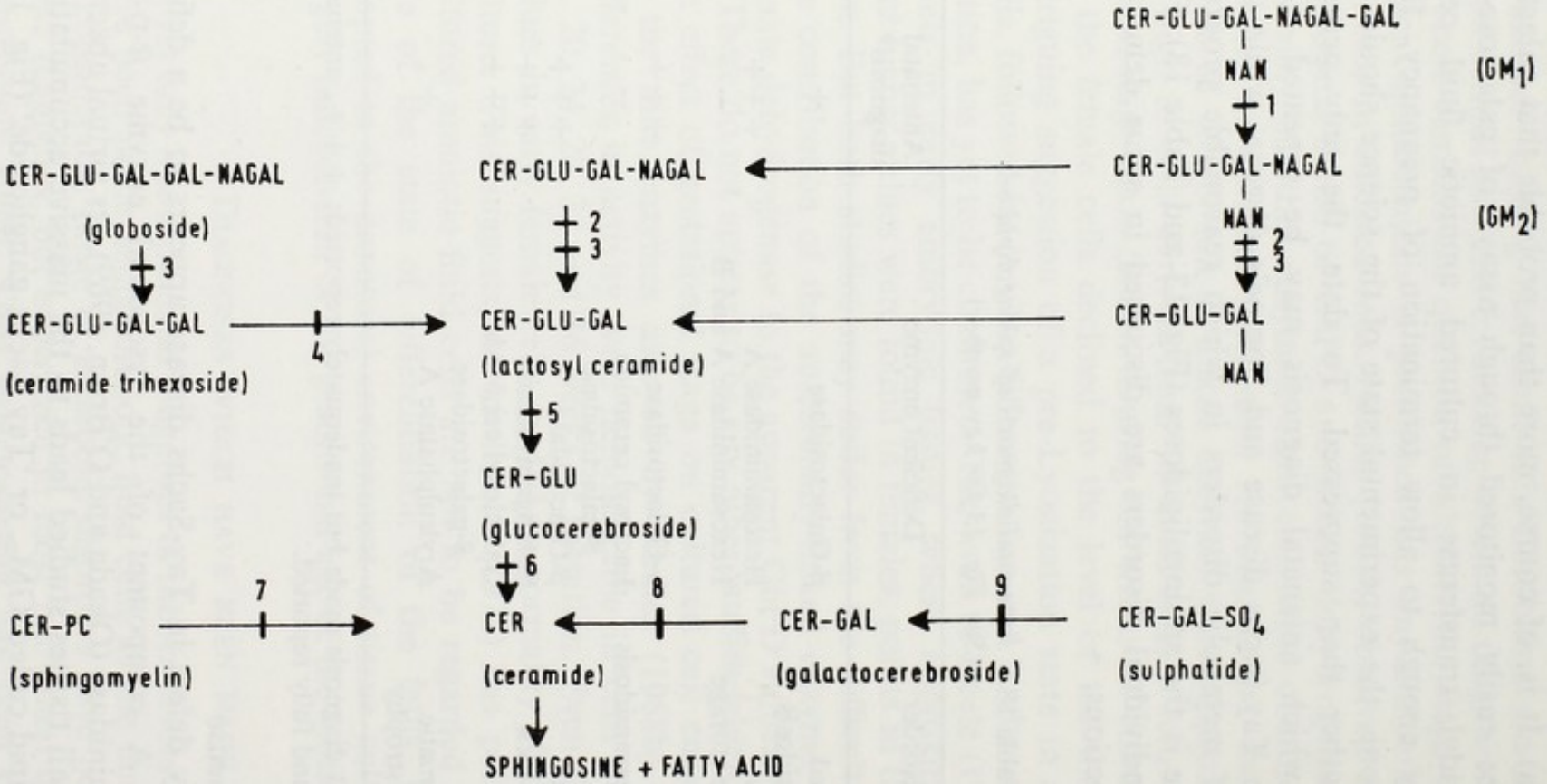

Fig. 13. Schematic representation of the sphingolipidoses. Abbreviations: CER, ceramide; GLU, glucose; GAL, galactose; NAGAL, N-acetylgalactosamine; NAN, N-acetylneuraminic acid. Enzyme block: at 1 in generalized gangliosidosis (β-galactosidase); at 2 in Tay-Sachs (hexosaminidase A); at 3 in globoside storage disease (hexosaminidase A and B); at 4 in Fabry's (ceramide trihexosidase/α-galactosidase); at 5 in lactosyl ceramidosis (lactosyl ceramide β-galactosidase); at 6 in Gaucher's (glucocerebrosidase/β-glucosidase); at 7 in Niemann-Pick (sphingomyelinase); at 8 in Krabbe's (galactosyl ceramide β-galactosidase); at 9 in metachromatic leucodystrophy (arylsulphatase A).

variant of Tay-Sachs in which there is a partial deficiency of hexosaminidase A (Suzuki and Suzuki, 1970a). Tay-Sachs disease itself is comparatively common in Ashkenazi Jews, and heterozygotes may be identified by a partial reduction of the enzyme in serum.

Hexosaminidase A is expressed both in cultured skin fibroblasts and in cultured and uncultured amniotic fluid cells (Okada and O'Brien, 1969). Schneck and his co-workers (1970) were the first to make an *in utero* diagnosis, employing both uncultured cells and cell-free amniotic fluid for their enzyme assays, and calculating the percentage of the A form in total hexosaminidase by acrylamide gel electrophoresis. In a high-risk pregnancy they found only trace amounts of hexosaminidase A in cells and fluid at 18 weeks and again at 20 weeks, made a tentative diagnosis of Tay-Sachs and performed an abortion. The diagnosis was confirmed by enzyme assays on the fetal liver and brain and by ganglioside analyses on the brain.

Within a year, O'Brien and his colleagues (1971) published a paper on the antenatal diagnosis of Tay-Sachs disease which may well become a landmark in the literature of this subject. From Israel, South Africa, Australia and four states of the U.S.A. they collected samples of amniotic fluid from 15 pregnancies in which a previous affected child had established the genetic risk. Despite the sometimes extensive travelling times of the samples, successful cultures were established in all cases, and only two taps had to be repeated. In 12 cases, amniocentesis had been performed early enough to allow for termination of pregnancy; within this group, five affected fetuses were identified and the diagnosis confirmed after abortion, four normal fetuses were identified and diagnosis confirmed after birth, while three presumed normal fetuses were still *in utero*.* To this impressive list may be added a further seven cases reported by Navon and Padeh (1971a) (five were early enough for action and of these one was affected and four normal), and four cases reported by Friedland, Perle, Saifer, Schneck and Volk (1971), all of which were normal. Assuming that there is no overlap in the cases reported by the different groups, experience with prenatal Tay-Sachs diagnosis is now quite extensive.

Both O'Brien *et al.* (1971) and Navon and Padeh (1971a) used a heat denaturation technique (O'Brien, Okada, Chen and Fillerup, 1970) to establish the proportion of the A component in total hexosaminidase activity, while Friedland *et al.* (1971) used electro-

* These diagnoses were subsequently shown to be correct (O'Brien, personal communication).

phoretic scanning. It would appear that both methods are equally reliable, though for the inexperienced the visual inspection of electrophoretograms for the absence of hexosaminidase A may be more reassuring. Without any doubt, cultured amniotic fluid cells are the material of choice for assays, though O'Brien *et al.* (1971) reported good correspondence between these and both uncultured cells and amniotic fluid itself, while Navon and Padeh (1971a) found correspondence between cultured and uncultured cells. Friedland *et al.* (1971) have confirmed that hexosaminidase A in cell-free amniotic fluid reflects the fetal state, an intriguing observation when set against earlier genetic studies showing the maternal

Table 19. *Reported experience on the prenatal detection of Tay-Sachs disease*

Investigators	Number of diagnoses made	Affected fetuses	Tissues used*	Method of assay
Schneck *et al.*, 1970	1	1	UAFC AF	Electro-phoresis
O'Brien *et al.*, 1971	15	5	CAFC UAFC AF	Heat denaturation
Navon and Padeh, 1971a	7	1	CAFC UAFC	Heat denaturation
Friedland *et al.*, 1971	4	0	AF	Electro-phoresis
Rattazzi and Davidson, 1970	1	0	UAFC	Electro-phoresis

* Abbreviations: UAFC, uncultured amniotic fluid cells; CAFC, cultured amniotic fluid cells; AF, amniotic fluid.

origin of another amniotic fluid protein, group-specific component (Ruoslahti, Tallberg and Seppälä, 1966; Usategui-Gomez and Morgan, 1966). However, it may be premature to generalize about the origin of proteins in amniotic fluid (see p. 113 *et seq.*). It must also be noted that a single misdiagnosis of Tay-Sachs disease has been made, based on enzyme levels in uncultured cells (Rattazzi and Davidson, 1970). Table 19 presents a summary of reported experience on the prenatal diagnosis of this disease.

Though Tay-Sachs is not usually clinically apparent until after the first six months of a child's life, examination of affected aborted material has abundantly demonstrated both chemical changes in brain tissue and also electron micrographic abnormalities in spinal cord and cortical neurones. Total gangliosides are elevated

and consist of an abnormal proportion of GM_2, easily demonstrated by thin-layer chromatography (Schneck *et al.*, 1970; Navon and Padeh, 1971a; O'Brien *et al.*, 1971). These findings, made in the second trimester of pregnancy, suggest that attempts to treat the disease in the neonatal period by infusion of hexosaminidase-rich serum, plasma or leucocytes are unlikely to be more than partially successful.

Pompe's disease

Pompe's disease (glycogen storage disease type II) was the first inborn error of metabolism to be attributed to an enzyme primarily localized in the lysosomal fraction of the cell. This enzyme, α-1,4-glucosidase, hydrolyses glycogen directly to glucose without the intermediacy of glucose-1-phosphate (the phosphorylase reaction). Its deficiency leads to massive accumulation of glycogen in intracellular vacuolated structures which are presumably abnormal lysosomes. The enzyme is widely distributed and has been demonstrated in cultured skin fibroblasts (Nitowsky and Grunfeld, 1967) and amniotic fluid cells (Nadler, 1968b).

Nadler (1968b) used cultured amniotic fluid cells to monitor a pregnancy in a mother who had had an earlier child with Pompe's disease and deferred action when α-1,4-glucosidase activities were found to be normal. Later, Nadler and Messina (1969) reported their extended experience in eight pregnancies at risk for Pompe's disease in one of which an affected fetus was detected, aborted and the diagnosis confirmed by fetal tissue assays. In this study, they found that α-1,4-glucosidase activities were consistent in cultured cells, uncultured cells. and amniotic fluid itself. This was challenged by Cox *et al.* (1970), who reported that they had found only cultured cells to be reliable material for monitoring the fetal state and pointed to the obvious difficulties when maternal blood contaminated the fluid and uncultured cells. Nadler, Bigley and Hug (1970) have now confirmed this opinion in a study in which α-1,4-glucosidase activity in cultured cells led to a correct diagnosis of Pompe's while activity in the fluid itself was quite misleading. Recently, Salafsky and Nadler (1971) have reported that amniotic fluid α-1,4-glucosidase has properties quite different to the enzyme from liver, fibroblasts and cultured amniotic fluid cells.

An alternative method of diagnosing Pompe's disease *in utero* was suggested by Hug, Schubert and Soukup (1970). Membrane-surrounded accumulations of glycogen were seen under the electron microscope in amniotic fluid cells taken in the 36th week of a pregnancy which led to an affected child. Similar findings in cultured cells obtained from a 16 week amniocentesis have con-

firmed that electron microscopy may be used to support diagnoses based on α-1,4-glucosidase activities (Nadler, Bigley and Hug, 1970).

Lysosomal acid phosphatase deficiency

A new familial metabolic disorder was reported in 1970 by Nadler and Egan. Three siblings were described with intermittent vomiting, hypotonia and lethargy leading to death in early infancy. But unlike so many other failure-to-thrive syndromes in the literature, a chance finding established the biochemical defect in one of the children and opened the possibility of antenatal diagnosis of the condition. Routine assay of a series of enzymes in fibroblasts cultured from the third child revealed a deficiency of acid phosphatase which was particularly marked in the lysosomal fraction of the cell. The enzyme was also profoundly deficient in a range of tissues obtained from the patient at autopsy, and diminished to approximately half-normal values in cultured fibroblasts and phytohaemagglutinin-stimulated lymphocytes of both parents. This established beyond reasonable doubt that acid phosphatase deficiency was the cause rather than an effect of the child's condition and that it was in all probability being transmitted as an autosomal recessive trait. Since failure-to-thrive has been attributed to a variety of causes, and since the cellular function of lysosomal acid phosphatase is obscure, demonstration of parental heterozygosity was particularly necessary in this condition.

Nadler and Egan were quick to use their finding in prenatal diagnosis. When the mother became pregnant again, amniocentesis was performed at 13 weeks and the pregnancy terminated on the basis of the acid phosphatase levels in the amniotic fluid cells. Unfortunately, the details of the investigation are cursorily reported; it appears that the enzyme was measured in uncultured cells (no values or control values cited) and the diagnosis confirmed in fibroblasts cultured from the skin of the abortus.

Mucopolysaccharidoses

Despite intensive investigations, the basic biochemical lesion has not been discovered in any of the six classical mucopolysaccharidoses—Hurler, Hunter, Sanfilippo, Morquio, Scheie and Maroteaux-Lamy.* There are, however, certain characteristic chemical abnormalities in these disorders which have assisted differential diagnoses and opened the way to antenatal detection. The two most important are the accumulation of mucopolysaccharides in cultured skin fibroblasts and the excretion of

*But see McKusick, V. A. *et al.* (1972) *Lancet* i, 993–996.

mucopolysaccharides in the urine of affected people. The latter is relevant to the *in utero* situation in that fetal urine may contribute to the amniotic fluid in early stages of gestation, so that analysis of fluid mucopolysaccharides may be expected to yield information on the fetal state (but see p. 120). The former has considerable bearing on the possibility of fetal cells displaying early mucopolysaccharide abnormalities.

The first observation that amniotic fluid cells might be useful in prenatal diagnosis of a mucopolysaccharidosis was made by Nadler (1968a). Cells cultured from a woman with a known history of rhesus incompatibility, but without any history of mucopolysaccharidosis, were observed to be larger and more granular than normal. Toluidine-blue staining revealed metachromasia, which is a typical finding in cultured skin fibroblasts of patients with Hurler's, Hunter's, Sanfilippo and Scheie syndromes (Danes and Bearn, 1967), but which has also been observed in lipid storage diseases and a variety of other miscellaneous disorders (Matalon and Dorfman, 1969). The pregnancy went to term and a male infant was delivered, who excreted increased amounts of mucopolysaccharide and who, at 5 months of age, had developed hepatosplenomegaly and bilateral inguinal hernias. There seems little doubt that metachromasia in the cultured amniotic fluid cells had given advance warning of a typical mucopolysaccharidosis.

In the past two or three years, the histochemical technique of toluidine-blue metachromasia as a diagnostic tool has been strongly criticized. It lacks specificity in that many anionic macromolecules, such as lipids, nucleic acids and proteins, will complex with the dye and induce the wavelength shifts which are all the hallmark of the microscopist's observation of metachromasia. However, if metachromasia were limited to homozygotes for lipid and mucopolysaccharide storage diseases, this lack of specificity would not matter very much. The problem arises when positive results are obtained with heterozygotes and those presumed to have no carrier status (Taysi, Kistenmacher, Punnett and Mellman, 1969; Matalon and Dorfman, 1969). Because of such findings, Danes, Scott and Bearn (1970) introduced an alternative histochemical technique for revealing mucopolysaccharide accumulation, which is based on the formation of complexes with alcian blue, and therefore termed 'alcianophilia'. This procedure seems more specific for the mucopolysaccharidoses and may be a valuable addendum to metachromasia in attempting to resolve the confusion caused by false positives.

A more detailed and specific attempt at intrauterine diagnosis of Hurler and Hunter syndromes has been made by Fratantoni,

Neufeld, Uhlendorf and Jacobson (1969). Their investigation was based on the earlier observation that mucopolysaccharide accumulation in cultured skin fibroblasts in these disorders could be assayed by following radioactive sulphate incorporation, a measure of the increased levels of stored dermatan and heparitin sulphates. In their first subject, a women who had had two previous children with Hurler's syndrome, cultured amniotic fluid cells showed the typical excessive sulphate incorporation seen in fibroblasts from an affected sibling. However, amniocentesis was not performed until the 25th gestational week and the cells were not ready for kinetic studies until they had been cultured for 10 weeks. Toluidine-blue metachromasia was negative at three weeks of culture and positive at the eighth week. The pregnancy went to term and a female infant delivered with signs of Hurler's syndrome. Diagnosis was confirmed by urinary mucopolysaccharide analyses, and by demonstration of metachromasia and abnormal sulphate incorporation in fibroblasts cultured from the umbilical cord. Interestingly, cells cultured from the amnion appeared quite normal.

