PRENATAL DIAGNOSIS OF CONGENITAL DISEASES

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INTRODUCTION

Congenital diseases

4 - 6% of the liveborns are affected with some form of congenital disease (Warkany, 1971; Trimble & Doughty, 1974). Accordingly, in The Netherlands each year 8000 - 12.000 children are born with a more or less severe handicap. In some cases morphological and/or functional abnormalities are apparent shortly after birth, as is the case in anencephaly, meningomyelocèle, congenital heart defects, several chromosomal aberrations and inborn errors of metabolism. In other congenital diseases the onset of clinical manifestations occurs in early or late childhood and some sex-chromosomal aberrations will not be detected before puberty.

Finally, a number of these diseases is not manifest before adulthood, like Huntington's chorea and a number of variants of inherited metabolic disorders.

The three main categories of congenital diseases are:

1. Chromosomal aberrations, with an incidence of 1 : 200 liveborns (Jacobs et al., 1974); about 1000 patients per year in The Netherlands.
2. Gene mutations, resulting in metabolic diseases, incidence 1 : 100 liveborns and accordingly 2000 patients per year.
3. Disturbances during embryonic development, 2 - 4 : 100 liveborns, and 4000 - 8000 patients per year.

Most numerical chromosomal aberrations are caused by nondisjunctions during gametogenesis or during the first divisions of the fertilized ovum. The most frequent examples are trisomy 21 (Down's syndrome), Klinefelter's syndrome (47 XXY), Turner's syndrome (45 XO) and 47 XYY. In some of these syndromes the risk of non-disjunction increases with maternal age, as in trisomy 21. This has an incidence of 1 : 3000 newborns from mothers younger than 30 years; the incidence rises to 1 : 280 in mothers of 35-39 years and to 1 : 40 in mothers older than 45 years. A minority of chromosomal disorders is caused by structural abnormalities of the chromosomes, like translocations, inversions, etc. Part of these are inherited from one of the parents, who is a phenotypic-

ally normal carrier of the balanced form of the rearrangement. The risk for affected offspring in carriers of balanced translocations is dependent on the type of translocation and the sex of the carrier-parent and may vary from 10-100%.

In McKusick's catalogue (1971) nearly 2000 different gene mutations of Mendelian inheritance are listed. Many of these result in metabolic disorders, but only in 10% of these the exact nature of the biochemical defect has been characterised (Stanbury et al., 1972). In about 50 severe inborn errors of metabolism the enzymatic defect or the storage of a specific metabolite can be demonstrated in _in vitro_ cultured skin fibroblasts from the patient (for reviews: see Milunsky et al., 1970; Milunsky and Littlefield, 1972). If the genetic trait shows recessive inheritance, both parents are heterozygous carriers and asymptomatic; the (recurrence) risk for an affected child is 25%. A son of a mother, who is heterozygous for an X-linked disease, has a chance of 50% to be affected. Most of these genetic metabolic diseases are relatively infrequent (varying from about 1 : 1500 to 1 : 100.000), but as a total group they are twice as frequent as chromosomal aberrations.

The major category of congenital diseases are the disturbances in embryonic development which may be the result of environmental and/or genetic factors. The most common types are congenital heart defects (± 1 : 100 live borns), neural tube defects (± 1 : 500), cleft lip and/or palate, club foot and several hundred other congenital deformities. The risk for such an anomaly increases in some types after the birth of an affected child; in other types this is possibly not the case.

The knowledge about the etiology is very incomplete in most cases of congenital disease. As a result causal prevention is nearly impossible, except for the avoidance of well-known teratogenic factors like ionizing radiation, certain viral infections and certain toxic drugs. Therapeutic methods are not available for chromosomal aberrations and most of the inherited metabolic di-

seases. Improved surgical techniques contributed to the management of many congenital malformations like cleft lip and palate, cardiac malformations, skeletal deformities, etc.

Genetic counseling and prenatal diagnosis of congenital diseases

Many of the congenital diseases from the three categories are associated with severe physical and/or mental abnormalities and most patients require intensive medical and psychosocial care, often during several decades. Besides the problems caused by a handicapped life, parents - and sometimes patients - are confronted with the fear of recurrence of the disease in their future offspring. The organization of genetic advice required in these situations received increasing attention during the last decade. An increasing number of couples, having a (distant) relative affected with some type of congenital disease, also ask for such an advice about their own risks and the methods for prevention. Genetic counseling will primarily be based upon the availability of a precise diagnosis and on information about the mode of inheritance of the disease involved. The physician giving genetic counseling should have sufficient knowledge of the principles of genetics, embryonic development, recent diagnostic methods for congenital diseases and of therapeutic and preventive means. He needs time to explain the risks (which may vary from negligeable to 100%) and to help the counselees to reach a decision which is appropiate in their individual situation. These decisions are often very difficult, since they may involve the acceptance of a high risk of another affected child or the decision to refrain from reproduction. In other cases artificial insemination, adoption, or abortion may be considered.

The interpretation of the risk-figures and the decision reached by the counselees will be determined by many factors, like the composition of their family, their age, the severity of the disease involved, their psychological tolerance and the opinions and attitudes in the society.

The development of methods enabling prenatal diagnosis of a number of congenital diseases offers new perspectives to many couples at risk. The first reports of succesfull analyses of the fetal karyotype in cultured amniotic fluid cells were published nearly 10 years ago (Steele & Breg, 1966; Jacobson & Barter, 1967). The first genetic metabolic disorders were detected soon afterwards by biochemical assays of amniotic fluid supernatant and cultured amniotic fluid cells (Nadler, 1968; Fujimoto et al., 1968). During the last five years more and more experience has been gained with transabdominal amniocentesis in the 14th - 16th week of pregnancy, the subsequent in vitro cultivation of fetal cells from the amniotic fluid and the karyotyping and biochemical analysis of these cells. An increasing number of parents at risk for a chromosomal aberration or a genetic metabolic disorder asked for prenatal diagnosis and several centers described the results on their first series of hundreds of cases (Nadler, 1972; Milunsky & Atkins, 1974; Philip et al., 1974; Hsu & Hirschhorn, 1974; Golbus et al., 1974; Wahlström et al., 1974; Niermeijer et al. 1975). It appears, that amniocentesis in early pregnancy is a relatively safe procedure and that the analytical results are very reliable.

It may be of great advantage for parents at risk to have certainty about the status of the fetus. Prenatal diagnosis will show in many cases that the fetus is not affected by the disease tested for and the parents can be reassured. On the detection of a fetal abnormality the parents may ask for interruption of the pregnancy, thus preventing the birth of a severely affected child.
The possibilities for prenatal detection were recently extended by the finding of Brock & Sutcliffe (1972) that closure defects of the neural tube (anencephaly and meningomyelocèle) are detectable in early pregnancy by elevated concentrations of alpha-fetoprotein in the amniotic fluid supernatant.

INDICATIONS FOR PRENATAL DIAGNOSIS

The main indications for prenatal diagnosis used at the moment are:

- mothers aged 38 years or over.
- mothers who previously gave birth to a child with a chromosomal aberration.
- pregnancies, where on of the parents is a balanced carrier of a chromosomal rearrangement (translocation, inversion).
- pregnancies at risk for one of those 50 inborn errors of metabolism, that can be detected in cultured amniotic fluid cells.
- pregnancies at risk for an open neural tube defect.

The indications will be briefly discussed below.

1. RISK FOR A NUMERICAL CHROMOSOMAL ABERRATION

The most frequent examples are the recurrence risk of trisomy 21 (Down's syndrome) and the risk for chromosomal aneuploidies at advanced maternal age (38 years and older).

The recurrence risk for trisomy 21 is 1% as calculated either from retrospective data (Pfeiffer et al., 1973) or from prospective series of prenatal diagnoses for this indication (Milunsky, 1973). The recurrence risk of other numerical aberrations is less well known. This is related to the question of the presence in man of a familial tendency to non-disjunction. Two types of evidence for this phenomenon are present:

a. A number of sibships have been described with aneuploidies for different chromosomes (Girardet et al.,1972; Bell & Cripps,1974; Holmgren & Ånséhn,1971; Hamerton, 1971).

b. Chromosomal studies on subsequent spontaneous abortions in the same family indicate that if the first abortion showed a chromosomal aberration, the following was likely to be caused by a chromosomal aberration, not necessarily being of the same type (Boué & Boué, 1973).

These factors are to be considered when giving recurrence risks for a trisomy others than trisomy 21. Bell & Cripps (1974) advise prenatal monitoring in

every pregnancy in families where a child with an aneuploidy has been born. Chromosomal mosaicism in one of the parents might give a considerably higher risk for recurrence of the same aneuploidy than in the previous group and several of these families were described (Beratis et al. 1972; Hsu et al., 1971). The recurrence risk of chromosomal mosaicism is probably in the order of 1% but cases of familial mosaicism were described, where higher risk figures are involved (Hsu et al. 1970; Shih et al., 1974).
Down's syndrome is not the only trisomy with an increased frequency at advanced maternal age; other autosomal trisomies tend to be more common as well. However, these will be found relatively rarely since there is a high early abortion rate in these cases (Laurence et al., 1974[a]). The risks for a chromosomal aberration in the maternal age-groups of 35-39 years and over 40 years were found to be 1.5% and 3%, respectively in a collected series of prenatal diagnoses (Hsu & Hirschhorn, 1974).

2. RISK FOR A STRUCTURAL CHROMOSOMAL ABNORMALITY

The risk for the carrier of a balanced chromosomal translocation for offspring with an unbalanced karyotype depends on the type of translocation and the sex of the carrier-parent. Hsu & Hirschhorn (1974) find a risk of 20% in 93 pregnancies monitored for this indication. Individuals carrying inversions in one of their chromosomes are also at an elevated risk for children with unbalanced karyotypes. In each patient with clinical symptoms suggestive for a chromosomal aberration karyotyping should be carried out to establish the exact nature of the aberration and its mode of inheritance. The birth of a child with an unbalanced karyotype may be prevented in time by family studies, identification of carriers, followed by genetic counseling and prenatal diagnosis. The birth of affected children in families of (distant) relatives and a high frequency of spontaneous abortions may be the indicators for the presence of such an inherited chromosomal translocation.

The recurrence risk of a de novo translocation is probably very small (Gardner & Veale, 1974) and at the moment this seems no indication for amniocentesis.

3. RISK FOR AN X-LINKED DISEASE

When the mother is a carrier of an X-linked disease, there is a 50% chance for her son to be affected. Prenatal sex determination by chromosome analysis may help these parents to limit their families to unaffected daughters (who will be carriers in 50% of the cases). The limitations of this approach are obvious, but it is the only possibility for these parents to have their own children, without being forced to accept a high risk for a severely affected child. This approach applies to those X-linked disorders where the basic defect is unknown. (e.g. Duchenne's muscular dystrophy, X-linked mental retardation, etc.) or is not yet detectable in amniotic fluid or cultured amniotic fluid cells (e.g. hemophilia). In a few X-linked diseases the biochemical defect is expressed in cultured amniotic fluid cells and this allows prenatal identification of an affected male fetus (Fabry's disease; Hunter's disease; the Lesch-Nyhan syndrome; for references: see Burton et al., 1974).

4. RISK FOR A METABOLIC DISEASE

About 50 severe recessive autosomal or X-linked diseases can now be detected in utero by biochemical assays of cultured amniotic fluid cells (for reviews, see: Milunsky et al., 1970; Milunsky & Littlefield, 1972; Burton et al., 1974). In autosomal recessive diseases the diagnosis of a first affected child usually is the first indication that both parents are carriers of the same gene mutation. The recurrence risk is 25%. Prenatal diagnosis in future pregnancies of these parents will only be reliable if cultured cells of the affected child are available. The prenatal diagnosis will be based on a comparison of the biochemical assays on amniotic fluid cells from the pregnancy at risk, control cells from a normal pregnancy cultured under identical conditions and fibro-

blasts from the patient with the metabolic defect.
The birth of a first affected child can be prevented only in those circumstances where the carriership of the parents has been identified before their first pregnancy. This is sometimes possible in X-linked diseases; for autosomal disorders this is generally not the case. Large-scale carrier testing for these diseases will only be useful in populations with a high frequency for certain gene mutations. The only example of such a program is the carrier-testing for Tay-Sachs' disease in certain Ashkenazi-Jew population groups in the United States, where prenatal diagnosis can be offered to couples identified as heterozygotes (Kaback et al., 1974).