The second case was a women who had earlier given birth to a son with Hunter's syndrome. Amniocentesis at 14 weeks yielded cells with a 46,XY karyotype. The parents elected to have the pregnancy terminated at the 25th week, but unfortunately no cells could be grown from the macerated abortus. However, by that time the cultured amniotic fluid cells showed both metachromatic and sulphate-accumulating abnormalities identical to those experienced in the Hurler's case. It seems reasonable to assume that if culture times can be accelerated, sulphate incorporation coupled to more sophisticated histochemical procedures (alcian blue staining), should be valuable in the intrauterine detection of both Hurler's and Hunter's syndromes, and possibly other mucopolysaccharidoses.

Lesch-Nyhan syndrome

The Lesch-Nyhan syndrome, or congenital hyperuricaemia, is an inborn error of purine metabolism caused by a profound deficiency of hypoxanthine-guanine phosphoribosyl transferase (HGPRT), an enzyme also referred to as IMP pyrophosphorylase. The main chemical feature of the disease is a gross overproduction of uric acid, which is associated with spasticity, choreoathetosis, psychomotor retardation and compulsory automutilation of hands and lips. It is inherited as an X-linked recessive trait and the enzyme deficiency is expressed in a wide variety of tissues including red cells and cultured fibroblasts. Heterozygous carriers of the Lesch-Nyhan gene cannot be identified through enzyme levels in

erythrocytes, for some form of cell selection appears to be operating (Kelley, Greene, Rosenbloom, Henderson and Seegmiller, 1969), to favour those with normal enzyme activity. However, it has proved possible to detect carriers by virtue of the fact that their cultured skin fibroblasts show cellular mosaicism with respect to HGPRT activity (Rosenbloom, Kelley, Henderson and Seegmiller, 1967).

The first pregnancy, in which there was a risk of an infant with Lesch-Nyhan syndrome, to be successfully monitored was described by Fujimoto, Seegmiller, Uhlendorf and Jacobson (1968). The proband was a woman who had already had two daughters and a normal son, but whose maternal aunt had had a son with hyperuricaemia. Her carrier status was established through demonstration of two cellular populations of cultured skin fibroblasts, one population being capable of incorporating hypoxanthine into nucleic acid and thus demonstrating HGPRT activity, while the other was not. Amniocentesis was performed at the 17th week of gestation and a female fetus demonstrated by sex-chromatin count on uncultured cells and later by a 46.XX karyotype on the cultured cells. This was sufficient to exclude the possibility of an affected child. However, Fujimoto *et al.* proceeded to show that it was possible to demonstrate HGPRT-positive and HGPRT-negative populations among the cultured amniotic fluid cells. This, of course, established that the fetus was itself a carrier of the condition, but more important, it suggested that since enzyme-deficient cells could be easily recognized by the autoradiographic technique used, there would be no difficulty in detecting affected males through amniotic fluid cell biochemistry.

This has proved to be the case. DeMars, Sarto, Felix and Benke (1969) reported a prenatal diagnosis of Lesch-Nyhan syndrome through cultured cells which failed to incorporate hypoxanthine. Their diagnosis was made too late for a termination to be performed and led to the birth of male twins, both of whom were affected. However, Boyle *et al.* (1970), working with amniotic fluid cells obtained at 18 weeks, established HGPRT deficiency in a male fetus at 22 weeks, terminated the pregnancy and confirmed their diagnosis by enzyme assay on various tissues from the abortus.

The prenatal diagnoses which have been made in the Lesch-Nyhan syndrome have all been based on an autoradiographic procedure which measures qualitatively the ability of cells to take up radioactively-labelled hypoxanthine. This assay can be used only with cultured cells for the uncultured amniotic fluid pellet contains too many non-viable cells which would be indistinguish-

able from those which were viable but HGPRT-negative. Cell culture has thus been an obligatory part of this particular prenatal diagnosis. However, Berman *et al.* (1969) suggest that there is an alternative method of scanning for HGPRT deficiency which may be applied to uncultured cells. When the activities of HGPRT and AMP pyrophosporylase are compared, an internal control is provided which offsets the large fluctuations of enzyme activity found in uncultured cells (p. 88). Their method has not yet been tested in a real diagnostic situation, but there seems no reason why it should not work.

Fabry's disease

Fabry's disease, or angiokeratoma corporis diffusum, is a storage disease in which the sphingolipid ceramide trihexoside (galactosyl-galactosyl-glucosyl-ceramide) accumulates in various tissues and organs (Fig. 13, p. 94). Symptoms are often primarily dermatological though impaired renal function may lead to death through kidney failure from the third decade onward. It is inherited as an X-linked recessive trait and the primary biochemical lesion is a deficiency of the enzyme responsible for the cleavage of the terminal galactose residue from ceramide trihexoside. This enzyme, usually referred to as ceramide trihexoside, appears to be located in the lysosomal fraction of the cell, and Fabry's disease may therefore be regarded, like Tay-Sachs, acid phosphatase deficiency and Pompe's as a 'lysosomal disorder'. An important development in the biochemical investigation of the disease was the discovery that the synthetic substrate, *p*-nitrophenyl-α-D-galactopyranoside, could be used in place of ceramide trihexoside to monitor the enzyme deficiency of Fabry's disease (Kint, 1970).

Using this α-galactosidase assay, Romeo and Migeon (1970) demonstrated the enzyme defect in fibroblasts cultured from the skin of an affected male and also confirmed the X-linkage of the disorder by showing that cells from heterozygous females could be cloned into enzyme-proficient and enzyme-deficient populations. Brady, Uhlendorf and Jacobson (1971) used this information to monitor a pregnancy at risk for Fabry's disease. An expectant mother, whose brother was affected, had approximately half-normal levels of α-galactoside in her fibroblasts, and was therefore deemed a probable carrier. Sex chromatin was absent in uncultured amniotic fluid cells; after three weeks a normal male karyotype was demonstrated in the cultured cells. After four weeks of culture a profound deficiency of α-galactosidase activity in the cells suggested that the male fetus was affected. The pregnancy was terminated and the diagnosis confirmed by α-galactosidase

assay on various tissues, and by analyses of ceramide trihexoside concentrations in brain and kidney.

Cystinosis

Cystinosis appears to be the only storage disease so far described which does not involve a macromolecule. The amino acid, cystine, builds up to high levels in bone marrow, cornea and internal organs of affected children; in leucocytes and fibroblasts an approximately 100-fold increase has been observed. This leads to the renal tubular defects of the Fanconi syndrome with progressive glomerular damage and death in late childhood. The primary biochemical defect is not known, though Schulman and Bradley (1970) have provided convincing evidence that lysosomes in cystinotic cells are deficient in their ability to dispose of cystine.

The characteristic cystine accumulation in cultured skin fibroblasts of cystinotic children suggested a method of prenatal diagnosis of the disorder (Schneider, Rosenbloom, Bradley and Seegmiller, 1967). Before this could happen, a more sensitive method of measuring cystine in small numbers of cells had to be developed. This was achieved by pulse-labelling growing amniotic fluid cells with ^{35}S-cystine and then measuring the incorporation of radioactivity into intracellular cystine compared to protein (Schulman, Fujimoto, Bradley and Seegmiller, 1970. The method was tested on a mother who had two earlier children with cystinosis. The cystine/protein ratio in the amniotic fluid cells was almost identical to that in fibroblasts from an obligate heterozygote, higher than that in amniotic fluid cells from normal pregnancies, but much lower than that in cystinotic fibroblasts. Schulman *et al.* (1970) concluded that the fetus was heterozygous and confirmed their diagnosis on umbilical cord fibroblasts at birth. Although this report hardly constitutes an antenatal diagnosis (amniocentesis at 27 weeks, diagnosis after 7 to 8 weeks of culture), it describes a method which may be developed by the use of cystine of higher specific radioactivity to apply to very small quantities of amniotic cells.

Niemann-Pick disease

Niemann-Pick disease is a sphingolipidosis characterized by the accumulation of sphingomyelin (Fig. 13, p. 94) throughout the body. Symptoms are hepatomegaly, splenomegaly and varying degrees of central nervous system damage which in the classical infantile form lead to severe mental retardation and death within the first two years. The basic deficiency is in the enzyme responsible for the cleavage of a phosphorylcholine residue from sphingomyelin,

usually referred to as sphingomyelinase. It is localized in the lysosomal fraction of the cell, and thus widely distributed among the body tissues, including cultured skin fibroblasts and amniotic fluid cells (Uhlendorf, 1970).

A single antenatal diagnosis of Niemann-Pick disease has so far been reported. Amniocentesis was performed on a mother who had had an earlier child with the disorder, and sphingomyelinase measurements made on the cultured amniotic fluid cells. Very low enzyme activity indicated a homozygous affected child, and this diagnosis was confirmed after termination of the pregnancy by sphingomyelinase determinations on the abortus (Epstein, Brady, Schneider, Bradley and Shapiro, 1971).

Krabbe's disease

Krabbe's disease (or globoid cell leucodystrophy) is a progressive neurological disorder of infants, beginning in the first few months of life and leading to death before the end of the first year. It is characterized morphologically by an absence of myelin and the presence of multinucleated 'globoid' cells in white matter, and chemically by an abnormally high galactocerebroside/sulphatide ratio (Fig. 13, p. 94). The primary defect has been shown to be a deficiency of the enzyme galactocerebroside β-galactosidase, which is found in a variety of tissues including brain, white cells and fibroblasts (Suzuki and Suzuki, 1970b).

An antenatal diagnosis of Krabbe's has been made by Suzuki, Schneider and Epstein (1971). Cells cultured from the amniotic fluid of a woman with a previous affected child had virtually no galactocerebroside β-galactosidase activity, even though non-specific β-galactosidase values were normal. The pregnancy was terminated and the diagnosis confirmed by enzyme determinations on brain and liver of the aborted fetus.

Other disorders

The antenatal diagnosis of several other disorders have been briefly recorded in the literature. Nadler and Gerbie (1970) discuss a case of *metachromatic leucodystrophy* detected by arylsuphatase A determinations (Fig. 13, p. 94) in cultured amniotic fluid cells which led to a therapeutic abortion. They also record the monitoring of a pregnancy in which there was a risk of *generalized gangliosidosis* and two pregnancies with a risk of *maple syrup urine disease*, in each of which the fetus was adjudged normal. Nadler (1968a) has also indicated that galactose-1-phosphate uridyl transferase activity in cultured cells may be useful for the *in utero* detection of *galactosaemia*, though in the case reported

cells were obtained at 33 weeks of gestation and the diagnosis not made until after the birth of the infant. Uhlendorf (1970) has discussed a pregnancy leading to the birth of a normal child where cystathionine synthetase assay of cultured cells was used to reassure a mother who had had two earlier children with *homocystinuria.* Nadler, Swae, Wodnicki and O'Flynn (1969) have investigated the possibility of diagnosing *cystic fibrosis* from metachromatic granules in cultured amniotic fluid cells, and concluded that at least as far as toluidine-blue metachromasia is concerned both false positives and false negatives can be found. Nadler (1971) claims to have used metachromatic granules in diagnosing *Marfan syndrome* but furnishes no details. Prenatal diagnoses of *Gaucher's disease** and *argininosuccinic aciduria* have been reported.†

Limitations and Prospects

The achievements of antenatal diagnosis at present fall a long way short of its potential. In principle, any protein-based Mendelian disorder, in which the biochemical lesion is known or which is associated with a distinctive marker substance, should be detectable *in utero.* (It is possible also that certain multifactorial conditions will reveal themselves through extrusion of characteristic molecules into the amniotic fluid, though this is less certain.) In practice, there are various limitations to this idea, some technical and some of a more fundamental nature, which may be summarized as: (1) the problem of distinguishing heterozygotes and homozygotes; (2) the range and heterogeneity of single-gene defects; and (3) variations in the tissue expression of different gene-products.

Heterozygotes and homozygotes

In both autosomal and X-linked recessive disorders the identification of clinically normal heterozygotes is important. Though there is as yet little direct experience of heterozygote detection from amniotic fluid cells, there is a great deal of indirect knowledge to be gained from the monitoring of genetic disorders in cultured skin fibroblasts. In most autosomal recessive disorders where the enzyme deficiency is known, dosage effects have been observed in a variety of tissues (red cells, white cells, fibroblasts; Hsia, 1970), so that intermediate enzyme levels distinguish the heterozygote from both the normal and abnormal homozygotes. In amniotic fluid cells, this has been found to be the case with

* Epstein, C. J. *et al.* (1972) *Am. J. hum. Genet.* **24**, 214–226.
† Jacoby, L. B. *et al.* (1972) *Am. J. hum.Genet.* **24,** 321–324.

hexosaminidase A levels in Tay-Sachs (O'Brien *et al.*, 1971), where a prenatal diagnosis of heterozygosity has been confirmed after birth by serum enzyme measurements. It is also apparently the case in Pompe's disease (Nadler and Messina, 1969), though here apparent heterozygosity was not confirmed.

Heterozygosity for cystinosis has been demonstrated by cystine concentration in cultured amniotic fluid cells (Schulman *et al.* 1970). In general, however, when measurements become remote from the level of the primary product of the gene, more uncertainty arises in the identification of genotype. This has been noted when metachromasia has been used to monitor for cystic fibrosis of the pancreas in cultured fibroblasts and amniotic fluid cells (Nadler *et al.*, 1969).

In X-linked recessive disorders, carrier detection is complicated by X-chromosome inactivation. It is not possible to know in advance what proportion of the cells of a given tissue of a female carrier will express the mutant gene. The only certain method of identifying a heterozygote is by demonstrating cellular mosaicism (the absence of cellular mosaicism is not in itself proof of homozygosity because of the possibility that positive and negative cells will interact to complement each other). With advances in tissue culture this has now been achieved in cultured fibroblasts in a number of X-linked disorders—G6PD deficiency, X-linked chronic granulomatous disease, the Lesch-Nyhan syndrome, Fabry's disease and Hunter's syndrome. Prenatal detection of female carriers is therefore possible in all of these, and has already been achieved in the Lesch-Nyhan syndrome (Fujimoto *et al.*, 1968). However, in more common disorders like the haemophilias A and B, even though the primary protein defect is known, carrier detection is imprecise because factors VIII and IX are not expressed in cultured cells. Detection of affected hemizygotes and heterozygous carriers is thus not yet possible. (though linkage studies may be helpful, see p. 125).

Range and heterogeneity

In *Mendelian Inheritance in Man* McKusick (1971) lists 1700 to 1800 genetic disorders which may be plausibly attributed to single genetic loci. If the gene–enzyme relationship discussed in the Introduction holds for all or a majority of these, they may be considered as candidates for antenatal detection. Multiple alleles at single loci have been documented for the α, β and δ chains of haemoglobin, glucose-6-phosphate dehydrogenase and in a more limited sense for pyruvate kinase, acid phosphatase, pseudocholinesterase α_1-antitrypsin, phosphohexose isomerase and methae-

moglobin reductase. Such heterogeneity, which has been a prominent feature of every inborn error which has been investigated in depth, when coupled to the range of genetic loci involved, suggests that in many cases it is going to be difficult for an investigator to establish any depth of experience in antenatal diagnosis. Requests will come in for single diagnoses in a variety of different disorders, each perhaps involving a different area of biochemistry. Variant forms of the disease may be involved; for example O'Brien *et al.* (1971) have demonstrated that Tay-Sachs may be detected antenatally with confidence but as yet there is no reported experience on the juvenile form of Tay-Sachs, which presumably derives from a distinct mutation at the same genetic locus (Suzuki and Suzuki, 1970a). Pompe's disease has been diagnosed *in utero* several times on the basis of a deficiency of acid maltase in amniotic fluid cells, yet it is becoming apparent that there is a form of maltase deficiency which leads not to the severe symptoms of Pompe's but instead to a comparatively mild myopathy (Hudgson, Gardner-Medwin, Worsfold. Pennington and Walton, 1968).

Tissue expression

At the present time antenatal diagnosis of inborn errors of metabolism is closely tied to the expression of enzyme defects in amniotic fluid cells, which in turn is intimately related to expression in cultured skin fibroblasts. This means that all the disorders in Table 14 are strong possibilities for *in utero* detection. Furthermore, even when the primary enzyme lesion is unknown, if the disorder has a characteristic marker substance in tissue culture (e.g. metachromasia, cystine accumulation) it is also potentially diagnosable.