5. RISK FOR AN OPEN NEURAL TUBE DEFECT

Open defects of the neural tube (anencephaly and meningomyelocèle) are relatively frequent congenital malformations of multifactorial origin. The recurrence risk after one affected child is about 5% and after two affected children it increases to ± 10% (Bonaiti-Pellié & Smith, 1974). The malformation in the subsequent child may be either identical or of a different type. An affected parent has a 3 - 4% risk for an affected child (Carter, 1974).
Prenatal detection of open neural tube defects is possible by analysis of the concentration of alpha-fetoprotein in the amniotic fluid (Brock & Sutcliffe, 1972; Allan et al., 1973); closed defects, some of which may have milder clinical manifestations, cannot be detected with this method (Laurence et al., 1973) and the parents should be informed accordingly.
This recent development will be a quite common indication for prenatal monitoring, and in most cases this will be for parents with an affected child.
Elevated levels of amniotic fluid alpha-fetoprotein can in some cases also be detected in maternal serum (Brock et al., 1973). In a number of studies the value of this maternal indicator for fetal neural tube defects was explored (Harris et al., 1974; Seller et al., 1974; Wald et al., 1974). The possibilities for large-scale screening so far are not very promising.

Future developments in prenatal diagnosis

The actual techniques of prenatal diagnosis by amniocentesis and analysis of amniotic fluid and cultured amniotic fluid cells seem to be preferable over proposed other approaches to fetal specimens for diagnosis. Recently, the use of placental biopsies (Kullander & Sandahl, 1973; Ogita et al., 1974) was advocated as a source for material for prenatal cytogenetic diagnosis. Valenti (1973) proposed intrauterine blood sampling by puncture of fetal blood vessels with an endamnioscope, which could enable intrauterine detection of hemoglobinopathies.

Preliminary results of techniques for fetal observation and biopsy through amnioscopes (Scrimgeour, 1974; Wheeless, 1974; Levine et al., 1974; Laurence et al., 1974[b]) warrant the conclusion, that as yet this procedure is too dangerous for clinical application.

Safe techniques for fetal observation and blood sampling might be a significant contribution to prenatal detection of some congenital malformations, fetal hemoglobinopathies and possibly in the future hemophilia, when analytical methods are available. Methods for the detection of hemoglobinopathies in small samples of blood have been described (Cividalli et al., 1974; Chang et al., 1974; Hobbins et al., 1974). Attempts are being made to detect HbS on individual erythrocytes occuring in the amnioting fluid (Boyer et al., 1974; Headings et al., 1974). In contrast to earlier reports (Fortune & Fox, 1973), the activity of factor VIII is present in fetal blood in early pregnancy and if appropiate sampling techniques become available, prenatal diagnosis of hemophilia could become possible (Holmberg et al., 1974).

The extension of possibilities for prenatal diagnosis in the near future will be mainly dependent on the further clarification of biochemical defects in genetic diseases. The recent finding of adenosine deaminase deficiency in cultured amniotic fluid cells from a fetus, postnatally shown to be affected with severe combined immonodeficiency disease (Hirschhorn et al., 1975) is an example of this rapidly expanding field.

Another important contribution would be the development of methods for the induction of the expression of genes normally not expressed in cultured cells.

The present state of prenatal diagnosis leaves much to be done. Although many papers appeared about this method, still too many people involved in medical care are unaware of its possibilities. Information about prenatal diagnosis, as well as on principles of genetics, recurrence risks and on modern methods for the diagnosis of chromosomal disorders and inborn errors of metabolism, should be incorporated into (postgraduate) medical education.
At this moment, genetic counseling is the most effective way for prevention of congenital diseases. For those parents at risk for a chromosomal aberration, one of some fifty metabolic diseases, a severe X-linked disease or a neural tube defect, prenatal monitoring may be of great help in the difficult choice between the acceptance of a (high) risk for an affected child or the impossibility of having their own healthy children. The discrepancy between the number of prenatal analyses made and the number of pregnancies that are at risk according to the present categories of indications shows that much improvement is possible in diagnosis and genetic counseling of congenital diseases.
The decisions by parents or patients at risk for affected offspring should not be influenced by fears for future generations. Several population geneticists (Motulsky et al., 1971; Fraser, 1972; Fraser and Mayo, 1974) calculated that even extensive use of prenatal diagnosis and selective abortion only have a minor effect on the gene frequencies of autosomal recessive diseases. For X-linked recessive diseases there may be an increase of female heterozygotes but not of an extend to cause any concern.
Prenatal diagnosis may therefore be considered as a useful means to prevent the birth of affected children in individual families and in this way it offers the possibility for parents at risk to extend their families with unaffected children.

INTRODUCTION TO THE PAPERS

This thesis deals with several methodological and practical aspects of prenatal diagnosis.

A genetic metabolic disease can only be detected in utero if the enzymatic defect or storage of a specific metabolite is expressed in cultured cells from an affected individual. This should be demonstrated for each separate disease. An example of such a study is presented in appendix paper I. The data indicate, that the enzymatic defect in the haemolytic anaemia caused by pyruvate kinase deficiency (an autosomal recessive disease) is not detectable in fibroblasts and cultured amniotic fluid cells, since these only produce the M-type of this enzyme, whereas the anaemia is related to a deficient activity of the L-type. As a consequence, prenatal diagnosis of this disease is not possible.

The prenatal diagnosis of enzymatic defects requires analysis of a sufficient number of amniotic fluid cell cultures cultivated under identical conditions and for a comparable period as the diagnostic sample. Sufficient amniotic fluid samples from normal pregnancies will generally not be available at the very moment these are required. To solve this problem a method was developped to store uncultured amniotic fluid cells without loss of cell viability. This method is described in appendix paper II and has enabled us to collect sufficient control material for later use in prenatal diagnosis of enzymatic defects. The possible detrimental effects of transport periods of up to a few days on the viability of amniotic fluid cells was tested simultaneously and the results indicate that transport during periods of up to seven days did not interfere with succesfull cell cultivation. This means that prenatal diagnosis can be offered to people living at long distances from the center performing the analysis.

The prenatal diagnosis of metabolic disorders usually required the biochemical analysis of a few million cells and accordingly the time to obtain these cells during cultivation varied from 4 - 8 weeks after amniocentesis. Such a long waiting period is a serious psychological disadvantage and also interruption of the pregnancy should for medical and ethical reasons - when needed - be carried out as early as possible. This problem was experienced in a number of groups (Littlefield, 1971; Galjaard et al., 1972; Milunsky et al., 1972; Epstein et al., 1972; Golbus et al., 1974). To reduce this waiting period the sensitivity of the analytical techniques had to be increased to enable the investigation of small numbers of cultured amniotic fluid cells. Our group has developped such methods for the microchemical analysis of several enzymes involved in genetic diseases (Galjaard, 1972; Galjaard et al., 1974[a]; 1974[b]). The methodology for one of these enzymes (acid α-1,4-glucosidase, deficient in Pompe's disease) is described in appendix paper III. Micromethods for incubation of small numbers of cells and small volumes of reagents were tested as well as different substrates either for spectrophotometric or fluorometric assays. Significant reductions in the number of cultured cells required for a diagnosis could be achieved by using microvolumes of the substrate 4-Methylumbelliferyl α-D-glucopyranoside. Analysis could be performed on homogenates containing the equivalent of about 1000 cultured amniotic fluid cells for each incubation; the total cell number for a prenatal diagnosis, including a protein assay, could be estimated as the equivalent of 4000 homogenised cultured cells. A further reduction could be realised by adaptation of preparation techniques developed for tissue sections by Lowry (Lowry, 1953; Lowry & Passoneau, 1972). Small groups (about 100) of freeze dried amniotic fluid cells are dissected under microscopic control from a culture grown on a thin transparant plastic foil.

The increasing use of cultured cells for diagnostic and genetic studies on human metabolic diseases warrants the study of a number of factors influencing

the expression of enzymatic activities in in vitro cultivated cells. Appendix paper IV reports a number of such studies on the causes of variation of lysosomal enzymatic activities. Wide variations in the activities of acid α-1,4-glucosidase in fibroblasts and cultured amniotic fluid cells from controls and heterozygotes for type II glycogenosis were observed. Part of these were caused by differences in cultivation conditions. It became apparent, that for a number of lysosomal hydrolases a proportional relation exists between the duration of the culture phase (passage) and the enzymatic specific activity. Variations of α-1,4-glucosidase residual activities observed in fibroblasts from different patients with Pompe's disease might be related to a genetic heterogeneity, i.e. different mutant genes causeating identical diseases. Levels of acid α-1,4-glucosidase in primary and later subcultures of control amniotic fluid cell cultures show considerable variations and this should be considered in comparative studies in actual prenatal diagnosis. Microassays of enzymatic activity in small cell numbers from the same culture show considerable variation of activities among individual cells and these might be caused by local differences in in vitro cell proliferation or differences in the cell cycle. Studies on genetic heterogeneity are possible by fusing (appendix paper IV) cellstrains from patients with apparently identical metabolic diseases and studying the occurrence of complementation, i.e. the restoration of enzymatic activity in hybrid cells. If the latter phenomenon occurs, this is an indication that the disease is caused by different gene mutations in the two patients. β-Galactosidase assays in individual binuclear hybrid cells were carried out to study the genetic background of different clinical variants of GM_1-gangliosidosis.

In appendix paper V the practical application is described of microchemical enzymatic analysis in 6 pregnancies at risk for Pompe's disease. Another example of application in prenatal diagnosis is described in appendix paper

VI in a pregnancy at risk for X-linked Fabry's disease. Microchemical analysis showed normal α-galactosidase activities in groups of 100-200 freeze-dried cultured amniotic fluid cells and this enabled a diagnosis 11 days after amniocentesis. At the same time fetal karyotyping of a replicate culture indicated the presence of a male fetus. The enzyme analysis was confirmed by a study on homogenates of larger numbers of cultured cells from the same pregnancy in two independent laboratories. This is an example of a prenatal diagnosis for a metabolic disease completed in a period comparable (or even shorter) to that for prenatal chromosome analysis.

Results of prenatal diagnoses in pregnancies at risk for different lysosomal storage diseases, including metachromatic leukodystrophy and Hunter's disease, are presented in appendix paper VII. Arylsulfatase A assays on homogenates of cultured amniotic fluid cells were completed in 16 and 18 days after amniocentesis, respectively. Studies of $^{35}SO_4$ incorporation and release in cells from 2 pregnancies at risk for Hunter's disease (mucopolysaccharidosis type II) were combined with cytogenetic analysis and could be completed within 4 weeks after amniocentesis.

A survey of prenatal monitoring of 350 pregnancies at risk for a chromosomal aberration, a sex-linked disease, a metabolic defect or a neural tube defect is presented in appendix paper VIII. The clinical and laboratory methods used are described. Amniocentesis was found to be a safe procedure both for the mother and the fetus. Prenatal chromosome analysis has now become a routine method and the use of banding techniques is essential for karyotyping.
Four chromosomal non-disjunctions were found in 87 pregnancies tested because of advanced maternal age. In 101 pregnancies with a recurrence risk of Down's syndrome, 2 fetuses with an abnormal karyotype were detected. In 11 cases, in which one parent was a carrier of a balanced translocation, 2 un-

balanced fetal karyotypes were found.

Fetal chromosome studies in 43 pregnancies at risk for an X-linked disease indicated the presence of a male fetus in 21 cases.

47 Analyses of alpha-fetoprotein in amniotic fluid from pregnancies at risk for a neural tube defect indicated the presence of 3 affected fetuses in the second half of pregnancy; these last fetuses were found to be anencephalic on ultrasound. In 34 pregnancies prenatal monitoring was carried out for 11 different metabolic diseases and 3 affected fetuses were detected. This type of investigation requires specific experience in the microchemical analysis of cultured amniotic fluid cells. As most of these diseases are infrequent, cooperation between different European centers to this purpose is needed; in the present study 40% of the samples for metabolic diseases was referred by different European centers.