This still leaves a very large number of individual genetic diseases where there is currently little prospect of antenatal diagnosis. This group includes disorders of obscure origin like Duchenne muscular dystrophy and Huntington's chorea, and those of known origin like Von Gierke's, phenylketonuria and β-thalassaemia, where the characteristic defect is not expressed in available tissue cells. For the former there is little to be done but to continue the search both for causal and for marker molecules. For the latter there are two possible courses of action: gaining access to fetal cells in which the enzyme or protein is known to be normally expressed (e.g. red cells for haemoglobinopathies), or causing the expression of the enzyme under study in amniotic fluid cells by *in vitro* manipulations.

The induction of an enzyme in cells where it is not normally expressed (or so weakly expressed as to be unmeasurable by current techniques) is often referred to as gene derepression. A great

deal has been written about the derepression of mammalian genes *in vitro*, and the problems this might solve in diagnosis of genetic disorders. There is, however, as yet no real evidence that mammalian genes are repressed in the first place. It is probably more correct to discuss the problem in terms of activation and inactivation, or switching on and switching off since this demands no knowledge of mechanisms. There seems little doubt that the entire genome is contained in the nucleus of each cell, and that a particular cell type is characterized by a set of active and inactive genes and the protein products of the active genes. What is required in antenatal diagnosis is the activation of designated genes in amniotic fluid cells so that they produce the enzymes needed to monitor a particular disorder. Thus the appearance in cultured cells of phenylalanine hydroxylase, normally found only in liver and kidney, would allow antenatal diagnosis of phenylketonuria, while the appearance of haemoglobin would open the way to diagnosis of a range of haemoglobinopathies.

Attempts at deliberate gene activation in tissue culture have been uniformly unsuccessful, and until the mechanism of gene control in eukaryotes is better understood the switching on of a gene can be little more than accidental. Developments in fetoscopy (p. 49) suggest that a far more profitable line of action is to attempt to obtain suitable tissues from the fetus in which the enzyme or protein under study is already expressed. Two recent reports emphasize the significance of this approach. Jakubovic (1971) has demonstrated that, contary to general belief, phenylalanine hydroxylase is synthesized in the fetal liver from as early as the 10th week of gestation; the antenatal diagnosis of phenylketonuria has now become a tissue-accessibility problem rather than a gene-activation problem, and though the prospect of fetal liver biopsy may seem daunting, it is probably a great deal easier than groping in the dark for gene switches.

The other report which has extended the dimension of antenatal diagnosis comes from Hollenberg, Kaback and Kazazian (1971). They have, again contary to accepted dogma, shown that haemoglobin A is synthesized in small quantities in the peripheral blood cells of fetuses from the ninth week on. This means that the gene controlling the haemoglobin β-chain is not switched on suddenly at birth, but begins to express itself very early in fetal life. It means also that haemoglobins with mutant β-chains (which include the more common abnormal haemoglobins—S, C, and E) may now be detected antenatally provided a sample of fetal blood can be obtained.* Indeed it may not even be necessary

* This has now been confirmed: Kan, Y. W. *et al.* (1972) *New Engl. J. Med.* **287**, 1–5.

to obtain fetal blood, for Hollenberg *et al.* (1971) observed that amniotic fluid cells are often contaminated by fetal erythrocytes. The sensitive methods of measuring β-chain formation which they have described may be capable of detecting Hb A synthesis by fetal cells (80 per cent reticulocytes) even in the presence of the Hb A of maternal erythrocytes.

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8

Biochemical Analysis of Amniotic Fluid

A. E. H. Emery

Introduction

The biochemical analysis of amniotic fluid as a possible means of diagnosing genetic disease *in utero* has obvious advantages over methods which depend upon the culture of amniotic fluid cells. Smaller volumes of fluid may be required, the technical difficulties involved in culturing amniotic fluid cells are avoided and results would be available much sooner. It may take as long as six or even eight weeks before there are sufficient cells on which the biochemist can establish a diagnosis. However, despite the obvious advantages of studying amniotic fluid itself there may be limitations to this approach. The aim of this chapter is to indicate some of the progress being made in this field as well as to point out some possible limitations. The discussion will be limited to disorders other than rhesus sensitization.

Origin of Amniotic Fluid

There is general agreement that fetal urine contributes largely to the formation of amniotic fluid in *later pregnancy,* there being little or no amniotic fluid when the fetus has absent ureters or kidneys (Bain and Scott, 1960; Potter, 1965). But other sources may also be involved, including the fetal respiratory tract (Biggs and Duncan, 1970). In the problem of antenatal diagnosis, however, we are more concerned with the origin of amniotic fluid in *early pregnancy* (before the 20th week of gestation) and here there is some controversy (Abramovich, 1970; Ostergard, 1970). Investigators have tackled the problem in a number of ways. The proportions of various protein fractions in amniotic fluid, fetal serum and maternal serum as revealed by electrophoresis have suggested to some that the fluid is of maternal origin (McKay, Richardson and Hertig, 1958), but to others that it is of fetal origin (Brzezinski, Sadovsky and Shafrir, 1964). Other investigators have studied the distribution of various metabolites in maternal and

fetal plasma, fetal urine and amniotic fluid in the hope of gaining a clearer understanding of the source of amniotic fluid in early pregnancy. At this stage of pregnancy the results of such studies suggest that amniotic fluid urea, uric acid and placental lactogen are of maternal origin (Friedberg, 1955; Serr, Czaczkes and Zuckerman, 1963; Tallberg, Ruoslahti and Ehnholm, 1965).* On the other hand, studies of α_1-fetoprotein (Gitlin and Boesman, 1966; Adinolfi and Gardner, 1967; Seppälä and Ruoslahti, 1972) suggest that this protein in amniotic fluid is of fetal origin. Casper and Benjamin (1970) have recently shown that immunoreactive insulin first appears in amniotic fluid at least by 16 weeks gestation, which corresponds to the time when the fetal pancreas is known to begin functioning, and the human placenta is impermeable to insulin (Adam, Teramo, Raiha, Gitlin and Schwartz, 1969). These observations suggest that amniotic fluid insulin is largely, if not entirely, of fetal rather than maternal origin.

Radioactive labelling experiments carried out in early pregnancy in humans have shown that most of the injected material which finds its way into the amniotic fluid does so via the fetus (Dancis, Lind, and Vara, 1960) though in the rhesus monkey the results of similar experiments suggested a different interpretation (Bangham, 1960). Cockburn and his colleagues (Cockburn, Robins and Forfar, 1970) have recently investigated the patterns of free amino acid concentrations in maternal and fetal plasma, fetal urine and amniotic fluid in 29 specimens between 15 to 20 weeks gestation. The highest number of significant positive correlations was between the amino acid concentrations in fetal urine and amniotic fluid suggesting that among the various fluids sampled the closest relationships exist between these two compartments. There are two possible interpretations for these results either fetal urine makes a significant contribution to the amniotic fluid amino acid composition or alternatively the chorioamnion may selectively transfer amino acids in a pattern similar to that maintained by the fetal kidney. Hydroxyproline and ethanolamine are found in amniotic fluid but not maternal serum (Emery, Burt, Nelson and Scrimgeour, 1970; Saifer, A'Zary, Valenti and Schneck, 1970).

From the results of these various studies it seems likely that, at least in early pregnancy, the various constituents of amniotic fluid are derived from both the mother and the fetus. There may be a placental barrier against certain proteins such as placental lactogen (Tallberg *et al.*, 1965), and the limited excretory function

* Group-specific component (Gc) has also now been shown to be of maternal origin in early pregnancy (Sutcliffe, R. G., Brock, D. J. H. & Scrimgeour, J. B. (1972) *Nature, Lond.* **238,** 400.

of the immature fetal kidney may account for the low concentrations of uric acid in early pregnancy (Serr *et al.*, 1963). It is also possible that some substances may find their way into the amniotic fluid without having to pass through the fetus (McKay *et. al.*, 1958; Gitlin and Boesman, 1966), i.e. from the amnion. Present evidence therefore suggests that it would be unwise to generalize about the source of the various constituents of amniotic fluid, at least in early pregnancy.

There seems little doubt, however, that fetal urine contributes to the formation of amniotic fluid from at least midpregnancy. This has been established from observations on the presence of urine in the fetal bladder (see Jeffcoate and Scott, 1959) and the progressive rise in the concentrations of urea and creatinine in amniotic fluid from around midpregnancy (Lind, Parkin and Cheyne, 1969). But urine has been expelled from fetal bladders as early as 11 weeks (Abramovich, 1968) and its composition studied (Gitlin and Boesman, 1966). Histological evidence of renal function has been reported by the 12th week (Hewer, 1924), and perhaps as early as the ninth week of gestation (Gersh, 1937) and Tähti (1966) has obtained radiographic evidence of renal function as early as 12 weeks gestation. Abramovich (1968) has estimated that the urinary output in the human fetus at 11 weeks is approximately 1·2 ml per day rising to 8·4 ml per day at 15 weeks.

It seems most likely that in the earliest weeks of pregnancy amniotic fluid is probably a transudate of maternal plasma. Later, as the fetus develops, and up to about midpregnancy, amniotic fluid may be considered in part an extension of the extracellular fluid of the fetus (Lind *et al.*, 1969; Lind and Hytten, 1970). However, around the end of the first trimester fetal urine begins to contribute to the formation of amniotic fluid and from around midpregnancy, when the fetal skin becomes keratinized and impervious (Parmley and Seeds, 1970), fetal urine is likely to be the main source of the fluid. In the macaque monkey if the fetus, but not the placenta, is removed from the uterus and the pregnancy allowed to continue to term there is a reduction of between 10 and 20 times in the volume of amniotic fluid, the composition of which then continues to resemble a transudate of maternal plasma (Behrman, Parer and Lannoy, 1967).

In summary, amniotic fluid and its various constituents are derived from different sources at different stages of gestation and this is reflected in changes in the composition of amniotic fluid throughout pregnancy. This is an important point if the analysis of amniotic fluid is to be considered in the antenatal diagnosis of genetic disease.

Biochemistry of Amniotic Fluid and Antenatal Diagnosis

Changes in the chemical composition of amniotic fluid at various stages of gestation have been the subject of a number of recent investigations (Bonsnes, 1966; Brown, 1968; Cherry, Dolger, Rosenfield and Kochwa, 1969; Gillibrand, 1969; Delecour, Monnier, and Codaccioni 1970; Doran, Bjerre and Porter, 1970; Queenan, Gadow, Bachner and Kubarych, 1970; Sinha and Carlton, 1970; Lind, Billewicz and Cheyne, 1971). Changes in the chemical composition of amniotic fluid (mainly creatinine) and the cytology of amniotic fluid cells have been used as a means of estimating fetal maturity (Parmley and Miller, 1969; Doran *et al.*, 1970; Lind and Billewicz, 1971), but a recent study suggests that ultrasound cephalometry is possibly more reliable than amniotic fluid studies (Underhill, Beazley and Campbell, 1971). Here we shall only be concerned with the composition of amniotic fluid in early pregnancy as it relates to antenatal diagnosis.

The protein content of amniotic fluid might be used as a unit of reference when investigating the concentrations of various compounds in amniotic fluid. For this reason we have determined normal amniotic fluid protein values at various stages of gestation (Fig. 14). The results are similar to those obtained by Queenan *et al.*, (1970) and show a rise in protein concentration in the first part of pregnancy with a progressive decrease after about the 24th week of gestation. The reason for these changes in protein concentration is not clear.

If the analysis of amniotic fluid is to be useful in antenatal diagnosis, it seems most likely to prove of value in disorders which are associated with changes in extracellular fluid and/or urinary composition which are present at birth, and which are not diet dependent. This approach to antenatal diagnosis presupposes that the biochemical compound in question is not absorbed to any great extent into the maternal circulation and metabolized by the mother.

For the sake of convenience, the analysis of amniotic fluid as a means of diagnosing genetic disease *in utero* will be considered under four headings: adrenogenital syndrome, other inborn errors of metabolism, secretor status and genetic linkage, and congenital abnormalities.

Adrenogenital syndrome

With the exception of rhesus incompatibility, the first attempt to diagnose an inherited metabolic error by the analysis of amniotic fluid was made by Jeffcoate and his colleagues in 1965 (Jeffcoate, Fliegner, Russell, Davis and Wade, 1965) in the case of the adreno-

genital syndrome. The adrenogenital syndrome (congenital adrenal hyperplasia) is a heterogeneous group of conditions inherited as autosomal recessive traits. All are due to defective synthesis of cortisol, usually as a result of 21-hydroxylase deficiency, but defects in other enzymes have been reported (Mitchell and Shackleton, 1969). The defective synthesis of cortisol results in increased pituitary secretion of ACTH, adrenal hyperplasia and excessive androgen secretion with consequent virilization. Some cases are associated with electrolyte disturbances which, if not treated promptly, may be fatal. There is excessive excretion of pregnanetriol and often also 17-ketosteroids in the urine. Jeffcoate *et al.* (1965) were the first to diagnose the adrenogential syndrome before birth by demonstrating increased levels of pregnanetriol (and 17-ketosteroids to a lesser extent) in amniotic fluid at term in an affected pregnancy. This observation has subsequently been confirmed by other investigators (Fuchs, 1967; Nichols, 1969). However, Merkatz, New, Peterson and Seaman (1969) could not

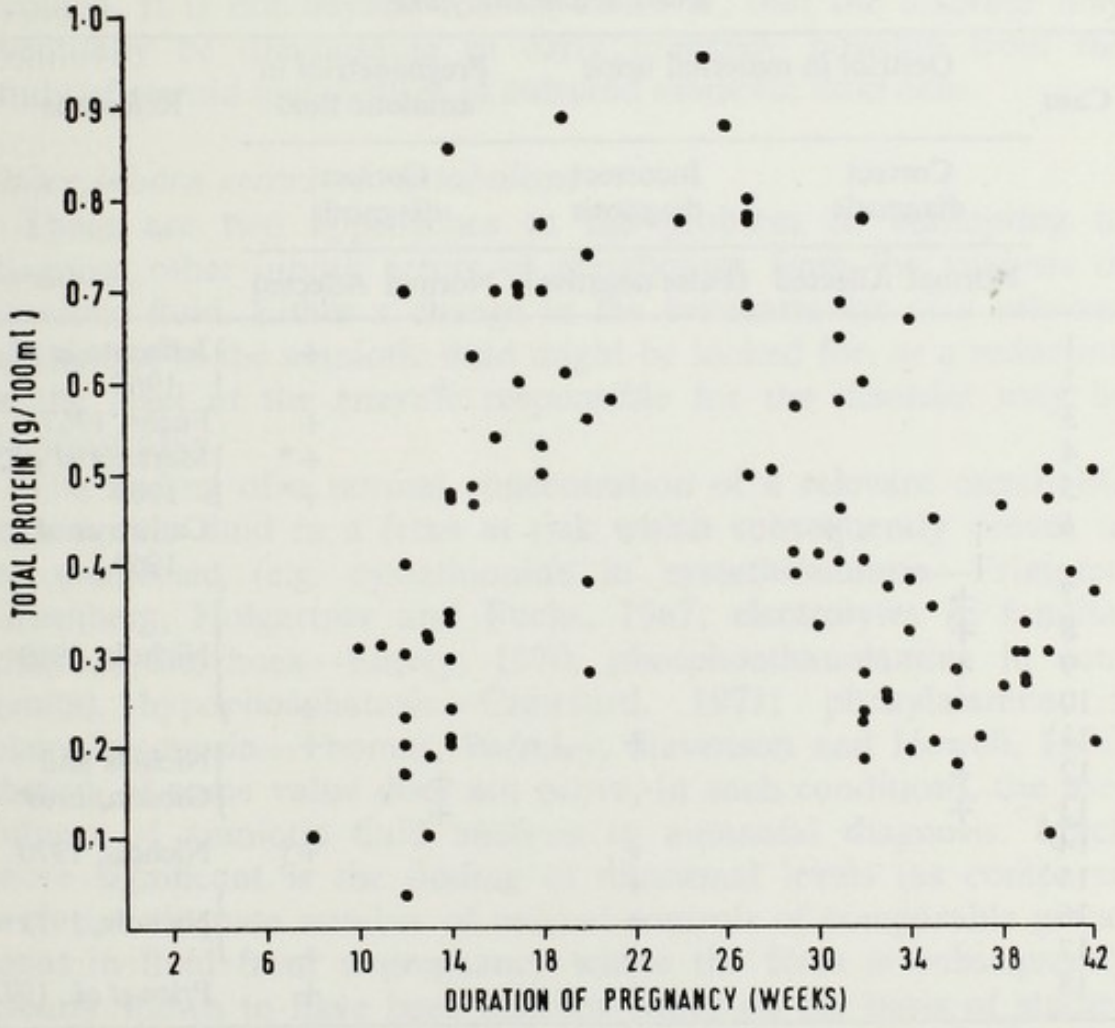

Fig. 14. Protein concentration (g/100 ml) of amniotic fluid in normal pregnancies at various stages of gestation.

detect the disease *in utero* by determining the 17-ketosteroid and pregnanetriol levels in amniotic fluid in early or midpregnancy, but only in late pregnancy (New, 1970). Nichols (1970) has shown that in the case of an affected fetus, previously high amniotic fluid levels of pregnanetriol decreased after injection of 25 mg of hydrocortisone into the fetus and has suggested this might be included as a means of diagnosing the adrenogenital syndrome *in utero,* as well as possibly being of therapeutic value.

Increased levels of oestriol in maternal urine have also been reported as possibly of value in diagnosing the adrenogenital syndrome *in utero* (Cathro, Bertrand and Coyle, 1969). These investigators reported 24 hour urinary excretions of oestriol well above the normal range (mean +2 s.d.) at 26, 29 and 34 weeks gestation in a mother who was subsequently delivered of an affected child. However, Nichols and Gibson (1969), Nichols (1970, 1971) and

Table 20. *The antenatal diagnosis of the adrenogenital syndrome on the basis of oestriol levels in maternal urine and pregnanetriol levels in amniotic fluid*

Case	Oestriol in maternal urine			Pregnanetriol in amniotic fluid		Reference
	Correct diagnosis		Incorrect diagnosis	Correct diagnosis		
	Normal	Affected	(False negative)	Normal	Affected	
1					+	Jeffcoate *et al.*, 1965
2				+		
3					+	Fuchs, 1967
4					+*	Merkatz *et al.*, 1969
5					+*	
6		+				Cathro *et al.*, 1969
7	+					Nichols, 1969
8	+					
9	+					
10					+	
11			+		+	Nichols and Gibson, 1969
12	+			+		
13	+			+		
14			+		+†	Nichols, 1970
15			+			Nichols, 1971
16			+			
17			+		+	
18			+		+	Price *et al.*, 1971

* Only at term; normal in early and midpregnancy.
† Pregnanetriol decreased after injection of hydrocortisone into fetus.

Price, Cone and Keogh (1971) have reported false negative results on the basis of maternal oestriol excretion rates in cases of affected fetuses which were correctly diagnosed on the basis of pregnanetriol levels in amniotic fluid (Table 20). There are considerable variations in normal levels of oestriol in maternal urine (Turnbull, 1970) and this may explain the discrepancy between Cathro's findings and those of others. Decreased excretion of oestriol in maternal urine in the last trimester of pregnancy has been reported by Frandsen and Stakemann (1964) in pregnancies with anencephalic fetuses associated with adrenal atrophy, but when the fetal adrenals are not atrophic, maternal oestriol excretion is normal (Frandsen and Stakemann, 1964).

Even if, as seems likely, the adrenogenital syndrome cannot be diagnosed *in utero* in early pregnancy, and later in pregnancy only by measuring steroid levels in amniotic fluid, this may still be of value. Knowing that a fetus is affected means that treatment can by instituted at birth (or perhaps even *in utero*) and so serious (and sometimes fatal) electrolyte disturbances might be avoided. It is not beyond reason, however, that the disorder may eventually be diagnosable in early pregancy possibly from the study of steroid metabolism in cultured amniotic fluid cells.

Other inborn errors of metabolism

There are two approaches to the problem of attempting to diagnose other inborn errors of metabolism from the analysis of amniotic fluid. Either a change in the concentration of a relevant metabolite in the amniotic fluid might be looked for, or a reduction in the level of the enzyme responsible for the disorder may be demonstrable.

The finding of a normal concentration of a relevant metabolite in amniotic fluid in a fetus at risk which subsequently proves to be unaffected (e.g. cystathionine in cystathioninuria—Frimpter, Greenberg, Holgartner and Fuchs, 1967; electrolytes in familial chloride diarrhoea—Emery, 1970; phosphoethanolamine in congenital hypophosphatasia—Crawfurd, 1971; phenylalanine in phenylketonuria—Thomas, Parmley, Stevenson and Howell, 1971) though of some value does not prove, in such conditions, the usefulness of amniotic fluid analysis in antenatal diagnosis. Much more significant is the finding of abnormal levels (as compared with an adequate number of normal controls of comparable gestation) in fluid from a pregnancy where the fetus is subsequently clearly shown to have been affected either on the basis of studies on the aborted material or on the infant delivered at term.

The first successful demonstration of an alteration in the com-

position of amniotic fluid in a genetic disorder was reported by Brzezinski *et al.* (1964). By electrophoresis these investigators diagnosed bisalbuminaemia *in utero* at 24 weeks gestation by demonstrating a double albumin peak in the amniotic fluid which was not present in the maternal serum. Bisalbuminaemia is inherited as an autosomal dominant trait, but unfortunately in this case the father was not available for testing.

Of greater interest is the recent report of Morrow, Schwarz, Hallock and Barness (1970) concerning the antenatal diagnosis of methylmalonicacidaemia, a rare recessive trait characterized by excessive urinary excretion of methylmalonic acid, ketoacidosis, vomiting, failure to thrive, hepatomegaly and affected individuals often die in early life. A variant of this disease is responsive to vitamin B_{12} and since these latter patients develop normally if treated, an early and correct diagnosis is particularly important. Morrow and his colleagues (Morrow *et al.*, 1970) reported the case of a pregnant woman heterozygous for this disorder. The fetus was suspected of being affected prenatally because of increased amounts of methylmalonic acid in amniotic fluid from the 17th week (though at this stage only trace amounts were present) and in maternal urine from the 25th week. Normally this metabolite is not found in amniotic fluid and only in very small amounts (less than 5 mg per 24 h) in urine. After birth the diagnosis of methylmalonicacidaemia was confirmed in the child. It is possible that, with refinements in biochemical techniques, abnormal levels of methylmalonic acid may be detected in amniotic fluid in affected pregnancies earlier than 17 weeks. However, if the changes in maternal urine are confirmed in other cases this would obviously be a preferable approach to the problem.

Matalon, Dorfman, Nadler and Jacobson (1970) reported an abnormally high concentration of acid mucopolysaccharide in amniotic fluid obtained at 14 weeks gestation from a pregnancy which subsequently resulted in an infant with Hurler's syndrome (8.7 mg per cent compared with 40 normal values from pregnancies of comparable gestation of 0.6 to 3.5 mg per cent). However, high levels of acid mucopolysaccharide in amniotic fluid can occur in cases of rhesus incompatibility (Danes, Queenan, Gadow and Cederqvist, 1970). What is more significant in Matalon's report is that approximately 63 per cent of the acid mucopolysaccharide in the affected pregnancy was heparitin sulphate—a compound apparently not detectable in normal amniotic fluid. As Danes and her colleagues point out, on this evidence qualitative rather than quantitative determination of mucopolysaccharides in amniotic fluid may be more informative. This conclusion may be premature, for Brock, Gordon, Seligman and Lobo (1971) have

reported finding normal levels of acid mucopolysaccharide, and with no qualitative abnormalities, in amniotic fluid obtained at 18 weeks gestation in a pregnancy which subsequently resulted in the birth of a child with undoubted Hurler's syndrome. Clearly more studies are required before the position of amniotic fluid analysis in the antenatal diagnosis of Hurler's syndrome can be assessed.

Apart from the adrenogenital syndrome, bisalbuminaemia, methylmalonicacidaemia and Hurler's syndrome, there have been no other reports to date of a positive antenatal diagnosis in a metabolic disorder being established on the basis of changes in the chemical composition of amniotic fluid. Certain aminoacidopathies might well be considered prime candidates for such an approach, and for this reason we have reported values for the amino acid composition of normal amniotic fluid throughout pregnancy (Emery *et al.*, 1970). The results of these studies have shown that there is a general tendency for the amounts of most of the amino acids to decrease as pregnancy progresses (Fig. 15) and at term, with the exceptions of taurine and ethanolamine, the concentrations in amniotic fluid are less than in plasma. There appear to be three patterns of change in the amino acid composition of amniotic fluid at various stages of pregnancy. In some the concentrations remain more or less the same throughout pregnancy (e.g. cysteic acid, phosphoethanolamine, ethanolamine and proline), in others the concentrations decrease throughout pregnancy (e.g. serine, glycine, phenylalanine, lysine and arginine), and finally the remaining amino acids appear to have a lower concentration in the first trimester than in the period 13 to 16 weeks, but thereafter their concentrations decrease as pregnancy progresses. In most cases concentrations rise in the last month of pregnancy (Fig. 15). For these reasons, values from normal individuals of the same gestational age must be taken into account if a genetic disorder associated with aminoaciduria is to be diagnosed *in utero*.

The offspring of mothers with untreated phenylketonuria are very often mentally retarded and microcephaly, cardiac defects, epilepsy, strabismus and various skeletal and other anomalies may also be present. Grossly elevated amniotic fluid levels of phenylaline have been reported in cases of uncontrolled maternal phenylketonuria (Thomas *et al.*, 1971; Emery, Farquhar and Timson, 1972) but only a slightly raised level in a treated mother who was subsequently delivered of a normal healthy child (Emery *et al.*, 1972). It is possible that amniotic fluid analysis might prove a useful guide to fetal prognosis in mothers with phenylketonuria and might also throw some light on the pathogenesis of the mental retardation and congenital anomalies in children of affected mothers.

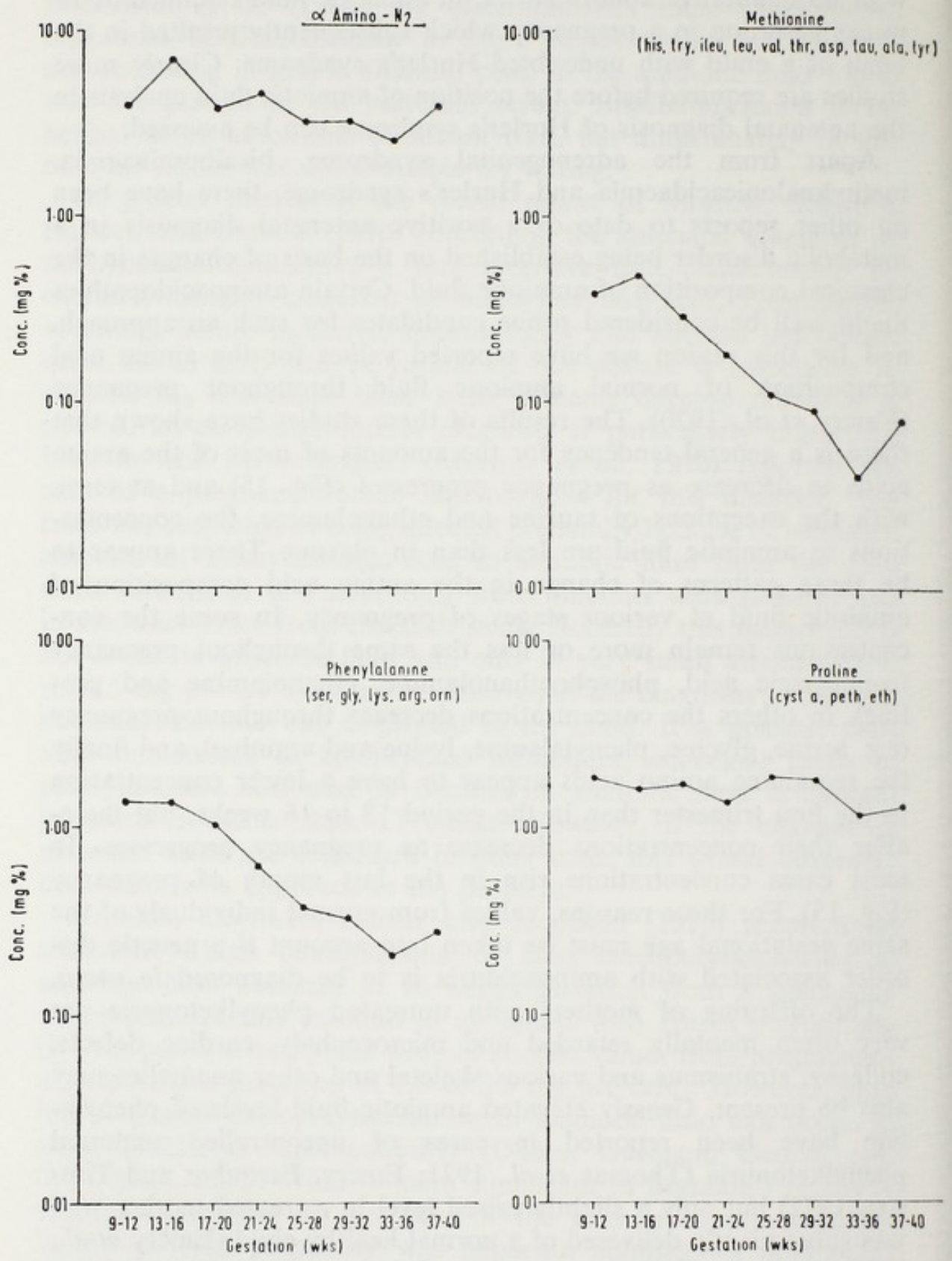

Figure 15. Patterns of amino acid concentrations in normal amniotic fluid at various stages of gestation.

It seems unlikely that the analysis of amniotic fluid will prove valuable in the antenatal diagnosis of conditions where the metabolic disorder is usually only manifest some time after birth and is affected by diet. It is therefore not surprising that no apparent abnormality in the amino acid composition of amniotic fluid at term was observed in a case of maple syrup urine disease (O'Neill, Morrow, Hammel, Auerbach and Barness, 1971). On the other hand, changes in the amino acid composition of amniotic fluid may prove diagnostic in such conditions as cystinuria, glycinuria and Hartnup's disease. Other conditions which for the same reasons might by diagnosed by changes in the composition of amniotic fluid include Lowe's syndrome since proteinuria (a β-globulin) is present at birth (Abbassi, Lowe and Calcagno, 1968) hypophosphatasia since hypercalcinuria is present at birth (Teree and Klein, 1968), the cerebro-hepato-renal syndrome since albuminuria is present at birth (Passarge and McAdams, 1967) and the neonatal variant of argininosuccinic aciduria which is associated with an increased urinary excretion of citrulline (Carton, de Schrijver, Kint, van Durme and Hooft, 1969).

With regard to the antenatal diagnosis of inborn errors of metabolism from enzyme studies on amniotic fluid, there have so far been two such reports: Pompe's disease and Tay-Sachs disease. Pompe's disease (type II glycogenosis) is due to a deficiency of the enzyme α-1,4-glucosidase. Nadler and Messina (1969) reported a case where there was reduced α-1,4-glucosidase activity in amniotic fluid and in cultured and uncultured amniotic fluid cells. The fetus was aborted at 19 weeks and the diagnosis confirmed by enzyme studies on the aborted material. However, subsequent experience by this same group (Nadler, Bigley and Hug, 1970) showed that normal levels of enzyme activity may be present in amniotic fluid (but not in cultured amniotic fluid cells) when the fetus is affected. The authors concluded that enzyme analysis on amniotic fluid is an unreliable method for establishing the *in utero* diagnosis of Pompe's disease. The reason for the discrepancy in these results is not clear. One possibility is that a false positive result might be a consequence of contamination of amniotic fluid with maternal blood with enzyme activity. In normal individuals α-1,4-glucosidase activity is found in leucocytes, but little or none in erythrocytes and plasma (Cox, Douglas, Hutzler, Lynfield and Dancis, 1970). A possible answer to the problem of the antenatal diagnosis of Pompe's disease might therefore be to remove all cellular elements by centrifugation before analysing the fluid.

Tay-Sachs disease is due to a deficiency of hexosaminidase A, and Schneck, Friedland, Valenti, Adachi, Amsterdam and Volk (1970) diagnosed the disease *in utero* at 18 to 20 weeks gestation by demonstrating only trace amounts of enzyme activity in amniotic fluid and uncultured amniotic fluid cells. More recent experience by this group has confirmed this finding (Friedland, Perle, Saifer, Schneck and Volk, 1971). In a recent and extensive study (O'Brien, Okada, Fillerup, Veath, Adornato, Brenner and Leroy, 1971), 15 pregnant women at risk of having a child with Tay-Sachs disease were monitored by amniocentesis with hexosaminidase A assays on amniotic fluid, cultured and uncultured amniotic fluid cells. The results of this study clearly demonstrated that the greatest difference in enzyme levels between normal and affected fetuses was with cultured amniotic fluid cells. In the case of uncultured cells and the fluid itself, the difference between the two groups was smaller but still clear cut with no apparent overlap. The results from the assay of uncultured cells and fluid were obtained within two days after amniocentesis but results from cultured cells were obtained only after 10 to 28 days. The advantage of using fluid and uncultured cells is therefore obvious provided there is no contamination with maternal blood.