The detection of a fetal abnormality or a male sex (in cases of X-linked disorders) was the indication for selective abortion in 36 of 350 pregnancies tested. However, in 90% of the pregnancies the result of prenatal testing excluded that the fetus was affected by the disease involved and as a result these pregnancies could be continued without further anxiety for the parents.

REFERENCES

Allan, L.D., Ferguson-Smith, M.A., Donald, I., Sweet, E.M. and Gibson, A.A.M. (1973) Amniotic-fluid alpha-fetoprotein in the antenatal diagnosis of spina bifida. Lancet, II, 522-525.

Bell, A.G. and Cripps, M.H. (1974) Familial aneuploidy: what risk to sibs? Canadian Journal of Genetics and Cytology, 16, 113-119.

Beratis, N.G., Kardon, N.B., Hsu, L.Y.F., Grossman, D. and Hirschhorn, K. (1972) Parental mosaicism in trisomy 18. Pediatrics, 50, 908-911.

Bonaiti-Pellié, C. and Smith, L. (1974) Risk tables for genetic counseling in some common congenital malformations. Journal of Medical Genetics, 11, 374-377.

Boué, J. and Boué, A. (1973) Chromosomal analysis in two consecutive abortuses in each of 43 women. Humangenetik, 19, 275-280.

Boyer, S.H., Boyer, M.L., Noyes, A.N. and Belding, T.K. (1974) Immunological basis for detection of sickle cell hemoglobin phenotypes in amniotic fluid erythrocytes. Annals New York Academy of Sciences, 241, 699-713.

Brock, D.J.H., Bolton, A.E. and Monaghan, J.M. (1973) Prenatal diagnosis of anencephaly through maternal serum-alphafetoprotein measurement. Lancet, II, 923-924.

Brock, D.J.H. and Sutcliffe, R.G. (1972) Alpha-fetoprotein in the antenatal diagnosis of anencephaly and spina bifida, Lancet, II, 197-199.

Burton, B.K., Gerbie, A.B. and Nadler, H.L. (1974) Present status of intrauterine diagnosis of genetic defects. American Journal of Obstetrics and Gynecology, 118, 718-746.

Carter, C.O. (1974) Clues to the aetiology of neural tube malformations. Developmental Medicine and Child Neurology, 16, supplement 32, 3-15.

Chang, H., Hobbins, J.C., Frigoletto, F.D., Mahoney, M.J., Kan, Y.W. and Nathan, D.G. (1974) In utero diagnosis of hemoglobinopathies. Hemoglobin synthesis in fetal red cells. New England Journal of Medicine, 290, 1067-1068.

Cividalli, G., Nathan, D.G., Kan, Y.W., Santamarina, B. and Frigoletto, F. (1974) Relation of beta to gamma synthesis during the first trimester: an approach to prenatal diagnosis of thalassemia. Pediatric Research, 8, 553-560.

Epstein, C.J., Schneider, E.L., Conte, F.A. and Friedman, S. (1972) Prenatal detection of genetic disorders. American Journal of Human Genetics, 24, 214-226.

Fortune, W.A. and Cox, J.R. (1973) Fetal factor VIII and IX levels in early pregnancy and their significance in prenatal diagnosis. Acta Haematologica, 49, 314-317.

Fraser, G.R. (1972) Selective abortion, genetic selection, and the X-chromosome. American Journal of Human Genetics, 24, 359-370.

Fraser, G.R. and Mayo, O. (1974) Genetical load in man. Humangenetik, 23, 83-110.

Fujimoto, W.Y., Seegmiller, J.E., Uhlendorf, B.W. and Jacobson, C.B. (1968) Biochemical diagnosis of an X-linked disease in utero. Lancet, II, 511-512.

Galjaard, H., Fernandes, J., Jahodova, M., Koster, J.F. and Niermeijer, M.F. (1972) Prenatal diagnosis of genetic disease. Bulletin of the European Society of Human Genetics, November, 79-91.

Galjaard, H., Hoogeveen, A., Keijzer, W., Wit-Verbeek, E. de and Vlek-Noot, C. (1974[a]) The use of quantitative cytochemical analyses in rapid prenatal detection and somatic cell genetic studies of metabolic diseases. Histochemical Journal, 6, 491-509.

Galjaard, H., Hoogstraaten, J.J. van, Josselin de Jong, J.E. de and Mulder, M.P. (1974[b]) Methodology of the quantitative cytochemical analysis of single or small numbers of cultured cells. Histochemical Journal, 6, 409-429.

Gardner, R.J.M. and Veale, A.M.O. (1974) De novo translocation Down's syndrome: risk of recurrence of Down's syndrome. Clinical Genetics, 6, 160-164.

Girardet, P., Grosset, L. and Juillard, E. (1972) Trisomie 21 et trisomie 18 dans une même fratrie. Helvetica Paediatrica Acta, 27, 583-589.

Golbus, M.S., Conte, F.A., Schneider, E.L. and Epstein, C.J. (1974) Intrauterine diagnosis of genetic defects: results, problems and follow up of one hundred cases in a prenatal genetic detection center. American Journal of Obstetrics and Gynecology, 118, 897-905.

Hamerton, J.L. (1971) Human Cytogenetics. Volume I, Academic Press, New York, p. 220.

Harris, R., Jennison, R.F., Barson, A.J., Laurence, K.M., Ruoslahti, E. and Seppälä, M. (1974) Comparison of amniotic fluid and maternal serum alpha-fetoprotein levels in the early antenatal diagnosis of spina bifida and anencephaly. Lancet, I, 429-433.

Headings, V., Bhattacharya, S., Anyaibe, S., Easton, L., Shukla, S., Scott, R. and Hopkins, E. (1974) Exploratory studies on detection of hemoglobins in the early human fetus in utero. Annals New York Academy of Sciences, 241, 714-721.

Hirschhorn, R., Beratis, N., Rosen, F.S., Parkman, R., Stern, R. and Polmar, S. (1975) Adenosine deaminase deficiency in a child diagnosed prenatally. Lancet, I, 73-75.

Hobbins, J.C. and Mahoney, M.J. (1974) In utero diagnosis of hemoglobinopathies. Technic for obtaining fetal blood. New England Journal of Medicine, 290, 1065-1067.

Holmberg, L., Henriksson, P., Ekelund, H. and Åstedt, B. (1974) Coagulation in the human fetus. Comparison with term newborn infants. Journal of Pediatrics, 85, 860-864.

Holmgren, G. and Ånséhn, S. (1971) The trisomy 21 and trisomy 17-18 syndromes in siblings. Human Heredity, 21, 577-579.

Hsu, L.Y.F., Hirschhorn, K., Goldstein, A. and Barcinski, M. (1970) Familial chromosomal mosaicism, genetic aspects. Annals of Human Genetics (London), 33, 343-349.

Hsu, L.Y.F., Gertner, M., Leiter, E. and Hirschhorn, K. (1971) Paternal trisomy 21 mosaicism and Down's syndrome. American Journal of Human Genetics, 23, 592-601.

Hsu, L.Y.F. and Hirschhorn, K. (1974) Prenatal diagnosis of genetic disease. Life Sciences, 14, 2311-2336.

Jacobs, P.A., Melville, M., Ratcliffe, S., Keay, A.J. and Syme, J. (1974) A cytogenetic survey of 11, 680 newborn infants. Annals of Human Genetics (London), 37, 359-376.

Jacobson, C.B. and Barter, R.H. (1967) Intrauterine diagnosis and managemt of genetic defects. American Journal of Obstetrics and Gynecology, 99, 796-807.

Kaback, M.M., Becker, M.H. and Ruth, M.V. (1974) In: D. Bergsma (editor) Ethical, social and legal dimensions of screening for human genetic disease, pp. 145-163. Stratton Intercontinental Medical Book Corporation, New York.

Kullander, S. and Sandahl, B. (1973) Fetal chromosome analysis after transcervical placental biopsies during early pregnancy. Acta Obstetrica Gynecologica Scandinavica, 52, 355-359.

Laurence, K.M., Turnbull, A.C., Harris, R., Jennison, R.F., Ruoslahti, E. and Seppälä, M. (1973) Antenatal diagnosis of spina bifida. Lancet, II, 860.

Laurence, K.M., Gregory, P.J. and Sharp, F. (1974[a]) Prenatal detection of D trisomy. Journal of Medical Genetics, 11, 398-400.

Laurence, K.M., Pearson, J.F., Prosser, J.F., Richards, C. and Rocker, I. (1974[b]) Fetoscopy followed by live birth. Lancet, I, 1120-1121.

Levine, M.D., Mc Neil, D.E., Kaback, M.M., Frazer, R.E., Okada, D.M. and Hobel, C.J. (1974) Second trimester fetoscopy and fetal blood sampling: current limitations and problems. American Journal of Obstetrics and Gynecology, 120, 937-943.

Littlefield, J.W. (1971) Problems in the use of cultured amniotic fluid cells for biochemical diagnoses. Birth Defects: Original Article Series, VII, no 5, 15-17.

Lowry, O.H. (1953) The quantitative histochemistry of the brain. Histological Sampling. Journal of Histochemistry and Cytochemistry, 1, 420-428.

Lowry, O.H. and Passoneau, J.V. (1972) A flexible system of enzymatic analysis. Academic Press, New York.

McKusick, V.A. (1971) Mendelian Inheritance in Man (3rd edition) The Johns Hopkins Press, Baltimore.

Milunsky, A., Littlefield, J.W., Kanfer, J.N., Kolodny, E.H., Shih, V.E. and Atkins, L. (1970) Prenatal genetic diagnosis. New England Journal of Medicine, 283, 1370-1381; 1441-1447; 1498-1504.

Milunsky, A. and Littlefield, J.W. (1972) The prenatal diagnosis of inborn errors of metabolism. Annual Review of Medicine, 23, 57-76.

Milunsky, A., Atkins, L. and Littlefield, J.W. (1972) Amniocentesis for prenatal genetic studies. Obstetrics and Gynecology, 40, 104-108.

Milunsky, A. (1973) The Prenatal diagnosis of hereditary disorders. Charles C. Thomas Publisher, Springfield, Ill.

Milunsky, A. and Atkins, L. (1974) Prenatal diagnosis of genetic disorders. An analysis of experience with 600 cases. Journal of the American Medical Association, 230, 232-235.

Motulsky, A.G., Fraser, G.R. and Felsenstein, J. (1971) Public health and long-term genetic implications of intrauterine diagnosis and selective abortion. Birth Defects: Original Article Series, VII, no 5, 22-32.

Nadler, H.L. (1968) Antenatal detection of hereditary disorders. Pediatrics, 42, 912-918.

Nadler, H.L. (1972) Prenatal detection of genetic disorders. In: Advances in Human Genetics, ed. by H. Harris and K. Hirschhorn, Vol. 3, pp. 1-37. Plenum Press, New York.

Niermeijer, M.F., Sachs, E.S., Jahodova, M., Tichelaar-Klepper, C., Kleijer, W.J. and Galjaard, H. (1975) Prenatal diagnosis of genetic disorders in 350 pregnancies. (this thesis: appendix paper VIII)

Ogita, S., Hasegawa, H., Matsumoto, M., Kamei, T. and Sugawa, T. (1974) Prenatal diagnosis of genetic anomalies by placental puncture. Obstetrics and Gynecology, 43, 709-712.

Pfeiffer, R.A., Dhadial, R. and Michelis, K. (1973) Das Wiederholungsrisiko in Familien mit einem mongoloiden Kind. Monatschrift für Kinderheilkunde, 121, 312-313.

Philip, J., Bang, J., Hahnemann, N., Mikkelsen, M., Niebuhr, E., Rebbe, H. and Weber, J. (1974) Chromosome analysis of fetuses in risk pregnancies. Acta Obstetrica et Gynecologica Scandinavica, 53, 9-14.

Scrimgeour, J.B. (1974) Fetoscopy. In: Motulsky, A.G. and Lenz, W. (editors) Birth Defects, Proc. 4th Intern. Conference, Excerpta Medica, Amsterdam, pp. 234-239.

Seller, M.J., Singer, J.D., Coltart, T.M. and Campbell, S. (1974) Maternal serum-alpha-fetoprotein levels and prenatal diagnosis of neural tube defects. Lancet, I, 428-429.

Shih, L., Hsu, L.Y.F., Sujansky, E. and Kushnick, T. (1974) Trisomy 18 mosaicism in two siblings. Clinical Genetics, 5, 420-427.

Stanbury, J.B., Wijngaarden, J.B. and Fredrickson, D.S. (1972) The metabolic basis of inherited disease, 3rd edition, Mc Graw Hill Book Company, New York, p. 4.

Steele, M.W. and Breg Jr., W.R. (1966) Chromosome analysis of human amniotic-fluid cells. Lancet, I, 383-385.

Trimble, B.K. and Doughty, J.H. (1974) The amount of hereditary disease in human populations. Annals of Human Genetics (London), 38, 199-223.

Valenti, C. (1973) Antenatal detection of hemoglobinopathies. American Journal of Obstetrics and Gynecology, 115, 851-853.

Wahlström, J., Bartsch, F.K. and Lundberg, J. (1974) Prenatal chromosome determination. A study of 219 cases. Clinical Genetics, 6, 184-191.

Wald, N.J., Brock, D.J.H. and Bonnar, J. (1974) Prenatal diagnosis of spina bifida and anencephaly by maternal serum-alpha-fetoprotein measurement. A controlled study. Lancet, I, 765-767 (1974).

Warkany, J. (1971) Congenital malformations. Year Book Medical Publishers, Chicago, pp. 38-43.

Wheeless Jr., C.R. (1974) Use of the laparoamnioscope for fetal observation and its effect on the human fetus. American Journal of Obstetrics and Gynecology, 119, 844-848.

SUMMARY

Prenatal diagnosis of a number of congenital diseases is possible by amniocentesis in the 14th - 16th week of pregnancy and subsequent analysis of cultured amniotic fluid cells or amniotic fluid supernatant. Parents at risk for a child with a chromosomal disorder, an X-linked disease, a neural tube defect or a metabolic disease may use this new tool in genetic counseling to limit their further offspring to unaffected children by requesting termination of a pregnancy when a fetal abnormality has been detected.
The present thesis describes methodological studies in the field of prenatal diagnosis of metabolic diseases and clinical applications of prenatal monitoring in a series of 350 pregnancies with an elevated risk for a chromosomal aberration, an X-linked disease, a metabolic genetic disorder or a neural tube defect.
One prerequisite for the prenatal diagnosis of a genetic metabolic disease is, that the enzymatic defect or metabolite specific for this disorder is detectable in cultured cells from an affected individual, since cultured (amniotic fluid) cells will be used in prenatal monitoring. This should be demonstrated for each separate disease. An example of such a study is presented in appendix paper I. The data indicate, that the enzymatic defect in the haemolytic anaemia caused by pyruvate kinase deficiency (an autosomal recessive disease) is not detectable in fibroblasts and cultured amniotic fluid cells, since these only produce the M-type of this enzyme, whereas the anemia is caused by a deficient activity of the L-type.
The availability of control amniotic fluid cell samples is important whenever a prenatal diagnosis of a metabolic disorder in cultured amniotic fluid cells is to be made: enzymatic data from the case at risk must be compared with those from amniotic fluid cells grown under identical conditions and for the same period of time. A method enabling the storage of control amniotic fluid cells with maintenance of cell viability is described in appendix paper II. Concurrently, a delay

of several days between amniocentesis and starting the cell culture of amniotic fluid samples showed to be not deleterious to the subsequent culturing of the cells. This makes prenatal diagnosis available to parents living at long distances from centers, where the analysis is carried out.
The detection of biochemical defects (enzyme activities or metabolites) in cultured amniotic fluid cells required relatively large numbers of cells (millions) in the earlier years of prenatal diagnosis and this prolonged the period between amniocentesis and completion of the diagnosis to 4 - 8 weeks in most instances. The application of microtechniques enables a significant reduction in the number of cells required for the prenatal diagnosis of a number of enzymatic defects. Appendix paper III describes the development of such techniques for the assay of acid α-1,4-glucosidase, the enzyme deficient in Pompe's disease. The use of homogenates of cultured cells enables diagnostic assays on ± 10.000 cells with maltose or p. nitrophenyl-α-D-glucopyranoside as the substrate; with 4 methylumbelliferyl-α-D-glucopyranoside this number could be reduced to ± 1000 cells. The use of groups of freeze dried cultured cells isolated from a culture grown on a thin transparant plastic film permits the analysis of 100 - 200 cells. These methods allow completion of a prenatal diagnosis in ± 2 weeks after amniocentesis.
Many factors, including genetic heterogeneity, may affect the level of enzymatic activities in cultured fibroblasts and amniotic fluid cells. Several of these were studied in appendix paper IV. A marked effect was found of the period of time in culture on lysosomal enzymatic activities in fibroblasts. In analyses on single or small groups of cultured cells variations were found which might be caused by local differences in cell proliferation of the culture or differences in the phase in the cell cycle of the cells used for analysis. β-Galactosidase assays in individual binuclear hybrid cells were carried out to study the genetic background of different clinical variants of GM_1-gangliosidosis.

The application of microchemical analyses in pregnancies at risk for Pompe's disease and Fabry's disease is presented in appendix papers V and VI. Prenatal monitoring for Pompe's disease was completed in 12 - 22 days in 6 cases. Differences were found in acid α-1,4-glucosidase activities between primary amniotic fluid cell cultures and later subcultures. Fabry's disease was excluded in a pregnancy at risk 11 days after amniocentesis by microchemical analysis of α-galactosidase activity in groups of 100 - 200 freeze dried cells. Analyses on homogenates of larger numbers of cultured cells, studied at a later moment after amniocentesis, confirmed these results.

These techniques allow a prenatal diagnosis of a number of lysosomal enzyme deficiencies in a period of about two weeks after amniocentesis, which is comparable to the time required for a prenatal chromosome analysis.

A summary of results on prenatal diagnosis of a number of lysosomal storage diseases in appendix paper VII indicates that monitoring for metachromatic leukodystrophy could be completed in 16 - 18 days and for some of the mucopolysaccharidoses (studied by metabolism of radioactive labelled sulphate in cultured cells) within 4 weeks after amniocentesis.

The results of prenatal monitoring in 350 pregnancies for a variety of genetic disorders are described in appendix paper VIII. Amniocentesis carries no appreciable risk to the mother and the risk to the fetus for a spontaneous abortion seems not to be elevated over the natural risk in this period of pregnancy. Prenatal diagnosis of chromosomal aberrations may be considered as a routine and the use of banding techniques is essential for a reliable analysis. This applies to mothers at advanced maternal age (38 years and older) who have an increased risk for non-disjunction. In a group of 87 pregnancies studied 4 non-disjunctions were detected.

The recurrence risk of trisomy 21 (Down's syndrome) may be estimated as 1%. In 101 pregnancies tested for this indication one trisomy 21 was found.

In 43 pregnancies at risk for an X-linked disease (haemophilia, Duchenne's muscular dystrophy, etc.) prenatal chromosome analysis was used to limit the

families of female carriers to unaffected daughters.
In 34 pregnancies prenatal monitoring was carried out for 11 different metabolic diseases; 3 abnormal fetuses were detected.
The detection of neural tube defects (anencephaly and meningomyelocèle) by elevated levels of alpha-fetoprotein in the amniotic fluid is a recent extension of the indications for prenatal diagnosis and it is the first congenital malformation of multifactorial inheritance that can be detected prenatally. This test was introduced in the present study in 1974 and besides pregnancies with a recurrence risk for a neural tube defect all amniotic fluids studied for other indications were tested as well. In 47 pregnancies at risk for an open defect of the neural tube three anencephalic fetuses were detected in the second half of pregnancy.
The results of prenatal diagnosis were the basis for selective termination in 36 of the 350 pregnancies tested. The value of this diagnostic approach was equally important for those parents, who obtained the reassurance that the genetic disorder tested for had been excluded.

SAMENVATTING

Prenatale diagnostiek van een aantal aangeboren ziekten is mogelijk door amniocentese (vruchtwaterpunctie) in de 14e - 16e week van de zwangerschap, gevolgd door onderzoek van gekweekte vruchtwatercellen of van het vruchtwater zelf. Het onderzoek stelt ouders met een verhoogd risico op een kind met een chromosoomafwijking, een sluitingsdefect van de neuraalbuis of een erfelijke stofwisselingsziekte in staat eigen kinderen te krijgen, die niet aan de betrokken afwijking lijden: wordt bij prenatale diagnostiek een afwijking vastgesteld, of een mannelijke foetus gevonden bij risico op een geslachtsgebonden erfelijke ziekte, dan kunnen zij om beëindiging van de zwangerschap vragen.
Dit proefschrift beschrijft een aantal methodologische onderzoeken op het gebied van de prenatale diagnostiek van erfelijke stofwisselingsziekten en de klinische toepassing van prenatale diagnostiek in 350 zwangerschappen, waarin een verhoogd risico bestond op een kind met een chromosoomafwijking, een geslachtsgebonden ziekte, een erfelijke stofwisselingsstoornis of een sluitingsdefect van de neuraalbuis (anencephalie of spina bifida aperta).
Er zijn thans ± 50 ernstige erfelijke stofwisselingsziekten, die prenataal zijn vast te stellen. Een voorwaarde daartoe is, dat het voor die ziekte specifieke enzymdefect of stapelingsproduct aangetoond kan worden in gekweekte cellen van een patiënt met de betrokken ziekte: bij prenatale diagnostiek zullen gekweekte (vruchtwater-) cellen worden gebruikt als bron van informatie over de foetus.
In artikel 1 van de appendix wordt aangetoond, dat deze voorwaarde niet vervuld kan worden bij de haemolytische anaemie ten gevolge van pyruvaat kinase deficiëntie, een autosomaal recessief erfelijke ziekte. De anaemie wordt veroorzaakt door een deficiëntie van het L-type van het enzym, terwijl in fibroblasten en in gekweekte vruchtwatercellen alleen het M-type van dit enzym wordt gemaakt.
Voor prenatale diagnostiek van erfelijke stofwisselingsstoornissen is steeds no-

dig een gelijktijdige analyse van vruchtwatercellen van de betrokken zwangerschappen èn cellen van normale zwangerschappen (verkregen bij abortus). Daar deze laatste slechts incidenteel beschikbaar zijn, werd een methode ontwikkeld (artikel II van de appendix) om vruchtwatercellen ongekweekt in te vriezen met behoud van levensvatbaarheid voor een latere celkweek. Tevens werd gevonden, dat transport van vruchtwatermonsters gedurende enkele dagen na amniocentese geen nadelige gevolgen behoeft te hebben voor de kweek van de cellen. Dit maakt prenatale diagnostiek bereikbaar voor ouders, die op grotere afstand wonen van centra, waar deze diagnostiek wordt uitgevoerd.
Bij de prenatale diagnostiek van erfelijke stofwisselingsziekten waren oorspronkelijk enkele miljoenen gekweekte vruchtwatercellen nodig voor de biochemische analyse.
Voor het verkrijgen van deze hoeveelheid cellen waren kweektijden nodig van 6 - 8 weken. Dergelijke lange wachttijden hebben niet alleen psychologische nadelen, maar maken het ook onmogelijk een eventuele zwangerschapsonderbreking zo vroeg mogelijk uit te voeren.
Een verkorting van deze wachttijd kon worden verkregen door gebruik te maken van gevoeliger meetmethodieken, die onderzoek van kleinere aantallen vruchtwatercellen mogelijk maken. De methodologie van de microchemische analyse van de activiteit van het enzym zure α-1,4-glucosidase, dat deficiënt is bij de ziekte van Pompe (glycogenose type II), wordt beschreven in artikel III van de appendix. Met 4-Methylumbelliferylglucopyranoside als substraat is analyse mogelijk van een homogenaat van ± 1000 gekweekte cellen. Een verdere reductie van dit celaantal is mogelijk door analyse van groepen van 100 - 200 gevriesdroogde cellen, die onder microscopische controle geïsoleerd zijn uit een celkweek die groeide op een dunne transparante plastic film. Met deze methoden is een prenatale diagnose van de ziekte van Pompe mogelijk in ongeveer 2 weken na amniocenteses.
Vele factoren kunnen van invloed zijn op de enzymactiviteiten in gekweekte

cellen. In artikel IV van de appendix werd een aantal daarvan onderzocht. Er. blijkt een duidelijke relatie te bestaan tussen de kweekduur en de enzymactiviteit van lysosomale enzymen. Bij analyse van kleine groepen cellen werden variaties gevonden, die te verklaren zijn uit locale verschillen in celproliferatie in een kweek of uit verschillen in fase in de celcyclus van de onderzochte cellen. De genetische achtergrond van klinisch verschillende typen van GM_1-gangliosidose werd bestudeerd door celfusie, gevolgd door analyse van de β-galactosidase activiteit in afzonderlijke binucleaire hybride cellen.