There is presumably more scope for antenatal diagnosis based on enzyme assays on amniotic fluid. Normal values for a number of enzymes in amniotic fluid have been published (Geyer and Schneider, 1970) and their origin discussed (Geyer, 1970).

Sutcliffe and Brock (1972) have studied the specific activities of five enzymes; acid phosphatase, heat-stable and heat-labile alkaline phosphatases, hexosaminidase and α-1,4-glucosidase in amniotic fluids obtained at various stages of gestation from 10 weeks to term. They found two major peaks of activity, one at about 15 weeks, and the other at term. Only α-1,4-glcosidase and heat-labile alkaline phosphatase have a strong early peak, while four enzymes (the exception being α-1,4-glucosidase) have strong late peaks. Heat-labile alkaline phosphatase is unusual in having both early and late peaks, probably reflecting its heterogeneity. The activity patterns are similar, whether results are expressed per millilitre amniotic fluid or per gram soluble protein, an important point when the uneven protein curve is considered (see p. 117). These are thus at least three enzyme profiles in amniotic fluid and this represents experience with only five enzymes, three of which are lysosomal. It seems obvious that more enzymes will have to be studied individually before conclusions are reached about patterns of activity and tissue origins.

Secretor status and genetic linkage.

The scope of antenatal diagnosis could be widened by the application of genetic linkage, a point first made by Edwards as long ago as 1956 (Edwards, 1956). This prophecy may now become a reality, since it has been demonstrated that the loci for myotonic dystrophy and ABH secretion are within measurable distance of each other (Renwick, Bundey, Ferguson-Smith and Izatt, 1971) and it has been shown that secretor status of the fetus can be determined from the amniotic fluid in early pregnancy (Harper, Bias, Hutchinson and McKusick, 1971). Unfortunately, this information can be useful only in certain family situations (see p. 149). Nevertheless, in such families the use of linkage information could be valuable in adding more precision to the estimate of the likelihood of a fetus having inherited the disorder from an affected parent. The fact that the glucose-6-phosphate dehydrogenase (G6PD) locus and the haemophilia A locus are closely linked (Boyer and Graham, 1965) may help not only in the detection of female carriers of haemophilia A in certain populations where G6PD variants are common (McCurdy, 1971), but could be extended to the antenatal diagnosis of this disorder since fetal G6PD phenotype can be determined in amniotic fluid cells. As more becomes known of the linkage relationships on the autosomes and X chromosome of man, it seems likely that this information will become increasingly useful in antenatal diagnosis. This approach would prove particularly valuable in disorders where the basic biochemical defect is not known, or is not expressed in amniotic fluid or its contained cells, such as Huntington's chorea and polyposis coli.

Congenital abnormalities

Congenital abnormalities not associated with any detectable chromosomal or biochemical defect present a problem in antenatal diagnosis, the answer to which may lie in the use of a fetoscope (see p. 49). However, it is possible that at least in certain congenital abormalities, biochemical changes in amniotic fluid may be demonstrable, and perhaps even diagnostic. This seems perhaps more likely in c.n.s. malformations than in other congenital abnormalities.

An increase in the optical density (Fig. 16) of amniotic fluid at 450 nm (ΔOD_{450}) was observed in six out of seven cases of anencephaly by Cassady and Cailliteau (1967) and in all four cases studied by Lee and Wei (1970). We have observed the same phenomenon in 16 out of 17 cases of anencephaly with or without spina bifida (Fig. 17). Some of the values fell within the

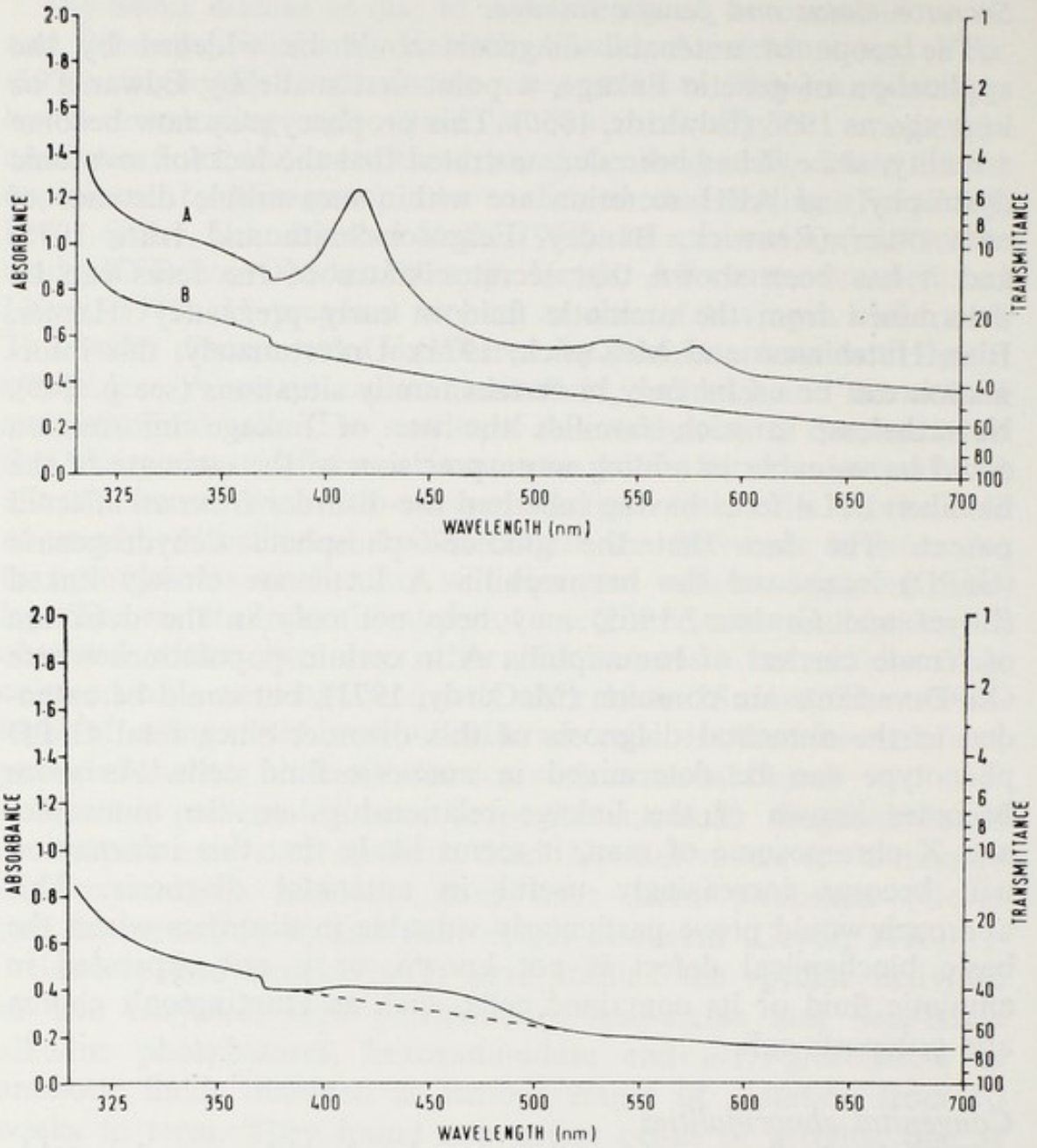

Figure 16. Spectrophotometric analysis of amniotic fluid. *Above* B, normal amniotic fluid at term; A, same specimen to which has been added haemolysate (1 in 1000), showing the absorption spectrum of oxyhaemoglobin. *Below* Anencephaly at 39 weeks gestation, showing an increase in optical density at 450 nm as determined by the method of Liley (1961).

range usually observed in fetuses with severe rhesus sensitization. In none of these cases was there evidence of rhesus incompatibility, and no apparent contamination with meconium or erythrocytes. The presence of meconium may invalidate the results of spectrophotometric analysis (Halitsky and Krumholz, 1970) though small amounts of oxyhaemoglobin appear to have little effect on the ΔOD_{450} (Halitsky and Krumholz, 1970; and Fig. 16).

The reason for the increase in ΔOD_{450} in pregnancies not

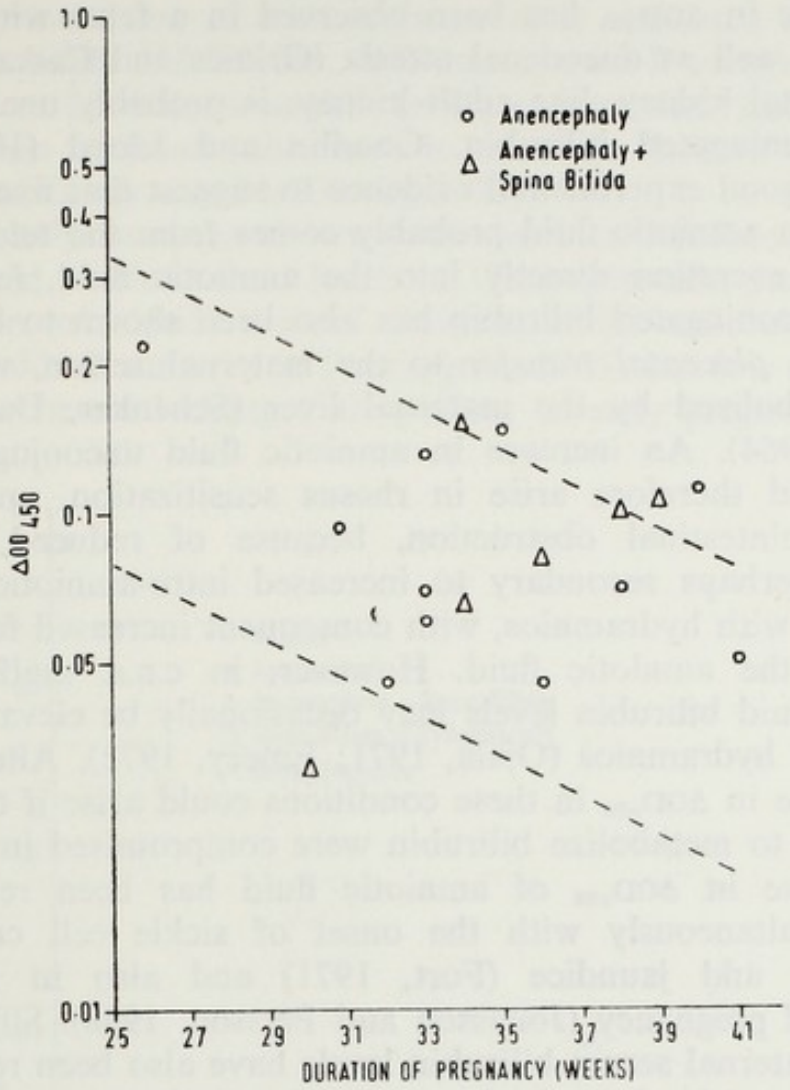

Figure 17. Optical density at 450 nm ($\triangle OD_{450}$) of fresh specimens of amniotic fluid in cases of c.n.s. malformations. The broken lines indicate Liley's zones; normal values usually lie in the lowest zone. (Liley, 1961.)

associated with rhesus sensitization is not clear. It is known that the ΔOD_{450} represents unconjugated (indirect) bilirubin, at least in rhesus sensitization (Brazie, Ibbott, Bowes, 1966; Mandelbaum and Robinson, 1966), which is bound to albumin (Cherry, Rosenfield, Kochwa, 1970; Fort, 1971). There is the possibility that the increase in ΔOD_{450} observed in anencephaly might be due to regurgitation of bilirubin, as a consequence of defective swallowing in this disorder, and this could also account for the phenomenon in cases of congenital atresia of the duodenum (Liley, 1963; Grimes and Cassady, 1970) and ileum (Grimes and Cassady, 1970). But this could not account for the increase in ΔOD_{450} observed in a case of multiple congenital abnormalities associated with pyloric stenosis, reported by Willoughby, Henry and Arronet (1969). Renal excretion of bilirubin is unlikely to be the explanation, for

an increase in ΔOD_{450} has been observed in a fetus with ureteral atresia, as well as dueodenal atresia (Grimes and Cassady, 1970), and the fetal kidney, like adult kidney, is probably unable to excrete unconjugated bilirubin. Goodlin and Lloyd (1968) have presented good experimental evidence to suggest that unconjugated bilirubin in amniotic fluid probably comes from the fetal trachea.

Besides excretion directly into the amniotic fluid, fetal excretion of unconjugated bilirubin has also been shown to be accomplished by *placental transfer* to the maternal serum, when it is then metabolized by the maternal liver (Schenker, Dawber and Schmid, 1964). An increase in amniotic fluid unconjugated bilirubin could therefore arise in rhesus sensitization, anencephaly and gastrointestinal obstruction, because of reduced placental transfer, perhaps secondary to increased intra-amniotic pressure associated with hydramnios, with consequent increased fetal excretion into the amniotic fluid. However, in c.n.s. malformations amniotic fluid bilirubin levels may occasionally be elevated in the absence of hydramnios (Ojala, 1971; Emery, 1972). Alternatively, the increase in ΔOD_{450} in these conditions could arise if the maternal ability to metabolize bilirubin were compromised in any way. An increase in ΔOD_{450} of amniotic fluid has been reported to occur simultaneously with the onset of sickle cell crises with haemolysis and jaundice (Fort, 1971) and also in idiopathic jaundice of pregnancy (Jonasson and Persson. 1968). Slight elevations in maternal serum bilirubin levels have also been reported in a case where the fetus had congenital pyloric stenosis, and the ΔOD_{450} of amniotic fluid was increased (Willoughy *et al.*, 1969). Finally, in pregnant rats experimentally produced obstructive jaundice has been shown to produce a significant elevation in the level of unconjugated bilirubin in amniotic fluid (Willoughby, Willis, Lee, Fuchigami, Kazeef and Desjardins, 1971).

Whatever the origin of the increase in ΔOD_{450} in certain congenital abnormalities, it is not known if this is so in early pregnancy when it might be of some value from the point of view of antenatal diagnosis. It can be concluded, however, that in the last trimester of pregnancy, an antenatal diagnosis of upper intestinal obstruction may be achieved if there is an increase in ΔOD_{450}, not associated with rhesus sensitization, and radiography or sonography has excluded anencephaly and particularly if visualization of the fetal gut is not possible with amniography.

With regard to the study of other metabolites in amniotic fluid in c.n.s. malformations, it was thought that the concentration of various catecholamine metabolites, (homovanillic acid and vanilmandelic acid) might be increased since it had been reported that

the urinary excretion of these substances is increased in children with spina bifida (McKibbin, O'Gorman, and Duckworth, 1969). However, we have been unable to detect any abnormality in the concentration of these substances in amniotic fluid in c.n.s. malformations (Emery and McKibbin, 1971).

Anencephaly has been shown to be associated in late pregnancy with reduced amniotic fluid levels of 17-ketosteroids, pregnanetriol (Jeffcoate *et al.*, 1965) oestriol (Michie, 1966) and certain other corticosteroids (Lambert and Pennington, 1965), but it is not known if these biochemical changes are present in early pregnancy. They

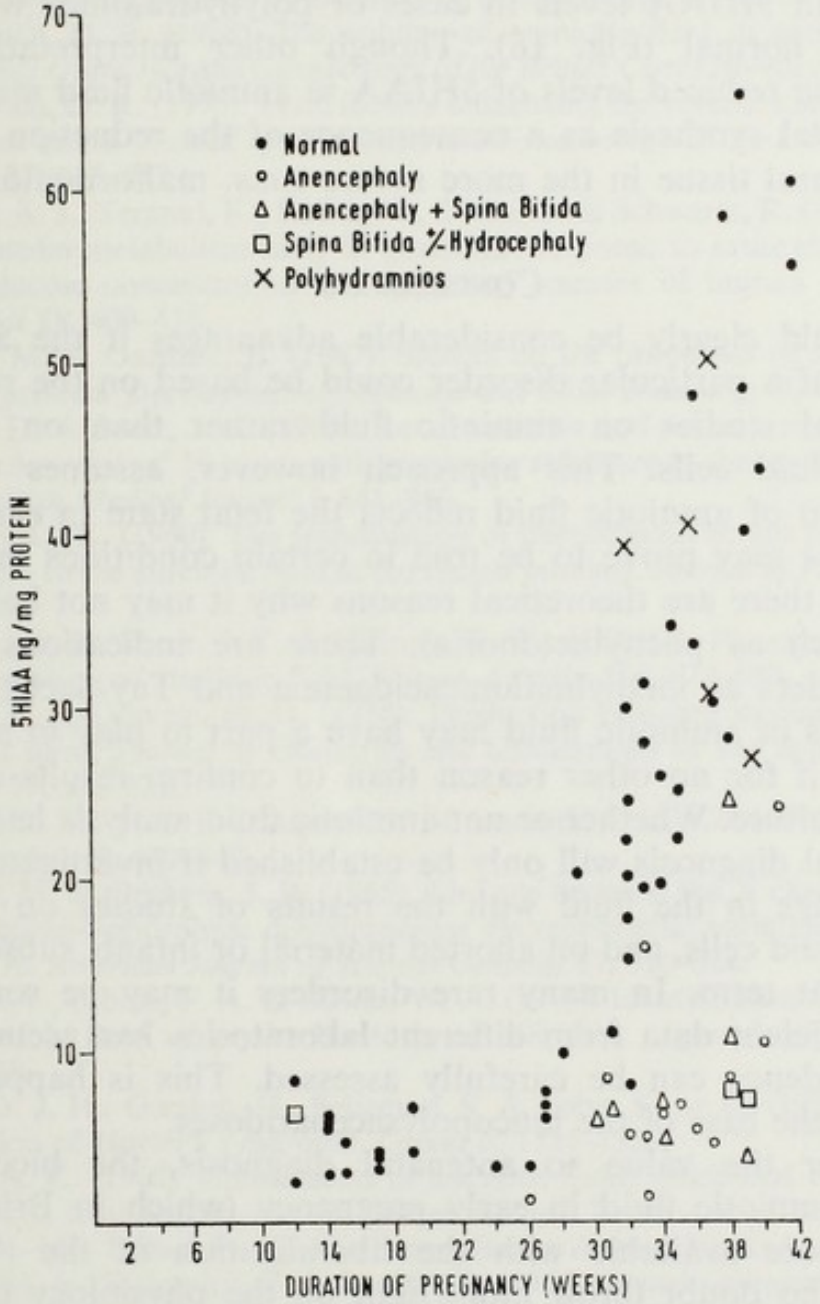

Figure 18. Levels of 5HIAA in amniotic fluid (ng/mg protein) at various stages of gestation in controls, c.n.s. malformations, and polyhydramnios where the fetus was normal.

are presumably a reflection of adrenal atrophy, which is an associated feature of anenecphaly. Nelson (1969) has reported a reduction in the proportion of lecithin in the phospholipid fraction of amniotic fluid at term in the respiratory distress syndrome, as well as in anencephaly, but again there is no information on amniotic fluid levels in early pregnancy in c.n.s. malformations.

Finally, we have observed, at least in the last trimester of pregnancy, a significant reduction in amniotic fluid levels of 5-hydroxyindoleacetic acid (5HIAA) in c.n.s. malformations (Emery, Eccleston, Scrimgeour and Johnstone, 1972). This reduction was not observed with a number of other substances present in amniotic fluid, nor in 5HIAA levels in cases of polyhydramnios where the fetus was normal (Fig. 18). Though other interpretations are possible, the reduced levels of 5HIAA in amniotic fluid may reflect reduced fetal synthesis as a consequence of the reduction in functioning neural tissue in the more severe c.n.s. malformations.*

Conclusions

There would clearly be considerable advantages if the antenatal diagnosis of a particular disorder could be based on the results of biochemical studies on amniotic fluid rather than on cultured amniotic fluid cells. This approach however, assumes that the composition of amniotic fluid reflects the fetal state in early pregnancy. This may prove to be true in certain conditions but as we have seen, there are theoretical reasons why it may not be true for others (such as phenylketonoria). There are indications that in such disorders as methylmalonicacidaemia and Tay-Sachs disease, the analysis of amniotic fluid may have a part to play in antenatal diagnosis, if for no other reason than to confirm results obtained with cell culture. Whether or not amniotic fluid analysis has a place in antenatal diagnosis will only be established if investigators compare findings in the fluid with the results of studies on cultured amniotic fluid cells, and on aborted material or infants subsequently delivered at term. In many rare disorders it may be some time before sufficient data from different laboratories has accumulated, so the evidence can be carefully assessed. This is happening at present in the case of the mucopolysaccharidoses.

Whatever the value to antenatal diagnosis, the biochemical study of amniotic fluid in early pregnancy (which in Britain has become more available with the liberalization of the Abortion Law) will no doubt throw more light on the physiology of amni-

* An increase in α-fetoprotein in amniotic fluid, at least in the last trimester of pregnancy, has been reported in anencephaly (Brock, D. J. H. & Sutcliffe, R. G. (1972) *Lancet* ii, 197–199).

otic fluid, and on fetomaternal relationships. It may even lead to a better understanding of pathogenesis, as might prove to be the case in certain congenital abnormalities.

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9

Implications of Antenatal Diagnosis

CHARLES SMITH

The preceding chapters have dealt with the various methods and techniques of antenatal diagnosis and considered where it might be indicated in medical practice. In this chapter, some of the statistical and population aspects of its application will be considered, concentrating on the results and their implications rather than on their derivation. The discussion will refer largely to diseases which are wholly or partly genetic in origin.

Firstly, the ways in which antenatal diagnosis might be applied in the population will be examined, and its scope for preventing genetic disease will be studied. Methods for evaluating risks to individuals, using information on their family history, will also be examined. Finally, the possible short-term and long-term effects of antenatal diagnosis on future generations will be considered.

SCOPE FOR ANTENATAL DIAGNOSIS

Extent and burden of genetic disease

As infectious diseases decline in frequency and environmental conditions improve, genetic disease becomes relatively more important as a cause of mortality and morbidity. For example, Carter (1963) has shown how congenital abnormalities have increased as a cause of infant mortality from about 5 per cent to 20 per cent in the last 70 years, and Roberts, Chavez and Court (1970) found that among deaths of children in hospital, over 40 per cent were genetic or partly genetic in origin. The total frequency of genetic disease in liveborn is usually taken to be about 3 to 5 per cent (Stevenson, 1961). In addition there are many common familial diseases, such as schizophrenia or diabetes, which manifest later in life.

In discussing genetic disease, it is important to keep in mind its diversity, not only in the modes of inheritance, but in its severity, the age at onset, its duration and the burden to the individual and to society and to relate all of these to preventive schemes. This

diversity is illustrated in Figure 19 showing the stage, extent and duration of the burden of several genetic diseases in terms of diminished abilities and early death of affected individuals, and of the burden to society in having to treat and support affected individuals. A severe disease with death at birth, like anencephaly, incurs little burden to society or to the family. On the other hand, if affected individuals survive and require continued care and treatment, as in spina bifida, the condition inflicts a severe burden on surviving individuals and is expensive to society. If a defect can be corrected by surgery, as for cleft lip (with or without cleft palate), there may be little cost to society but some cosmetic burden to the

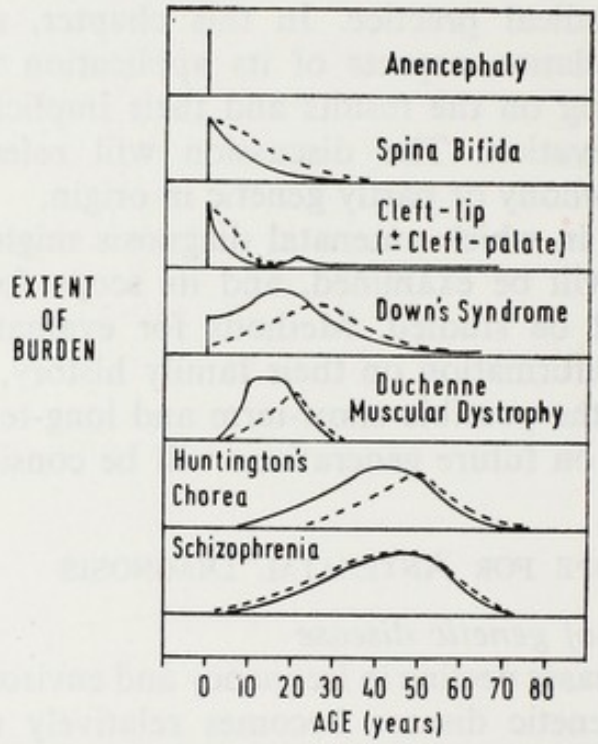

Figure 19. Diagram to illustrate the burden of various genetic diseases to affected individuals and their families (solid lines) and to society (broken lines) in terms of onset, severity and duration of the disease. (After Murphy, 1969, with permission.)

individual throughout life. With severe incapacitating diseases, such as Duchenne muscular dystrophy and Down's syndrome, the burden to the individual and to the family is high, but the cost to society is low while the child remains at home, but is high if he is institutionalized. Similarly, the severe late onset diseases, such as Huntington's chorea and schizophrenia, cause considerable burdens to affected individuals, to their families and to society alike.

Cost-benefit to society

The cost of a case of a disease to society will depend on many factors such as the extent of the disability incurred and the consequent loss in productivity, the cost of treatment and care required, and on the duration of the disease, as illustrated in Figure 19. Since the costs will accumulate throughout the duration of the disease, they should be discounted to the equivalent current value by an appropriate discount rate. The cost figure will then represent the average benefit to society of preventing one case of the disease. This benefit will tend to stay constant per case prevented.

The cost of preventing a case of a disease by antenatal diagnosis will also depend on several factors such as the costs of screening and testing, the frequency of the disease and the proportion of cases prevented. It may be easy to prevent a few cases, for example in pregnancies at high risk, but the law of diminishing returns may set in and the cost per case prevented is likely to increase as the proportion of cases prevented increases. Any risks to the mother or the fetus in antenatal diagnosis must also be considered and any such iatrogenic costs included.

As an example, cost-benefit analysis might be used to seek the age above which mothers might be screened for Down's syndrome in their offspring. In Figure 20 the risk of Down's syndrome by maternal age is shown (Penrose and Smith, 1966). The cost of detection per case will be inversely proportional to the risk of the

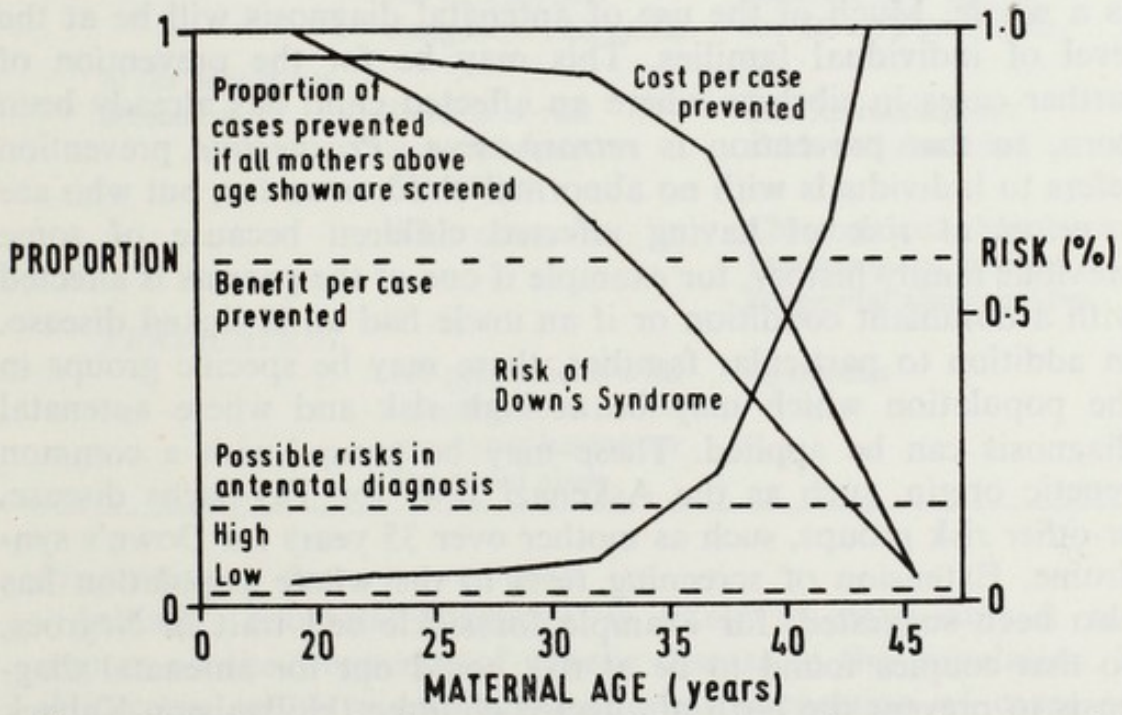

Figure 20. Diagram showing the risks of Down's syndrome by maternal age, and illustrating the risks, benefits and costs per case detected by antenatal diagnosis.

disease. The benefit to society of preventing a case will tend to be constant, as discussed above. There will be some risks in antenatal diagnosis, and in terminations late in pregnancy (see Chapter 3). If the risk in antenatal diagnosis is of abortion, there would be little cost to society, but if the risk were, say, of mental retardation with normal life expectancy, then the iatrogenic cost to society would be high. In practice, estimation of the various cost, benefit and risk functions may be very difficult. For example, Milunsky *et al.* (1971) and Friedman (1971) differ by a factor of four in their estimates of the lifetime cost of a case of Down's syndrome. Since there is usually some uncertainty, the benefits of a procedure should be very much higher than the costs, before it is widely adopted. In general, the object of cost-benefit analysis is not to derive a fixed cost-benefit equation for decision making, but to review and consider the various factors (economic, social and personal) associated with alternatives to help arrive at an informed and considered policy.

Levels of application

Currently antenatal diagnosis is largely restricted to high-risk pregnancies. However, the application at other levels in the population have been discussed (Fraser and Motulsky, 1968) and so the broad possibilities of application, summarized in Table 21, are considered here. Distinction is made between application to particular families, to groups at special risk and to the population as a whole. Much of the use of antenatal diagnosis will be at the level of individual families. This may be for the prevention of further cases in sibships where an affected child has already been born, so that prevention is *retrospective. Prospective* prevention refers to individuals with no abnormal children so far, but who are *a priori* at risk of having affected children because of some previous family history, for example if one of the parents is affected with a dominant condition or if an uncle had an X-linked disease. In addition to particular families, there may be specific groups in the population which may be at high risk and where antenatal diagnosis can be applied. These may be groups with a common genetic origin, such as the Askenazi Jews for Tay-Sachs disease, or other risk groups, such as mother over 35 years for Down's syndrome. Extension of screening tests to the whole population has also been suggested, for example for sickle cell trait in Negroes, so that couples found to be at risk could opt for antenatal diagnosis to prevent the birth of affected children (Hollenberg, Kaback and Kazazian, 1971). It is also possible to contemplate screeening all pregnancies, for example for chromosomal aberrations by karyo-

typing or for certain external congenital abnormalities by fetoscopy. However, the costs and the risk would then have to be very low indeed. Finally, the virtual elimination of certain simple Mendelian diseases by antenatal diagnosis and abortion of all individuals with abnormal alleles has been considered (Fraser and Motulsky, 1968). Though possible, this application is very unlikely, since most individuals are heterozygous for several deleterious recessive genes and because there are appreciable risks to mothers from termination, especially if late in pregnancy (see Chapter 3).

Table 21. *Possible levels for application of antenatal diagnosis*

Application level	Antenatal diagnosis applied to	Examples
PREVENTION		
In families		
Retrospective	Parents with an affected child	Xeroderma pigmentosum. Duchenne muscular dystrophy
Prospective	Parents with a previous family history	Parent with myotonic dystrophy. Uncle with haemophilia
In groups		
Screening	Parents at risk screened from a special genetic group	Carrier matings for Tay-Sachs in Ashkenazi Jews
	All parents in a designated risk group	Mothers over age 35 for Down's syndrome
In the population		
Screening	Parents at risk screened from the population	Carrier matings for sickle-cell anaemia
	All pregnancies	Chromosomal aberrations. Fetoscopy for external congenital abnormalities
ERADICATION		
	All pregnancies with selective abortion of genotypes having an abnormal allele	Cystinosis

Proportion of cases preventable

Initially, antenatal diagnosis is likely to have only a small impact on the frequency of genetic disease in the population. But if it came to be widely applied, what proportion of cases of a genetic disease might be prevented? This question has been studied to assess the value of genetic counselling in preventing genetic

Table 22. *The maximum proportion of cases preventable through antenatal diagnosis for different preventive schemes*

Percentage of cases preventable	Prevention in families at risk		Prevention by screening groups at risk	Prevention by parental screening	Prevention by antenatal diagnosis for all pregnancies
	Retrospective	Prospective			
100			Simply inherited disorders in genetic 'isolates'	Recessive	All modes of inheritance
80		Mild dominant		Mild dominant Mild X-linked	
50	Dominant	Mild X-linked	Large groups at low risk		
20	Recessive X-linked	Severe dominant Severe X-linked		Severe X-linked Severe dominant	
10			Small groups at high risk		
0	Multifactorial Chromosomal	Others		Others	

disease (Fraser and Motulsky, 1968; Smith, 1970), and similar results apply here. These are summarized in Table 22 showing the effectiveness of different methods. The results represent maximum levels of prevention, for they assume full application of the tests and methods available.