De toepassing van microchemische analysemethoden in prenatale diagnostiek van de ziekten van Pompe en Fabry wordt beschreven in artikel V en VI van de appendix. Bij 6 prenatale diagnosen voor de ziekte van Pompe werd het resultaat bekend in 12 - 22 dagen na amniocentese; de ziekte van Fabry kon worden uitgesloten 11 dagen na amniocentese door microchemische analyse van α-galactosidase activiteit in kleine groepen gevriesdroogde vruchtwatercellen. Deze resultaten werden bevestigd door onderzoek van homogenaten van gekweekte cellen op een later tijdstip.

Een samenvatting van prenatale diagnostiek voor een aantal lysosomale stapelingsziekten is beschreven in artikel VII van de appendix. Prenataal onderzoek voor metachromatische leukodystrofie (arylsulfatase A deficiëntie) werd voltooid in 16-18 dagen en voor de mucopolysaccharidose type II (ziekte van Hunter) (onderzocht door meting van metabolisme van radioactief gemerkt sulfaat in vruchtwatercellen) binnen 4 weken na amniocentese. De toepassing van microchemische analysemethoden heeft bijgedragen om de lange wachttijden bij prenatale diagnostiek van erfelijke stofwisselingsziekten te verkorten.

Artikel VIII van de appendix beschrijft de toepassing van prenatale diagnostiek in 350 zwangerschappen, waarin een verhoogd risico bestond op een in vruchtwater of vruchtwatercellen aantoonbare afwijking. Het risico van amniocentese voor de moeder is verwaarloosbaar klein, voor de foetus lijkt het risico op een spontane

abortus na amniocentese niet duidelijk verhoogd t.o.v. het normale risico op een miskraam in de betreffende zwangerschapsperiode.
Prenataal chromosomenonderzoek is een routine geworden voor zwangeren van 38 jaar en ouder, die een verhoogd risico hebben op een kind met een chromosoomafwijking (1,5 -3%); in 87 van de onderzochte gevallen werd 4 maal een chromosoomafwijking vastgesteld. Hetzelfde geldt voor moeders, die reeds een kind met een trisomie 21 (Down's syndroom) kregen. Het herhalingsrisico van deze afwijking bedraagt 1%. In 101 onderzochte zwangerschappen werd éénmaal een foetus met een trisomie 21 gevonden.
In 43 zwangerschappen bestond een risico op een geslachtsgebonden erfelijke ziekte bij een mannelijke foetus (spierdystrofie van Duchenne, haemofilie, etc.). Via prenataal chromosomenonderzoek konden deze draagster-moeders hun gezin beperken tot meisjes, die niet aan de betreffende ziekte zullen lijden. In 34 gevallen werd prenataal onderzoek gedaan naar één van 11 verschillende stofwisselingsziekten; drie maal werd een afwijkende foetus gevonden.
Sluitingsdefecten van de neuraalbuis (anencephalie en spina bifida aperta) zijn de eerste aangeboren afwijkingen met multifactoriële erfelijkheid, die prenataal zijn aan te tonen door een verhoging van het gehalte van het foetale eiwit α-foetoproteine in het vruchtwater. Deze test werd in 1974 ingevoerd in dit onderzoek en sedertdien wordt het onderzoek gedaan niet alleen in zwangerschappen met een herhalingsrisico voor een defect van de neuraalbuis, maar ook in alle andere vruchtwatermonsters van zwangeren, die om andere indicaties worden onderzocht. In 47 gevallen, waarin een verhoogd risico bestond, werd 3 maal in de tweede helft van de zwangerschap een sterk verhoogd α-foetoprotein gehalte gevonden. In deze laatste gevallen werd bij ultrasonografisch onderzoek een foetus met anencephalie aangetoond.
Op grond van de resultaten van prenatale diagnostiek werd in 36 gevallen door de ouders beëindiging van de zwangerschap gevraagd. De waarde van deze diagnostische mogelijkheid was even duidelijk voor de overige ouders, die de zekerheid kregen, dat in de onderzochte zwangerschap het verhoogde risico was uitgesloten.

NAWOORD

Curriculum vitae: eindexamen gymnasium 1958. Studie geneeskunde te Leiden (doctoraal examen 1967; semi-arts examen 1969) en te Rotterdam (arts-examen 1972). Van 1960 - 1965 student-assistent op de afdeling Sociale Hygiëne van het Nederlands Instituut voor Praeventieve Geneeskunde te Leiden. Vanaf mei 1968 verbonden aan de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam, waar eind 1969 begonnen werd met het in dit proefschrift beschreven onderzoek.

De prenatale diagnostiek kon in Rotterdam tot stand komen door de nauwe samenwerking tussen de afdelingen Celbiologie en Genetica, Verloskunde-Gynaecologie, Biochemie I, Kindergeneeskunde en Neurologie. Deze samenwerking en die met vele andere collega's in Nederland en daarbuiten is essentieel om prenatale diagnostiek in het bereik te brengen van die zwangeren, bij wie daartoe een indicatie bestaat.

Prof. Dr. H. Galjaard heeft zich als promotor ten zeerste ingezet voor de introductie van prenatale diagnostiek in Nederland. Ik ben hem erkentelijk voor zijn grote betrokkenheid bij dit onderzoek en zijn aandacht bij de totstandkoming van de publicaties.
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In de dagelijkse samenwerking met mevr. M. Jahodova, gynaecologe, mevr. Dr. E.S. Sachs en Dr. W.J. Kleijer ontstond een hecht team, waarin de

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Daarnaast is dit onderzoek verbonden met het enthousiasme van de mede-auteurs, werkzaam op verschillende afdelingen, waarvoor ik hen dankbaar ben.
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PAPER I

Pyruvate kinase in cultivated amniotic fluid cells

After glucose-6-phosphate dehydrogenase deficiency L-type pyruvate kinase deficiency is the most common erythrocyte metabolic error. Although the clinical pattern is reported to be quite variable, a rather high rate of mortality in early life has been found[1]. Recently, pyruvate kinase was purified from human erythrocytes[2] and it was found that the enzyme exhibits allosteric properties. These properties were compared with the partially purified enzyme from pyruvate kinase-deficient patients.

In three of four unrelated patients the mutant enzyme did not show allosteric properties, while in all patients an increased thermolability was found[3].

The properties of red blood cell pyruvate kinase is very much like the (liver) L-type pyruvate kinase. Besides the L-type the liver also contains the (muscle) M-type pyruvate kinase which in deficient patients remains unchanged. It has been shown that in pyruvate kinase deficiency there is also a decrease of total liver pyruvate kinase activity[4]. However, to perform antenatal diagnosis of this deficiency it is necessary to establish which type (L- or M-type) of pyruvate kinase is present in human cultured fibroblasts and amniotic fluid cells.

Pyruvate kinase activity was measured according to the method of Bücher and Pfleiderer[5]. Starch gel electrophoresis was performed with the method of Bigley *et al.*[6], the pyruvate kinase activity was detected with the fluorescent technique[7].

Fig. 1 shows the saturation curve for phosphoenolpyruvate (PEP) of fibroblast pyruvate kinase with [ADP] = 2 mM. The curve is hyperbolic and fructose-1,6-diphosphate (Fru-1,6-P_2) does not stimulate the enzymatic reaction. The same result was obtained with cultivated amniotic fluid cells. It is known that the L-type pyruvate kinase of liver and erythrocyte exhibits an S-shaped saturation curve, while the M-type does not. Furthermore, Fru-1,6-P_2 stimulates the L-type and transforms the sigmoidal curve into a hyperbolic one. The enzymatic activity of the M-type is not influenced by Fru-1,6-P_2.

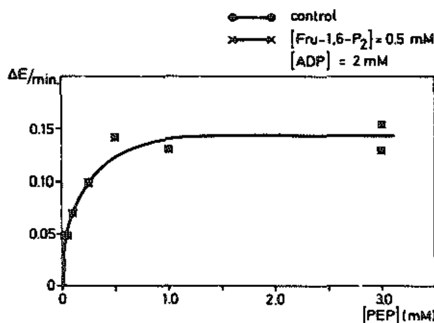

Fig. 1. PEP saturation curve for fibroblast pyruvate kinase in the absence and presence of Fru-1,6-P_2. Buffer 0.1 M Tris–HCl (pH 7.6), [ADP] = 2 mM, reaction medium contains 0.08 mg protein. ●–●, control; x–x, in the presence of Fru-1,6-P_2 (0.5 mM).

Table I summarizes the apparent K_m values for PEP at fixed ADP concentration. It shows that the apparent K_m values obtained for pyruvate kinase in fibroblasts

and cultivated amniotic fluid cells are in the same order as those for the M-type from various origins.

TABLE I

THE APPARENT K_m VALUES FOR THE SUBSTRATE PHOSPHOENOLPYRUVATE AT [ADP] = 2 mM FOR RAT LIVER (L- AND M-TYPE), HUMAN ERYTHROCYTE, HUMAN FIBROBLAST, HUMAN AMNIOTIC FLUID CELLS, AND HUMAN LEUCOCYTES PYRUVATE KINASE

	Type	K_m *(mM)*
Liver (rat)*	L	0.80
	M	0.07[5]
Erythrocyte (human)	L	0.45
Muscle (human)	M	0.02
Leucocytes (human)	M	0.05
Cultured amniotic fluid cells (human)	M	0.10
Cultured fibroblasts (human)	M	0.10

* Obtained from Tanaka *et al.*, *J. Biochem. (Tokyo)*, 62 (1967) 71.

Furthermore, electrophoresis showed that the mobility of fibroblast pyruvate kinase is the same as that of the M-type, but quite different from the erythrocyte L-type pyruvate kinase.

From these data, it can be concluded that cultivated fibroblasts and amniotic fluid cells contain the M-type pyruvate kinase. As the diagnosis pyruvate kinase deficiency is based on a decreased activity of the L-type, fibroblasts nor amniotic fluid cell cultures can be used in diagnosis of this disease. Therefore, antenatal diagnosis of this disease by using cultured amniotic fluid cells is impossible.

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1 H. S. Bowman and F. Procopio, *Ann. Internal Med.*, 58 (1963) 567.
2 G. E. J. Staal, J. F. Koster, H. Kamp, L. van Milligen-Boersma and C. Veeger, *Biochim. Biophys. Acta*, 227 (1971) 86.
3 G. E. J. Staal, J. F. Koster and L. van Milligen-Boersma, *Biochim. Biophys. Acta*, 260 (1970) 613.
4 R. H. Bigley and R. D. Koler, *Ann. Human Genet.*, 31 (1968) 383.
5 T. Bücher and G. Pfleiderer, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. I, Academic Press, New York, 1965, p. 435.
6 R. H. Bigley, P. Stenzel, R. T. Jones, J. O. Campos and R. D. Koler, *Enzymol. Biol. Clin.*, 9 (1968) 10.
7 R. von Fellenberg, R. Richterich and H. Oebi, *Enzymol. Biol. Clin.*, 3 (1963) 240.