After one affected child has been born in a family, other affected children may be prevented retrospectively. Fraser (1972) has derived expressions to estimate the maximum percentage of cases that might be prevented in this way. Depending on the distribution of family size, this percentage is about 30 to 40 per cent for dominant conditions and about 20 to 30 per cent for X-linked and recessive conditions. The percentage of cases prevented is likely to be much less in practice; if diagnosis is not made early, if counselling is not effective, or if a proportion of cases are due to new mutations. Because the recurrence risks are not very high for most chromosomal abnormalities (apart from translocation carriers nor for multifactorial disorders (conditions due to many genetic and environmental factors), the proportion of cases preventable will be quite small. Prospective prevention will be effective for mild dominant and X-linked conditions where female relatives may be carriers of the abnormal allele (Smith, 1970).

The proportion of cases prevented by screeening specific groups will depend on the nature of the group and the risks. If the group at risk has a common genetic origin, such as the Ashkenazi Jews for Tay-Sachs disease, a high proportion of cases may be prevented. But for a group at risk for some general condition (such as Down's syndrome in mothers over a certain age), the proportion of cases prevented will be small, because the condition will not be confined to the high-risk group. Parental screening and antenatal diagnosis could theoretically prevent all cases of a recessive condition, for example sickle-cell anaemia, and all cases for X-linked and dominant conditions, except for those due to new mutations. However, parental screening will not be effective in preventing multifactorial or chromosomal disorders. Finally, 'fetal screening' (antenatal diagnosis applied to all pregnancies) could theoretically prevent all cases of any disease, if antenatal tests were available. However, such extreme applications seem very unlikely in practice.

Ascertainment for Antenatal Diagnosis

For antenatal diagnosis to be applied effectively to prevent genetic disease, there must be some means of finding matings at risk of having affected children, and various methods to do this are now

considered. As before, a distinction is made between working with particular families and screening the whole population or groups at risk. Families with a specific disorder will be especially motivated to prevent further cases occurring.

Families at risk

When a disease occurs in a family, the family can be ascertained and studied. Risks to all members of the family can be estimated and appropriate treatment, counselling and preventive measures instituted. Those at risk of having affected children may then opt for antenatal diagnosis if this is possible for the disease concerned.

In practice it will be difficult to ensure that all those at risk are contacted, counselled and informed about the possibility of antenatal diagnosis. Currently there is no facility to provide such a service. Rather than rely on *ad hoc* recording and recall, a system is required to collate information on families at risk with the genetic and medical services available. Such a system is being developed in this Department (Emery and Smith, 1970) and is referred to by the acronym RAPID—Register for the Ascertainment and Prevention of Inherited Disease. A similar system (Fomers)—a Family Oriented Medical Records System—but stressing treatment rather than prevention, is being developed by McKusick (1968) and co-workers. Antenatal diagnosis provides new impetus to these systems because it may offer the prospect of normal children to parents at risk of having affected children, and so provides a strong motive for their co-operation in prevention of genetic disease.

The object of the RAPID system is to prevent genetic disease by ascertaining individuals and families who are at risk from genetic disease, by evaluating the risks involved, by ensuring adequate provision for genetic counselling and medical care and by continuing the follow-up and supervision of the families over time. Central to the system, as shown in Figure 21, is the genetic register, which records, processes and utilizes the information on the family. Details on the disease, status, risks, counselling, follow-up and other factors are recorded. Families at risk are ascertained through genetic counselling clinics and through hospital, general practitioner and public health records by record linkage (Newcombe, 1967). For antenatal diagnosis, record linkage to the genetic register of women attending maternity clinics could be used to detect women at risk. Also, all pregnant women might be questioned about any family history of genetic disease and any at risk could be considered for antenatal diagnosis.

A genetic register system for prevention of genetic disease will be most useful for simply inherited Mendelian conditions where the risks are high. Initial results with RAPID (Emery and Smith, 1970) show that only a small proportion (under 20 per cent) of those at risk in ascertained families had been counselled about the risk of affected children. Many affected children were born to parents who were *a priori* at risk but had never been counselled. In some 445 families with serious genetic disease, attending a genetic clinic, the average number of relatives per family at risk (risk greater than 10 per cent, age less then 40) of having affected children was 3.2 for dominant, 2.8 for X-linked, 0.5 for recessive, but less than 0.1 for chromosomal and 0.2 for multifactorial conditions (Smith, Holloway and Emery, 1971). These results show that there is ample scope for antenatal diagnosis in families with Mendelian conditions, but less for other modes of inheritance.

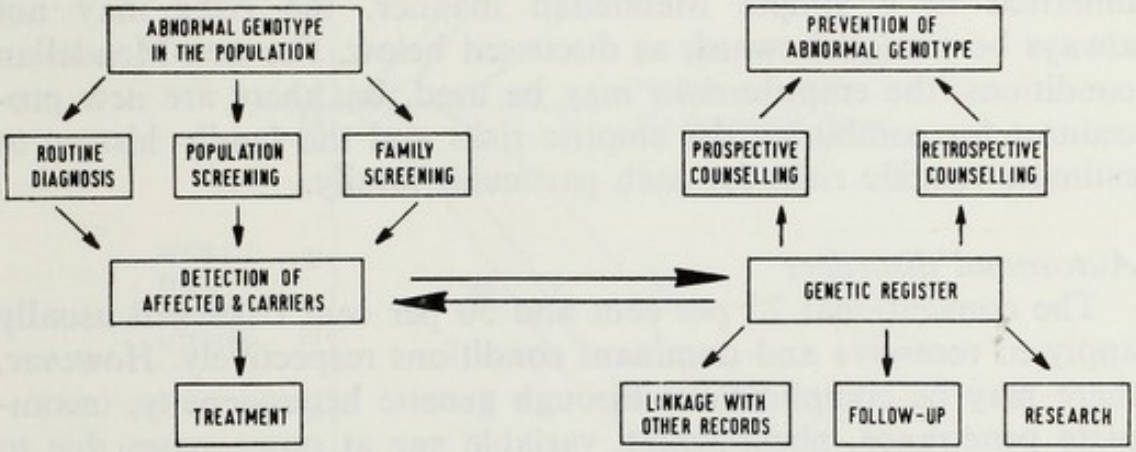

Figure 21. A genetic register for the ascertainment and prevention of genetic disease.

Parental screening

Full parental screening could be an effective means of preventing some genetic diseases, since all matings at risk of having affected children might be detected. However, in practice the value of population screening is usually limited (McKeown, 1968), especially by the low return on the effort expended. Since individual genetic diseases are usually at low frequency in the population, the returns from screening will be correspondingly lower for genetic diseases. The number of parental screening tests required per case prevented can readily be estimated knowing the mode of inheritance and the frequency of the disease. For example, Motulsky, Fraser and Felsenstein (1971) show that for a recessive disease with a frequency of about 1/400, such as sickle cell anaemia in Negroes,

some 200 parental screening tests would be needed per case of disease prevented. If the disease is very rare as for many of the inborn errors of metabolism, some 10,000 or more tests may be required per case prevented, though this may be modified if different tests are made concurrently. Of course, the number of antenatal diagnoses needed, depends on the number of matings found to be at risk. In practice, some balance between the effort and costs of screening and the benefits from prevention of disease will determine the level of screening applied.

Risk Estimation

An important statistic in antenatal diagnosis is the risk of the fetus being affected. This may determine whether antenatal diagnosis is medically indicated and is acceptable to the parents. In many cases the risks will be easy to derive, depending only on the mode of inheritance of the condition. However, even for conditions inherited in a simple Mendelian manner, the risks may not always be straightforward, as discussed below. For non-Mendelian conditions, the empiric risks may be used, but there are new procedures for combining the empiric risks and the family history to estimate specific risks for each particular family.

Autosomal disorders

The conventional 25 per cent and 50 per cent risks will usually apply to recessive and dominant conditions respectively. However, there may be complications through genetic heterogeneity, incomplete penetrance, phenocopies, variable age at onset, cases due to mutation and matings of affected parents. A common problem in late-onset dominant conditions is that it is not known whether an individual with an affected parent is normal or will yet manifest the disease. The probability of still being a carrier can be derived from a graph of the distribution of age at onset of the disease in the population. For example, in Figure 22 the cumulative distributions of cases by age at onset are given for three dominant conditions, and below the corresponding risks by age of becoming affected, if a parent was affected. This procedure has been put on a more formal basis in the ENCU (Equivalent Normal Child Units) scoring system (Chase, Murphy and Bolling, 1971).

X-linked disorders

If a mother is known to be a carrier of an X-linked disorder then the risk of an affected son is, of course, 25 per cent. However, if the mother's status is in doubt, predicting her risk may be quite

complex. Details about the family history (including normal males) and about any carrier tests on female relatives can be combined to give a more accurate estimate of risk. Murphy and Mutalik (1969) have shown how Bayesian methods can be used to estimate the risks, and Murphy (1970) has formalized the methods in an ENSU (Equivalent Normal Son Unit) scoring system. Information on biochemical tests of carrier status of the mother and other female relatives can also be included, and formulae and graphs for estimating the risks are available for lethal X-linked conditions (Emery and Morton, 1968).

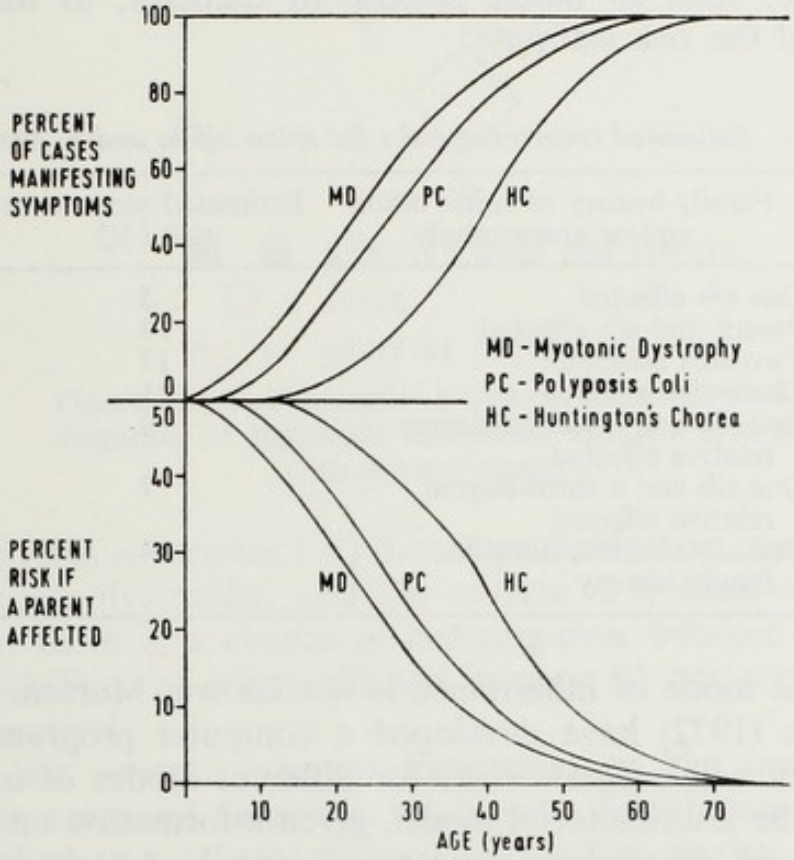

Figure 22. Percentage of cases manifesting clinical symptoms by age and the risks to children of an affected parent by age, for three autosomal dominant disorders.

Multifactorial disorders

For multifactorial disorders, with many genetic and environmental factors involved, empiric (observed) recurrence risks have been used in the past. These are average risks obtained from a large number of families. Recurrence risks in particular families can now be estimated (Smith, 1971; Curnow, 1972) using the multifactorial model with two parameters, the frequency of the disease and the heritability of liability to the disease (Falconer, 1965). These methods take account of the number and relationship of affected and unaffected relatives, their sex, age at onset, current

age and other relevant variables for the particular family at risk. A series of risk tables have been prepared and a computer program (RISKMF) has been developed (Smith, 1972) to calculate the recurrence risk in any family presented. For example, the estimated recurrence risks for spina bifida and/or anencephaly are given in Table 23 for several different family histories. These methods are likely to be most useful: (1) in common familial conditions: (2) if there are sex or age effects; and (3) if there is a complex family history of the disease. It may be possible also to include information on correlated continuous traits associated with the disease, such as blood glucose in diabetes, to improve the accuracy of the risk estimate.

Table 23. *Estimated recurrence risks for spina bifida and/or anencephaly*

Family history of spina bifida and/or anencephaly	Estimated recurrence risk (%)
One sib affected	5
Parent and sib affected	13
Two sibs affected	13
Three sibs affected	21
One sib and a second-degree relative affected	9
One sib and a third-degree relative affected	7
One sib affected, no other family history	4

Often the mode of inheritance is not known. Morton, Stevenson and Harris (1972) have developed a computer program (COUNSEL) which will estimate risks for different modes of inheritance, including the multifactorial model, given information on incidence, ascertainment frequency, penetrance, family history and other relevant factors.

Genetic Linkage and Antenatal Diagnosis

The use of genetic linkage in genetic counselling and in antenatal diagnosis has recently been discussed by Mayo (1970). In principle, if the disease locus is linked to a genetic marker, then information about the genotype of a fetus at the marker locus may give information about the genotype at the disease locus. As an example, consider use of the secretor locus (Se) in the diagnosis of myotonic dystrophy, the recombination fraction between these loci being about 0.07. An informative pedigree is given in Figure 23. To make a diagnosis, the linkage phase of the affected relatives

must be known. Since secretor status is a dominant (SeSe and Sese both type +), the secretor status of the grandparents must also be known to establish if the affected parent is heterozygous at the secretor locus.

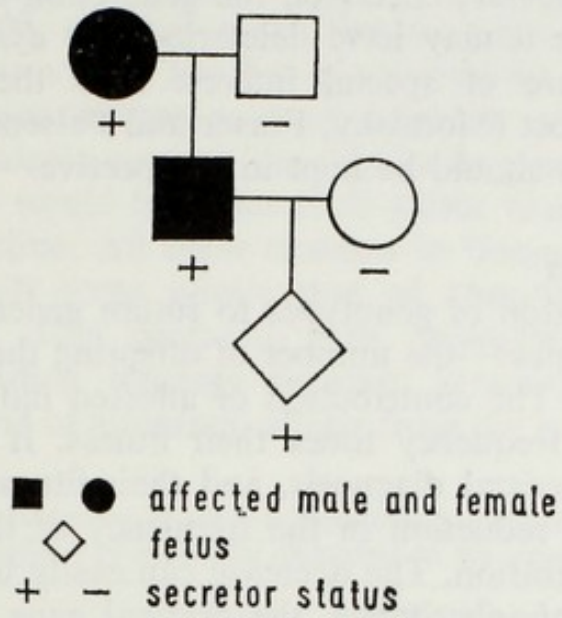

Figure 23. An informative pedigree for the antenatal diagnosis of myotonic dystrophy through linkage with secretor status.

As the fetus is secretor (+) then it probably also carries the gene for myotonic dystrophy, and the reverse if it were secretor (−). However, there is a chance of crossing-over between the loci, so the probabilities of being affected become 93 per cent and 7 per cent respectively.

There are several inherent limitations to the use of genetic linkage in antenatal diagnosis. At present, few diseases are known to be linked to marker loci, and as yet few marker loci can be typed *in utero*. Other methods of antenatal diagnosis can be used in all pregnancies at risk and provide a definite result. But antenatal diagnosis through linkage is possible in only a proportion of pregnancies at risk. For dominant and X-linked disorders, the affected or carrier parent must be heterozygous and his linkage phase must be known. For recessive conditions the same applies, but for both parents. If the marker locus acts as a dominant, then the proportion of informative pedigrees may be quite small. Finally, due to crossing-over, there will always be an error rate in classification. Thus antenatal diagnosis through linkage is unlikely to become a routine or accurate procedure, and may only be useful if there are close linkages and if no other tests are available, and then in only a proportion of families presented.

Eugenic Effects of Antenatal Diagnosis

Antenatal diagnosis will lower the frequency of a genetic disease in the current generation, but what effects will it have on subsequent generations? In most circumstances antenatal diagnosis will have beneficial, or *eugenic,* effects on the gene pool, but there are some situations where it may have deleterious, or *dysgenic,* effects, and because they are of special interest it is the latter that have been studied most (Motulsky, Fraser and Felsenstein, 1971). These opposing effects should be kept in perspective.