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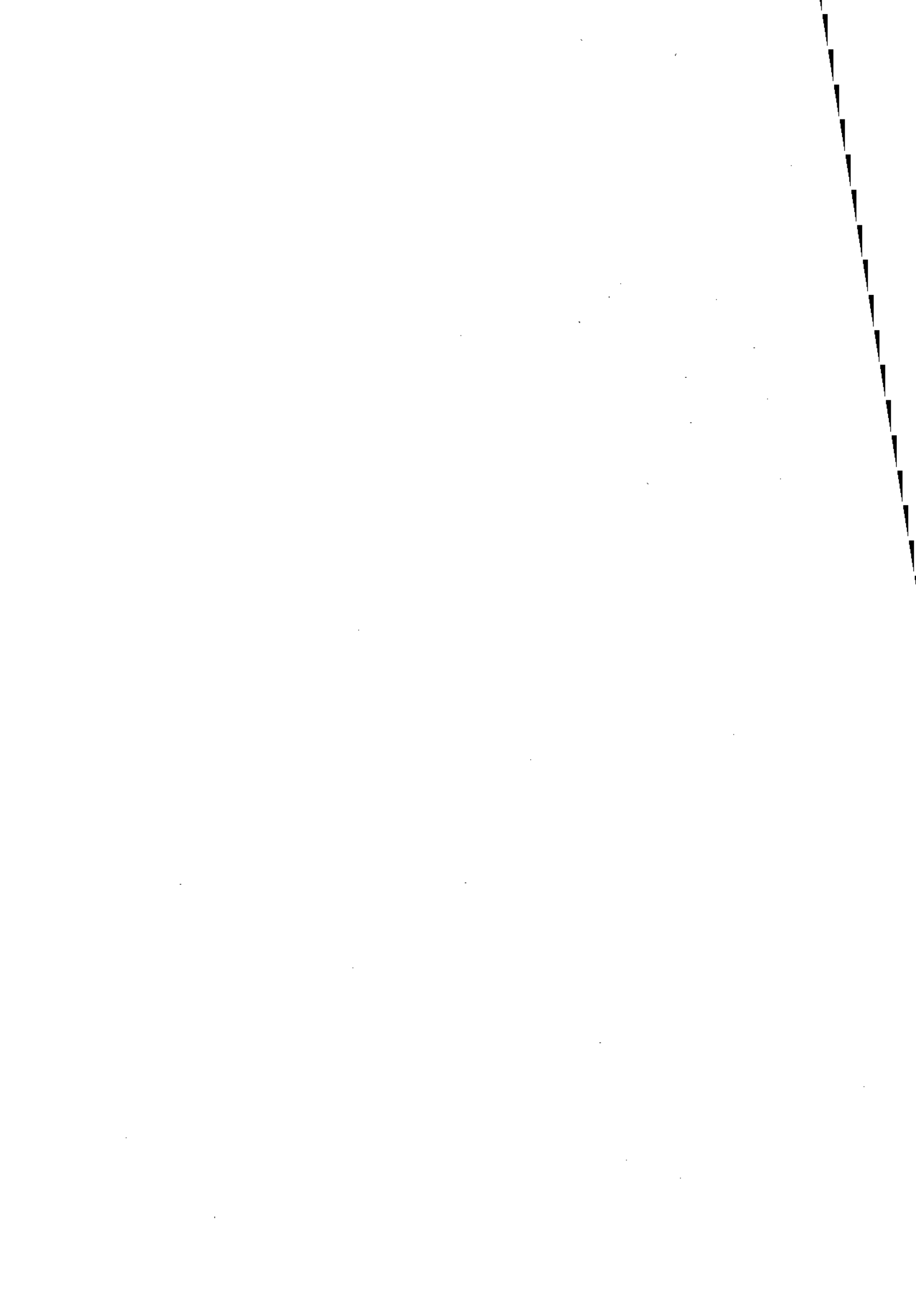

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Transport and Storage of Amniotic Fluid Samples for Prenatal Diagnosis of Metabolic Diseases

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Summary. 1. A method for freezing uncultured amniotic fluid cells is presented, which allows their use as pregnancy-age matched controls in prenatal diagnosis of metabolic diseases. 2. Amniotic fluid cells were successfully cultured after up to 7 days in transport, which makes prenatal diagnosis available to parents living a long way from specialized centers.

Zusammenfassung. 1. Eine Einfriermethode für nichtkultivierte Fruchtwasserzellen wird beschrieben, wodurch man diese wie Kontrollmuster anwenden kann bei pränataler Diagnostik von Stoffwechselkrankheiten. 2. Fruchtwasserzellen wurden erfolgreich kultiviert nach langzeitigem Transport (7 Tage); dieser Befund bringt eine pränatale Diagnostik im Bereich entfernt von spezialisierten Zentren lebender Eltern.

An increasing number of inborn errors of metabolism can be detected in early pregnancy (Milunsky *et al.*, 1970; Milunsky and Littlefield, 1972). Until now metabolic defects have been determined by biochemical analysis of cell homogenates obtained from large numbers (millions) of cultured amniotic-fluid cells. As a consequence the period of time required for prenatal diagnosis in most instances has been very long (4—10 weeks) (Brady *et al.*, 1971; Fratantoni *et al.*, 1969; Epstein *et al.*, 1972; Cox *et al.*, 1970).

This period can be substantially reduced for a number of metabolic diseases by using microchemical techniques allowing the analysis of enzyme activities in clones of a few hundred cells (Galjaard *et al.*, 1972). Knowledge of the variation in enzyme activity among heterozygotes for the disease concerned and also a proper selection of control material is of great importance for a reliable prenatal diagnosis, as there is increasing evidence that the "biochemical behavior" of cultured cells can be influenced by the duration of cell culture (Robbins *et al.*, 1970), the phase in the growth curve (DeMars, 1964) and the conditions of cell culture (Ceccarini and Eagle, 1971; Lie *et al.*, 1973). It therefore seems preferable to compare analytical data from the pregnancy at risk with those from control amniotic fluid samples at the same stage of pregnancy and grown in identical

conditions at the same time. This can only be realized when control amniotic fluid is available at any moment when a pregnancy at risk is to be monitored, which will rarely occur. In this communication the transport of amniotic fluid samples is discussed and a method is presented for long-term storage of these samples with preservation of cell viability.

Samples of amniotic fluid are centrifuged for 5 min at $80 \times g$ and the cell pellets ($0.2 - 1.4 \times 10^6$ cells) are resuspended in 1 ml cell culture medium (Ham's F 10) supplemented with 15% fetal calf serum, 4% dimethyl sulfoxyde (as cryoprotective agent) and antibiotics. Cell suspensions are transferred into ampoules which, after sealing, are frozen at a rate of 1 degC/min over liquid nitrogen vapor down to —70°C and subsequently stored under liquid nitrogen. To test the viability of the cells ampoules are taken after 7—22 days and rapidly thawed (under running tapwater). The cell suspensions are then washed by resuspending in 5 ml of serum-free medium, centrifuged for 5 min at $80 \times g$, and resuspended

Table 1. Amniotic fluid cell culture: Direct culture versus culture after freezing of uncultured cells[a]

Sample	Pregnancy (weeks)	Direct culture: First dividing cells (days) + type[b]	Frozen cells: First dividing cells (days) + type[b]
1	20	5	10
2	17	10 (E, F)	4 (F)
3	18	4	6
4	17	—	4
5	13	6	5
6	16	6 (E, I, F)	7 (E, I, F)
7[c]	18	4 (E, I, F)	6 (I)
8[c]	19	3 (E, I, F)	7 (E, F)

[a] Suspensions of uncultured amniotic fluid cells ($0.2 - 1.4 \times 10^6$ cells/ml).
[b] F = Fibroblast-like, E = Epitheloid, I = Intermediate.
[c] Samples in transport for 7 days.

Table 2. Amniotic fluid cell culture: Culture of samples after 5—7 days in transfer[a]

Sample	Pregnancy (weeks)	Days in transport	Cell count/ml	Primary growth (days)	Cell type[b]	Karyotyping (days)[c]
1	13	7	76500	6	F	20
2	14	5	26100	6	E/I	17
4	21	5	55600	4	F	6
5	16	5	36500	7		12
6	?	5	121000	3	E + F	6
7	18	5	69000	4	F	11
9	18	?	21000	7	E + I	10

[a] Transport by air mail in siliconised glass tubes at ambient temperature.
[b] E = Epitheloid, F = Fibroblast-like, I = Intermediate type.
[c] Interval (days between initiating culture and obtaining sufficient cells for chromosome analysis.

in F 10 supplemented with 25% fetal calf serum and antibiotics. Aliquots (0.75 ml) of cell suspensions are carefully pipetted onto 22 × 22 mm coverslips in 35 mm Falcon Petri dishes. After 12—24 hrs for cell attachment additional medium is added. A fully humidified incubator with 5% CO_2 in the gaseous phase is used.

The results of culturing of the stored samples were compared with those of aliquots from the same sample cultured immediately (Table 1). No difference was observed in the time interval for the appearance of dividing cells or the type of proliferating amniotic fluid cells. It may be concluded that samples of uncultured amniotic fluid cells treated according to the method described can serve as reliable controls in prenatal diagnosis of metabolic disorders.

Two of the samples (Nos. 7 and 8, Table 1) had even been in transport for 7 days without detrimental effects on *in-vitro* cell growth afterwards. This finding is also illustrated by results of cell cultures of amniotic fluid samples from various periods of gestation air-mailed from the USA to Europe during different transfer times (Table 2). First mitoses were observed after 3—7 days and sufficient cells for karyotyping were obtained after 6—20 days of *in-vitro* growth. These results indicate that a transport time of up to 7 days does not affect the possibility for prenatal karyotyping.

The main conclusions from these results are:

1. Storage of unprocessed amniotic fluid cell samples is possible with preservation of cell viability, thus allowing the use of optimal control material in the prenatal analysis of a pregnancy at risk for a metabolic disease.

2. Even a few days of transportation have no detrimental effect on *in-vitro* cell growth, offering the possibility of prenatal diagnosis for parents at risk for a genetic disease living a long way from specialized centers.

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References

Brady, R. O., Uhlendorf, B. W., Jacobson, C. B.: Fabry's disease: antenatal detection. Science **172**, 174—175 (1971)

Ceccarini, C., Eagle, H.: pH as a determinant of cellular growth and contact inhibition. Proc. nat. Acad. Sci. (Wash.) **68**, 229—233 (1971)

Cox, R. P., Douglas, G., Hutzler, J., Lynfield, J., Dancis, J.: In-utero detection of Pompe's disease. Lancet **1970 I**, 893

DeMars, R.: Some studies of enzymes in cultivated human cells. Nat. Cancer Inst. Monogr. **13**, 181—195 (1964)

Epstein, C. J., Schneider, E. L., Conte, F. A., Friedman, S.: Prenatal detection of genetic disorders. Amer. J. hum. Genet. **24**, 214—226 (1972)

Fratantoni, J. C., Neufeld, E. F., Uhlendorf, B. W., Jacobson, C. B.: Intrauterine diagnosis of the Hurler and Hunter syndromes. New Engl. J. Med. **280**, 686—688 (1969)

Galjaard, H., Fernandes, J., Jahodova, M., Koster, J. F., Niermeijer, M. F.: Prenatal diagnosis of genetic disease. Bull. Europ. Soc. Hum. Genet. Nov. 79—91 (1972)

Lie, S. O., Schofield, B. H., Taylor, H. A., Jr., Doty, S. B.: Structure and function of the lysosomes of human fibroblasts in culture: dependence on medium pH. Pediat. Res. **7**, 13—19 (1973)

Milunsky, A., Littlefield, J. W., Kanfer, J. N., Kolodny, E. H., Shih, V. E., Atkins, L.: Prenatal genetic diagnosis. New Engl. J. Med. **283**, 1370—1381, 1441—1447, 1498—1504 (1970)

Milunsky, A., Littlefield, J. W.: The prenatal diagnosis of inborn errors of metabolism. Ann. Rev. Med. **23**, 57—76 (1972)

Robbins, E., Levine, E. M., Eagle, H.: Morphologic changes accompanying senescence of cultured human diploid cells. J. Exptl. Med. **131**, 1211—1222 (1970)

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PAPER III

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A METHOD FOR RAPID PRENATAL DIAGNOSIS OF GLYCOGENOSIS II (POMPE'S DISEASE)

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SUMMARY

A method is described enabling prenatal diagnosis of glycogenosis II (Pompe's disease) within 7–10 days after amniocentesis in the 14th–16th week of pregnancy. The procedure is based on amniotic fluid cell cultivation on thin plastic foil, freezing and freeze-drying of the cells and subsequent microdissection of pieces of plastic foil each containing 70–250 lyophilized cells. These are incubated in microliter volumes of substrate. The extinction or fluorescence values are measured in microcuvettes adapted to a normal spectrofluorometer or in microcapillaries using a microscope spectrofluorometer design. The methods developed are compared with conventional biochemical analyses.

INTRODUCTION

Hers[1] delineated the basic defect in type II glycogenosis (Pompe's disease) as a deficient activity of the lysosomal acid α-1,4-glucosidase. In patients with Pompe's disease this enzyme deficiency has been demonstrated in various tissues and organs[2,3]. Nitowsky and Grunfeld[4] showed that the acid α-glucosidase deficiency is also expressed in leucocytes and in cultivated skin fibroblasts. This not only facilitated the diagnosis of patients and heterozygous carriers but also formed the basis for prenatal detection of this autosomal recessive disease. The first examples of prenatal diagnosis of Pompe's disease were based on a deficiency of α-glucosidase in amniotic fluid, in uncultivated and cultivated fluid cells[5,6]. Later, it became apparent that the use of amniotic fluid and uncultivated cells could result in erroneous interpretations (refs. 7, 8), because of the possibility of contamination with maternal components and because of the presence in amniotic fluid of an α-glucosidase with different properties than the lysosomal acid glucosidase which is deficient in Pompe's disease[7,9].

The necessity to use cultivated amniotic fluid cells for a reliable prenatal diagnosis implied a long waiting period. So far 4–6 weeks of *in vitro* cultivation (after amniocentesis in the 14th–18th week of pregnancy) was necessary to obtain sufficient cell material for α-glucosidase analysis[5,6,10]. Such long waiting periods not only mean

a psychological stress for the parents concerned but also in the case of an abnormal fetus interruption of a pregnancy at later stages may result in more obstetrical difficulties. The use of microchemical techniques[8,11–15] enables a reduction of the number of cultured cells required for analysis and preliminary data on the application of such microtechniques in the prenatal analysis, of a number of lysosomal enzymes in cultured amniotic fluid cells have been presented[8,16].

In this paper a micromethod is presented which allows prenatal diagnosis of Pompe's disease within 7–14 days after amniocentesis in the 14th–16th week of pregnancy. The method developed has been compared with other assay procedures and some problems encountered in the practical use of the various methods will be discussed. The application of this micromethod in the prenatal monitoring of several pregnancies at risk for Pompe's disease will be described separately[17].

MATERIAL AND METHODS

In vitro cultivation and preparation of cell material

Control amniotic fluid was obtained by transabdominal amniocentesis between the 14th–16th week of pregnancy after ultrasound localization of placenta and fetus. 5–15 ml amniotic fluid was centrifuged in siliconised glass tubes and the cell pellet was resuspended in Ham's F10 medium supplemented with 20% fetal calf serum. When microchemical studies were to be performed on dissected small clones of freeze-dried cells, *in vitro* cultivation was carried out on "mylar dishes" consisting of a 5 cm diameter glass ring and a bottom of thin plastic (Melinex polyester film type 0, I.C.I. Holland). For (micro)biochemical studies on cell homogenates cultivation was performed in 35 mm falcon dishes. Established fibroblast strains from control individuals, patients with Pompe's disease and heterozygous carriers were cultured under similar conditions, using Ham's F10 supplemented with 15% fetal calf serum.

Amniotic fluid cells were either directly used for cell cultivation and subsequent analysis or were cultivated after storage of the unprocessed cells, using a new method (ref. 18). Cell homogenates were prepared by trypsinization with 0.25% trypsin solution and washing of the cells which were then suspended in 0.001% bovine albumin. After counting the cell number in a hemocytometer, homogenization was carried out by sonic vibration (2×30 sec) or by repeated freezing and thawing. In most experiments a concentration of about 10^6 cells per ml was used (corresponding to a protein concentration of about 0.3 mg/ml). Analysis of cell homogenates was either performed directly or after storage of the cell sonicate at $-70°$. Depending on the type of experiments 1–10 μl aliquots of cell homogenate were used for α-1,4-glucosidase and protein analyses.

For direct assays on freeze-dried cells (Fig. 4), the "mylar" dishes containing groups of cultured amniotic fluid cells or fibroblasts were frozen in CO_2–acetone ($-70°$) or in isopentane–liquid N_2 ($-170°$) after removing the medium by three times rinsing in isotonic salt solution. The dishes were then transferred to a freeze-drying apparature (W.K.F., type 05, Wetzlar Instr.) while kept at low temperature. Lyophilization of the cultured cells was carried out *in vacuo* (10^{-2} mm Hg) at $-45°$ during about 15 hours and the vacuum was released after the dishes were allowed to warm to $20°$. Freeze-drying of the cells not only allows storage for long periods (at $-70°$) but it also enables isolation of cells without any loss of enzyme activity.

In a conditioned room (20° ± 0.5 and relative humidity ≤40%) pieces of plastic foil containing small numbers (tenths–hundreds) of freeze-dried cells were isolated using free hand microdissection under a stereomicroscope (zoomobjective, total magnification 60–120×). The number of cells on each dissected piece of plastic was counted using a raster specially designed for this purpose (Micropure, Arnhem, Holland) which is placed under the culture dish during microdissection. The dissected pieces of foil, containing a known number of cells (70–250) were used for further microchemical analyses.

(Micro)biochemical assays

Protein analysis. The method described by Lowry *et al.*[19] was adapted to a smaller final volume to decrease the amount of cell material required for analysis. To 5 μl of cell homogenate 50 μl copper sulphate is added and after 10 min 5 μl of Folin reagent is added. After 45 min in the dark the extinction at 750 nm is read in ultramicrocuvettes (volume of about 20 μl with an optical path of 10 mm) adapted to a Zeiss PMQ 2 spectrophotometer. Different concentrations of bovine albumin in distilled water served as standards.

α-1,4-glucosidase assays

(1) Maltose as a substrate. The procedure described by Nitowsky and Grunfeld[4] was adapted for use in small final volumes to reduce the number of cells required for analysis. 10 μl cell homogenate was incubated with 10 μl substrate (5 mg maltose per ml in 0.05 M acetate buffer pH 4.0) during 30 min, 1 and 2 hours at 37°. After heating 2 min at 100°, cooling in ice and 5 min centrifugation at 3000 rev./min 10 μl of the incubation mixture was added to 50 μl glucose oxidase reagent and incubated at 37° during 1 hour. The extinction was read at 440 nm in Zeiss ultramicrocuvettes. Concentrations up to 200 μg/ml glucose were used as standards.

(2) p-Nitrophenyl α-D-glucopyranoside was also used as a substrate using various final volumes depending on the amount of cell material to be analyzed. 5 μl cell homogenate has been incubated with 25 μl 4 mM *p*-nitrophenyl α-D-glucopyranoside in 0.1 M citrate buffer, pH 4.0. Incubation times varied from 0.5–6 hours at 37° and the reaction was stopped with 30 μl 0.2 M NaOH in ice. Extinction at 405 nm was read in different ways depending on the final volume. Down to 60 μl extinctions were recorded in microcuvettes adapted to a Zeiss spectrophotometer. Extinction measurements in final volumes of 1 μl were carried out in glass microcapillaries (Drummond microcaps of 1 mm diameter) using a Leitz microspectrophotometer type MPV obj. 2.5× Pl. and an E.M.I., type: 9558 QA photomultiplier with a Knott power supply.

(3) 4-Methylumbelliferyl α-D-glucopyranoside was used as a substrate in a concentration of 2.2 mM acetate buffer, pH 4.0. To obtain maximal sensitivity of the α-glucosidase analysis incubation was carried out in microliter volumes[8]. In most experiments incubation of cell homogenate (0.1–1 μl) or of dissected pieces of plastic foil containing a small number of lyophilized cultured cells was carried out in 0.3–2 μl of substrate during 1–2 hours at 37°. To prevent evaporation incubation in such small volumes was performed under paraffin oil using teflon rack with small wells (oil well technique) according to principles described by Matchinsky[20] and Lowry[12] (Fig. 4). Cell samples and microdroplets of reagents were introduced into the "oil well" under microscopic control using quartz constriction pipettes which were kindly made for us

by Dr. O. H. Lowry. After incubation the reaction volume was pipetted into 500 μl 0.5 M carbonate buffer (pH 10.7) and the fluorescence of methylumbelliferone was measured in a Perkin-Elmer fluorometer (extinction 365 nm, fluorescence 448 nm). The fluorescence of smaller volumes was measured in capillaries using the same microspectrofluorometer as described, but with a Ploem Opak illuminator[21].

All experiments on cell homogenates were performed in triplo and in the microassays on freeze-dried cells at least 10 pieces of plastic containing a varying number of cells (70–250) were dissected. In the latter experiments empty pieces of plastic dissected adjacent to the lyophillized cells were incubated as blanks. When low cell concentrations are incubated addition of bovine albumin (0.2 mg/ml) is required.

RESULTS

(1) Microchemical analyses on cell homogenates

(a) Maltose as substrate. When the analysis of α-1,4-glucosidase activity is carried out according to the original procedure of Nitowsky and Grunfeld[4] about 10^6 cultivated control fibroblasts are required to yield accurate extinction values. For a reliable diagnosis sufficient cell material must be available for triplo measurements, a protein assay and also the sensitivity of the assay should enable a clear distinction between low enzyme activities in cells from heterozygous carriers and deficient cells from patients with Pompe's disease. This means that at least 10^7 cultured amniotic fluid cells must be available for a prenatal diagnosis when conventional procedures are used.

In this assay, like in each spectrophotometric analysis, the amount of cell material required, decreases proportionally when the final volume of the assay is reduced. We have combined such a reduction of the incubation and measuring volume with a simplification of the procedure. 10 μl of several dilutions from a homogenate prepared from cultured amniotic fluid cells has been incubated for 2 hours at 37° in 10 μl maltose substrate. After 2 min heating to 100° 10 μl of the incubation mixture was pipetted into 50 μl glucose oxidase reagent and kept for 1 hour at 37°. The ex-

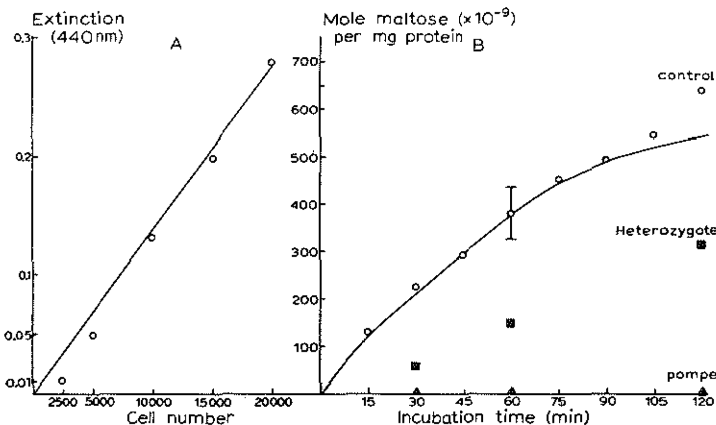

Fig. 1. α-1,4-Glucosidase activity in cultured amniotic fluid cells (A) and fibroblasts (B) using maltose as a substrate (60 μl final volume).

tinction at 440 nm was read in 20 μl microcuvettes fitting to a specially designed cuvet housing for a Zeiss spectrophotometer. Several steps of the original procedure (addition of water, centrifugation, addition of strong acid in the glucose assay step) could be omitted thus enabling more easy analysis in microvolumes.

The results illustrated in Fig. 1A show that under those conditions a minimum of about 10 000 normal amniotic fluid cells is required for one single measurement. Incubation of less cells results in unreliable extinction values. As mentioned earlier in prenatal diagnosis sufficient cell material must be available for triplo measurements and a Lowry protein assay which requires a few thousand cells even when performed with a 60 μl final volume. Furthermore low α-glucosidase activities as present in cells from heterozygotes should be detectable and results in Fig. 1B illustrate that this will require more than twice the amount of cell material compared to normal controls. The α-glucosidase activity in normal cultured fibroblasts is in the order of $6 \cdot 10^{-9}$ mole $\cdot$ min^{-1}mg^{-1} protein or 1–$2 \cdot 10^{-13}$ mole $\cdot$ h^{-1} per cell. As a result of these factors at least 70 000 cultured amniotic fluid cells must be available for prenatal diagnosis of Pompe's disease when maltose is used as a substrate even when the assay is carried out in the smallest final volume that can be measured with microcuvettes adapted to commercially available spectrophotometer.