Beneficial effects

The contribution of genotypes to future generations depends on their relative fitness—the number of offspring they produce relative to the average. The contribution of affected individuals is proportional to their frequency times their fitness. If their frequency is reduced by antenatal diagnosis, and their fitness is not zero, then there will be a reduction in the frequency of the abnormal allele in the next generation. The decrease can easily be predicted, knowing the mode of inheritance, the original gene frequency (q) and the proportion (x) of affected individuals whose birth has been prevented and their previous fitness (f). The new gene frequency will be a proportion ($1-fx$), ($1-fx/3$) and ($1-qfx$) of the previous gene frequency for dominant, X-linked and recessive diseases respectively. Thus if affected individuals can reproduce, there will be a substantial reduction in the gene frequency for dominant and X-linked conditions, but a much smaller reduction for recessive conditions. This is likely to be the most important factor in changing the gene frequency of abnormal alleles, as shown in Table 24, and the effects will be beneficial to the gene pool.

Deleterious effects

Deleterious effects of antenatal diagnosis may arise if families tend to replace fetuses diagnosed as affected with other children. Such dysgenic effects might already be occurring if families compensate for the loss of affected children born, so-called reproductive compensation.

For dominant conditions there should be no dysgenic effects because antenatal diagnosis and compensation for affected genotypes should lead to only normal children being born in families using antenatal diagnosis.

In autosomal recessive conditions, two thirds of children compensating for affected genotypes will be carriers and so an increase in the frequency of the abnormal allele may result. Motulsky *et al.*

(1971) have studied the situation in detail. They show that the gene frequency, and the frequency of carriers, will increase to a new equilibrium frequency from 1.2 to 1.5 times the original frequency, but the change will take many generations. For example, the frequency of carriers of galactosaemia might rise from 6.3 per 1000 to 7.3 per 1000, and for cystic fibrosis from 5 per cent to 7.5 per cent, but over a large number of generations. Alternatively, if a previous selective advantage were relaxed, as in sickle cell anaemia through malaria control, the decline in gene frequency of the abnormal allele would be reduced to about two thirds of the previous rate of decline. All these changes in frequency are small because there is still some elimination of abnormal alleles by antenatal diagnosis. This contrasts with prevention of affected individuals by avoiding matings between heterozygotes, where there is no elimination of abnormal alleles from the gene pool.

Table 24. *Summary of eugenic (beneficial) and dysgenic (deleterious) effects of antenatal diagnosis on the gene pool*

	Effects: eugenic (+) or dysgenic (−)	
	Affected individuals may reproduce (fitness > 0)	Affected individuals do not reproduce (fitness = 0)
1. No reproductive compensation	+++	No effect
2. Reproductive compensation		
Mode of inheritance		
Dominant	+++	No effect
Recessive		
Maintained by		
(a) mutation	+	−
(b) selection	+	−
X-linked		
Abortion of		
(a) affected males	++	−
(b) all males	+	−−
(c) affected males and carrier females	++	+
Chromosomal aberrations	(+)	(−)
Multifactorial disorders	(+)	(−)

Deleterious effects of antenatal diagnosis for X-linked diseases have been considered by Motulsky *et al.* (1971) and Emery, Nelson and Mayo (1971). They examined three possible applications of antenatal diagnosis, namely (1) abortion of affected males, (2) abortion of all males and (3) abortion of affected males and carrier females. The last case is clearly eugenic, reducing the frequency

of abnormal alleles to a new equilibrium depending on the mutation rate and on the proportion of carrier women ascertained. Abortion of affected males, with full reproductive compensation, will lead to a new equilibrium with the frequency of carrier females being about 1.5 times the original rate (Motulsky *et al.*, 1971). The abortion of all males from carrier mothers with full reproductive compensation is more dysgenic, since carrier mothers will contribute an equal proportion of normal and abnormal genes to their daughters in the next generation and there will be no elimination of abnormal genes. But mutation will continually add new abnormal genes to the gene pool, and so the gene frequency will rise linearly with time at a rate equal to the mutation rate. If the fitness of affected males were zero, this will correspond to doubling the gene frequency every two generations.

The effects of antenatal diagnosis on the frequency of chromosomal and multifactorial conditions will be small since the recurrence risks are normally low. Full reproductive compensation for affected genotypes corresponds to within-family selection and the consequences for multifactorial traits have been discussed by King (1965).

An attempt is made to summarize the various eugenic and dysgenic effects of antenatal dignosis in Table 24. It is apparent that dysgenic effects are usually small and arise only when the previous fitness of affected individuals was very low or zero and when there is full reproductive compensation. In practice, full reproductive compensation is unlikely, since each pregnancy at risk must undergo antenatal diagnosis and there will be many other factors limiting family size in families at risk. Moreover, it is likely that only a proportion of the matings at risk will be ascertained, and so any trends in frequency caused by antenatal diagnosis will be correspondingly moderated. In general we can conclude that dysgenic effects of antenatal diagnosis are likely to be small and doubtful, and of a very much lower order than other possible dysgenic effects acting on the gene pool.

Conclusions

It is apparent that antenatal diagnosis has considerable potential in preventing genetic disease, both in particular families and in the population as a whole. Currently its use is limited and its impact small, but as experience grows and as new techniques are developed to diagnose more genetic diseases antenatally, it could

be used more widely and its impact on genetic disease may increase. The chief restrictions to its widespread use in practice are likely to be the cost of hospital and laboratory services, and the risks involved to the mother and the normal fetus. It is important that clinicians should record the nature and extent of any risks involved by different techniques and procedures of antenatal diagnosis, so that the net effect on health can be assessed. An amniocentesis register has already been established at the National Institute of Child Health and Human Development in the United States (Friedman, 1971), and another is being set up by the Medical Research Council in Britain. Such measures of 'quality control' should quickly point to any drawbacks in antenatal diagnosis, so that they may be avoided. If the technique proves safe and reliable, patients can be reassured and clinicians encouraged to apply the procedure in practice.

In general, antenatal diagnosis is likely to have beneficial (eugenic) effects on the gene pool. Only if there is full reproductive compensation for the loss of abnormal fetuses and if abnormal individuals do not reproduce, will there be deleterious (dysgenic) effects on the population and even then they will be small and readily modifiable.

To extend the use of antenatal diagnosis in practice, procedures for ascertaining those at risk, either in particular families or by screening all parents, need to be developed. This would change the emphasis in human genetics from genetic counselling as a retrospective practice to a genetic prevention service on a prospective basis. New attitudes about the responsibility of parents in procreation might be engendered, which, together with acceptance of family planning, would ensure, as far as is possible, for the wellbeing of their children, both genetically and environmentally. It is salutary to note that further liberalization of legislation to allow euthanasia of defective newborn would make antenatal diagnosis largely unnecessary. However, this is unlikely for some time, so that antenatal diagnosis should remain a useful tool in the prevention of genetic disease.

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Appendix

Laboratory Methods of Use in Antenatal Diagnosis

M. M. Nelson

Cell Counting Methods

Trypan blue (after Steele and Breg, 1966)

Stain: 0·4 per cent aqueous trypan blue.

Method: 0·1 ml unspun amniotic fluid is mixed with 0·02 ml trypan blue solution in a small container. The mixture is incubated at 36° to 37°C for 5 min.

The cells are counted under low power in a Neubauer counting slide. All big squares (18) are examined and the blue cells and the clear unstained cells counted. Each total multiplied by 667 gives the numbers per ml of amniotic fluid of that cell type. The total number of unstained cells represents the total number of viable cells.

Nile blue sulphate (after Gordon and Brosens, 1967)

Stain: 0·1 per cent aqueous nile blue sulphate.

Method: 1 drop unspun amniotic fluid is mixed with 1 drop nile blue sulphate solution, on a clean slide.

The mixture is covered with a cover slip. The slide is warmed gently, e.g. on the microscope stage, for 2 min. The cells are examined under low power. If orange cells are present, a total of 500 cells is counted and the percentage of orange cells in the total is calculated. (N.B. After 37 to 38 weeks gestation, the orange cells may be present in large clusters, which are difficult to count.)

Results: 2 per cent orange cells indicates a pregnancy of 32 to 34 weeks; 10 per cent one of 34 to 36 weeks; 50 per cent and over, one of at least 38 weeks gestation. (N.B. Occasionally, counts of less than 10 per cent may be obtained at 38 weeks and later.)

Cell Fixation

One to 2 ml of amniotic fluid is spun at 900 rev/min for 10 min. The supernatant is decanted and the cells resuspended in 2 ml of fixative (methanol + glacial acetic acid, 3:1) which has been chilled. The cells are allowed to stand at room temperature for 30 min, and then respun for 10 min. The supernatant is discarded and the cells resuspended in 0·25 ml fixative. The cells are then ready for use and may be stored in the refrigerator at 4°C.

Nuclear Sexing

Sex chromatin

Stain: 0·5 per cent or 1·0 per cent aqueous solution of cresyl echt violet.

Method: 1 or 2 drops of the fixed-cell suspension are dropped onto a clean slide and air dried. The slide is then immersed in the stain for 5 min.

Then: Rinse in tap water; dip in two separate changes of 95 per cent alcohol; dip in absolute alcohol; clear in two changes of xylene for 15 min each; air dry

Cover stained cells with coverslip mounted with DPX or equivalent. Examine cells under X 100 magnification. Count 100 nuclei and calculate percentage of nuclei with sex-chromatin bodies. (N.B. Count only nuclei with well-marked membrane outline. Do not include any folded nuclei or those with heavy chromatin-stained masses. Include only nuclei in which the detail can be clearly seen. The sex-chromatin body is a dark triangular or planoconvex mass seen at the periphery of the nucleus and measures about 7μm.)

Results: 0 per cent = male (N.B. some laboratories accept up to 2 per cent); 5 per cent and over = female.

F body (after Polani and Mutton, 1971)

Stain: Atebrin (quinacrine hydrochloride) 0·5 per cent aqueous solution.

Method: 2 drops of fixed-cell suspension are dropped on to a clean slide and air dried.

Then: rinse in deionized water; stain in Atebrin for 5 min; wash in running tapwater for 3 min; air dry.

Mount in deionized water or buffer at pH 5·5. Examine under ultraviolet light. (We use a Leitz Ortholux with exciter filter BG12 and barrier filter combination 510 and 530 with a X 54 oil immersion flat-field objective.) Count at least 50 nuclei. The F body is a minute, very bright fluorescent spot seen anywhere in the nucleus: two close together = duplex; two far apart = 2 Y chromosomes.

Results: 0 per cent to 10 per cent = female; 25 per cent and over = male. (N.B. Each laboratory has its own normal range and this must be worked out first.)

Amniotic Fluid Cell Culture

Primary culture

Culture dishes: 35 mm Falcon plastic petri dishes containing one 22 × 22 mm glass coverslip.

Medium: Eagle's minimal essential medium with 30 per cent fetal calf serum added. Antibiotics added as follows: penicillin 0·12 mg per ml final concentration; streptomycin 0·1 mg per ml final concentration ('Fungizone' 0·002 mg per ml final concentration—optional).

Method: gently agitate the amniotic fluid in the original container. Decant into centrifuge tube; spin at 900 rev/min for 10 min; decant supernatant.

Resuspend cells in remaining amniotic fluid (approximately 0·5 ml) and add 0·25 ml pure fetal calf serum. Mix gently and drop on to coverslip in the petri dish.

Stand for a minimum of 5 min. at room temperature or in the incubator if over 15 min. Add 1·5 ml culture medium. Place in incubator with 95 per cent air + 5 per cent CO_2 atmosphere at 36°C. Leave undisturbed for 7 days. (N.B. All procedures must be carried out under sterile conditions and all equipment must be carefully sterilized before use.)

Subculture

Examine the culture for confluence of cells and when ready, detach the cells as follows: Wash the culture dish twice with tris buffer solution (pH 7·4); add 0·5 to 2 ml trypsin solution (0·25 per cent in triple distilled water); incubate for 5 to 10 min till the cells have detached.

Add 1, 2 or 5 ml medium, according to size of dish.

Resuspend cells in medium–trypsin mixture, transfer to centrifuge tube and spin at 900 rev/min for 10 min.

Decant supernatant all but 1 ml and resuspend cells in this. Divide cells between number of new dishes required. Add appropriate amount of medium to new dishes, incubate and medium change at 3 days. (N.B. Cell counts on the cell suspension after trypsinization will allow the optimum inoculum required to be estimated for each new dish.)

Subculture method for small quantities of cells

Wash the culture dish twice at least in Dulbecco solution (phosphate-buffered saline, Dulbecco A). Add 2 to 5 ml trypsin (0·25 per cent trypsin in Dulbecco A). Swirl the trypsin over the culture and then remove as much as possible, leaving only a film.

Incubate for 15 to 30 min till cells are detached.

Add medium to correct amount for the size of the dish if the aim is to re-distribute the cells in the original culture dish. If new dishes are to be set up, add sufficient medium to resuspend the cells and divide between the required number of new dishes.

Chromosome Preparations

Coverslip chromosome preparations

When cells are almost confluent and are noted to be in mitosis with light microscopy or phase, add colchicine to dish to give final concentration of 1/40,000 (w/v). Incubate for 4½ hours.

Remove medium and replace with hypotonic solution (0·075 M-KCl). Leave at room temperature for 50 min.)

Remove half the hypotonic solution and replace with fixative (methanol+ acetic acid, 3:1). Leave for 5 min. Repeat twice more. Finally remove all mixture and replace with fixative only. Leave 10 min.

Air or flame dry.

Stain with appropriate stain, e.g. Giemsa—10 min in 10 per cent Giemsa; or Atebrin—as for Y bodies.

Rinse in tap water, dehydrate in acetone and clear in xylene.

Cell suspension for chromosome studies

When cells are ready for examination, remove medium and trypsinize as for subculture. Spin cell suspension and decant trypsin-medium mixture. Resuspend cells in hypotonic solution (0·075 M-KCl). Leave for 30 min at room temperature.

Spin for 5 minutes at 800 to 900 rev/min. Decant hypotonic solution and resuspend cells in 2 mls fixative (methanol+acetic acid, 3:1). Stand for 30 min. Spin for 5 min and resuspend in 0·25 ml fixative. The cells are now ready for use and may be stored at 4°C. Stain as required.

Metachromasia

When growth is confluent enough for study, remove cover-slip from culture dish. Wash well in normal saline. Fix cells by either of the following methods:

(a) Leave for 30 min in the following fixative:

10 ml Pb NO_3 2 per cent solution
10 ml formalin
80 ml ethanol

100 ml

(N.B. the solution may turn cloudy when poured on—agitate gently.) Wash in 70 per cent, 80 per cent and absolute ethanol. Air dry.

(b) Leave for 10 min. in methanol. Air dry.

Toluidine blue

Stain: 0·1 per cent toluidine blue in 30 per cent methanol.

Leave cover-slip in stain for 5 min. Dehydrate in acetone and clear in xylene. Mount in DPX.

Results: metachromatic cells show red-purple cytoplasm; cytoplasm otherwise blue.

Alcian blue

(a) Stain: 1 per cent alcian blue in distilled water saturated with thymol.

Leave cover-slip in stain for 1 min. Wash in distilled water. Place in 0·5 per cent borax in 80 per cent ethanol for 24 hours. Dehydrate in 95 per cent and absolute alcohol. Clear in xylene. Mount in DPX.

Results: dark blue/green granules on pale background in positive cells.

(b) Stain (after Danes and Bearn, 1970): 0·05 per cent alcian blue in 0·025 M-acetate buffer at pH 5·7 with added 0·3 M-magnesium chloride.

Leave cover-slip in stain overnight. Dehydrate in 70 per cent and 100 per cent alcohol. Clear in xylene. Mount in DPX.

Results: Dark blue/green granules against paler background in positive cells.

(c) Stain (after Swift and Finegold, 1969): 1 per cent alcian blue in 3 per cent acetic acid, pH 2·5.

Leave cover-slip in stain for 15 min. Rinse in running tapwater. Counterstain with 1 per cent chromotrope 2R for 10 min. Dehydrate in butanol. Clear in xylene. Mount in DPX.

Results: Dark blue granules against pale red background in positive cells. Best results are obtained with lead-formalin fixative.

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GENDER DIFFERENCES

Their Ontogeny and Significance

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Few books approach their subject by such diverse paths, few will arouse such conflicting questions in the minds of their readers. Geneticists and psychologists, epidemiologists and paediatricians here examine the fundamental mechanics of gender differentiation and the consequences of gender differences in human development. They assemble a wealth of research material, much of it new, and suggest ways by which gender differences may be explored: these include social behaviour and development of intellect in young children, relationship between gender identity and sexual behaviour of adults, development of spatial and linguistic skills, development of disease, and sex chromosomal errors.

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