Theoretically, further reduction in the number of cells required for analysis could be accomplished by combining longer incubation periods with a further decrease of the final volume using microscopic spectrophotometry to measure the extinction in submicroliter volumes. As is shown in Fig. 1B the enzyme reaction already shows some deviation from linearity during the first 2 hours of incubation and after longer incubation periods no reliable results were obtained. A further reduction in the incubation volume and final volume (down to 1 μl) implicate the necessity of incubation undei paraffin oil to prevent evaporation and of extinction measurements in microcapillaries using a microspectrophotometer. It was found that the various steps required in the α-glucosidase assay when maltose is used as a substrate, could not easily be combined with the manipulation of submicroliter volumes. For this purpose single step enzyme assays should be preferred.

(b) p-Nitrophenyl-α-D-glucopyranoside as substrate. The α-glucosidase assay using *p*-nitrophenyl-glucoside as a substrate has several advantages when the analysis

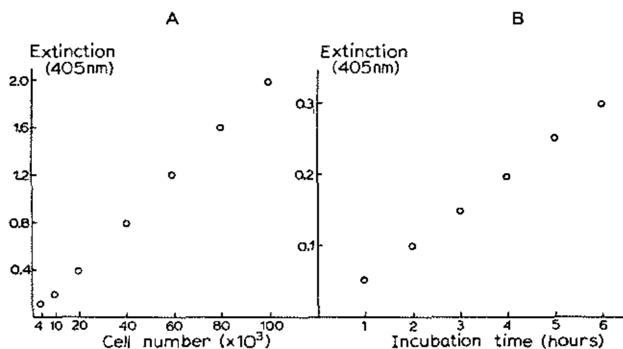

Fig. 2. α-1,4-Glucosidase activity in cultured fibroblasts using *p*-nitrophenyl-α-D-glucoside as a substrate (60 μl final volume).

is to be carried out on little cell material. Linearity of the enzyme reaction with this artificial substrate was observed even during very long incubation periods (Fig. 2B). Again for this spectrophotometric assay a reduction of the incubation and final volume will enable extinction measurements with less cell material. In Fig. 2A results are illustrated of incubations of decreasing numbers of cultured control fibroblasts using a 60 μl final volume and an incubation time of 6 hours. A minimum of about 4000 cells is required to yield extinction values of about 0.1. The activity in normal cultured fibroblasts was found to be about 3–4$\times$ less than with maltose as a substrate. In order to be able to perform triplo measurements, a protein assay and to detect low enzyme activities in cells from heterozygous carriers at least 30000 cultured amniotic fluid cells should be available for a prenatal diagnosis of Pompe's disease when *p*-nitrophenyl-glucoside is used as a substrate in a final volume of 60 μl.

Compared to the assay with maltose as a substrate the single step reaction with *p*-nitrophenyl-glucoside will more easily enable a further reduction of the final volume. When incubation is performed in microliter volumes the reaction should be performed under paraffin oil to prevent evaporation. The extinction measurements of the *p*-nitrophenol formed can be carried out with a microscope spectrophotometer design. To investigate the reliability of such measurements various dilutions of *p*-nitrophenol in 0.1 *N* NaOH were measured in a normal 3 ml cuvet (10 mm optical path) in a Zeiss spectrophotometer and the results were compared with those of 1 μl samples measured in microcapillaries using a Leitz microspectrophotometer. From each dilution 10 samples were measured and the results are shown in Table I. A remarkable agreement is observed over the whole range of extinction values. The fact that the 1 μl samples show ten fold lower extinction values is the result of the ten fold smaller optical path in the microcapillaries. The results also show that in microspectrophotometry much lower extinction values (about 10$\times$) can still be measured accurately compared with a normal spectrophotometer. The standard deviation of the measurements is quite small over the whole extinction range. These results indicate that the use of 1 μl final volume enables the analysis of 30–60 fold less cell material than is possible with the smallest microcuvettes adapted to normal spectrophotometers.

TABLE I

ACCURACY OF EXTINCTION MEASUREMENTS OF *p*-NITROPHENOL IN 1 μl VOLUMES USING MICROSPECTROPHOTOMETRY

Extinction in normal spectrophotometer (3 ml cuvet, 10 mm optical pathway)	*Mean extinction in microspectrophotometer* (1 μl capillary, 1 mm optical pathway)*	
	$\bar{X}$	*S.D.*
0.630	0.0621	0.0003 (0.5%)
0.535	0.0531	0.0004 (0.7%)
0.448	0.0446	0.0006 (1.2%)
0.350	0.0346	0.0005 (1.4%)
0.261	0.0255	0.0001 (0.5%)
0.074	0.0075	0.0004 (5%)
0.064	0.0064	0.0001 (2%)
0.044	0.0044	0.0001 (2%)
0.030	0.0030	0.0002 (7%)

* Measuring times up to 30 min did not influence the extinction values, indicating that no evaporation of the microdroplets covered with paraffin oil in the microcapillaries occurred.

For the prenatal diagnosis of Pompe's disease this means that a few hundred-thousand cultured cells would be sufficient for α-glucosidase analysis if a final volume of 1 μl is used. However, a protein determination in triplo microvolumes requires at least 3000 cells and hence a total of about 4000 amniotic fluid cells must be available.

(c) Methylumbelliferyl α-D-glucopyranoside as substrate. Incubations with this substrate have been carried out at 37° and a substrate concentration of 2.2 mM which is about the maximal solubility. The K_m for cultured fibroblasts was found to be 3.6 mM. Methylumbelliferyl-glucopyranoside yields a fluorescent product which can be measured with greater sensitivity than a coloured substance. The minimum amount of methylumbelliferone which could be measured in a 500 μl final volume under experimental conditions is about 10^{-12} mole. However, this does not necessarily mean that this amount of product can be accurately determined in an enzyme assay on cultured cells. Like in any other fluorometric reaction, the sensitivity which can be attained for a given final volume and fluorometer design mainly depends on the blank value. This latter can be reduced by decreasing the incubation volume as this results in less nonspecific fluorescent material as well as less fluorescence from spontaneously hydrolysed substrate. The influence of a decrease in incubation volume and final volume on the minimum amount of methylumbelliferone that can be detected has been investigated in the following way. Different amounts of methylumbelliferone were dissolved in varying volumes of 2.2 mM methylumbelliferyl-α-D-glucopyranoside and incubated during 1 hour at 37°. Subsequently the samples were diluted with 0.5 M carbonate buffer to different final volumes. In 5 ml and 500 μl volumes the fluorescence at 448 nm was measured with a conventional fluorometer and in volumes of 50,

TABLE II

INFLUENCE OF INCUBATION VOLUME AND FINAL VOLUME ON THE SENSITIVITY OF THE FLUOROMETRIC ASSAY OF METHYLUMBELLIFERYL SUBSTRATES

	Amount of methylumbelliferone in 2.2 M M.U.-glucoside	*Incubation volume μl*	*Final volume μl*	*Blank % transm.*	*Measurement % transm.*
Conventional fluorometry	750 10^{-13} mol.	30	5000	15	37
	750 10^{-13} mol.	30	500	117	353
	75 10^{-13} mol.	30	500	117	42
	75 10^{-13} mol.	3	500	15	37
	7.5 10^{-13} mol.	0.3	500	3	4
Microscope fluorometry	7.5 10^{-13} mol.	0.3	50	21	42
	2 10^{-13} mol.	0.3	10	83	26
	2 10^{-13} mol.	0.05	5	33	56

10 and 5 μl by microfluorometry. The results in Table II indicate that for measurements with a conventional fluorometer the incubation volume must be reduced to 0.3–3 μl in order to be able to detect 10^{-11}–10^{-12} mole of methylumbelliferone. Even then the difference between the measurement and the blank value is very small. Greater sensitivity can be obtained by also reducing the final volume and thus increasing the concentration of the fluorescent product. As shown in Table II the ratio between measurement and blank value improves when the reduction in final volume is combined with a further decrease in incubation volume. By combining incubation in 0.05 μl of substrate under paraffin oil and measurement of the fluorescent

product in a 5 μl final volume using microfluorometry less than 10^{-13} mole methylumbelliferone can be detected.

As the α-glucosidase activity in normal cultured fibroblasts is in the order of 10^{-14} mole·hour^{-1} per cell it can be predicted that only a few tenths–hundreds cultured cells are sufficient for a reliable assay provided that the incubation volume is kept small. In Fig. 3 the relation between the amount of methylumbelliferone formed, and the number of cultivated amniotic fluid cells is illustrated. In agreement with the expectation based on the model experiments from Table II the α-glucosidase activity could be detected in as little as 200–300 cells when 1 μl cell homogenate was incubated in 2 μl substrate. After incubation during 1 hour measurement of methylumbelliferone was performed after dilution to a final volume of 500 μl. The activity found for normal cultured amniotic fluid cells was about 10 fold less than with maltose as a substrate.

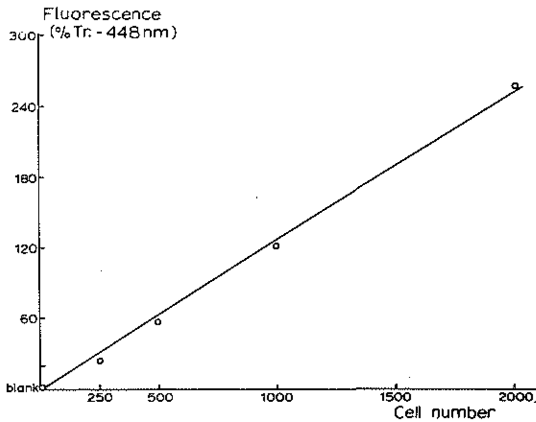

Fig. 3. α-1,4-Glucosidase activity in cultured amniotic fluid cells using methylumbelliferyl-α-D-glucoside as a substrate (3 μl inc. vol., 500 μl final volume).

Again, for a reliable prenatal diagnosis more cell material will be required to enable triplo measurements and to make a proper distinction between an enzyme deficiency and a decreased α-glucosidase activity as present in cells from heterozygous carriers. As a consequence, using methylumbelliferyl substrate about 1000 amniotic fluid cells are required for a prenatal analysis using microliter volumes for incubation and 500 μl final volume. For a parallel protein assay another 3000 cells must be available.

Further increase in the sensitivity of the methylumbelliferyl assay can be obtained by longer incubation periods as it was found that the reaction is linear up to over 8 hours. Another possibility is a further reduction of the incubation volume (down to 0.05 μl) and measurement of the fluorescence in small final volumes (about 10–50 μl) using microcapillaries and a microfluorometer design. According to the model experiments in Table II this procedure nearly allows α-glucosidase analysis down to the single cell level. However, in prenatal diagnosis based on studies of cell homogenates such extreme sensitivity of the enzyme assay is irrelevant, because of the necessity of a parallel protein determination which requires at least a few thousand cells. To avoid this disadvantage a method has been developed for a direct α-gluco-

sidase analysis on isolated groups of freeze-dried cells thus enabling the expression of the enzyme activity per cell instead of per unit weight protein.

(2) Microchemical analysis on microdissected clones of freeze-dried cells

(a) Preparation and microchemical analysis of normal freeze-dried cultured cells. For those enzyme assays with a sufficient sensitivity to allow the analysis of a few tenths–hundreds cells the enzymatic acitvity can in principle be expressed per single cell. This requires the isolation of a counted number of cultivated cells without any detrimental effect on the activity of the enzyme to be analyzed. Methods originally described by Lowry[11] for the isolation and microanalysis of tissue structures have been adapted for the investigation of small groups of cultured cells. The procedures followed are described in the paragraph MATERIAL AND METHODS and Fig. 4 illustrates the main steps.

In vitro cultivation of established amniotic fluid cell strains or fibroblasts used as controls in prenatal diagnosis should be done in such a way that the cell density is not too large to avoid erroneous cell counting. On the other hand the cell density should not be too small because the minimum number of cells (about 70–120 cells) required for a reliable α-glucosidase assay must be present on an area of about 0.2–1 mm^2 of plastic foil on which the cells have grown. A piece of plastic foil with larger dimensions does not fit into the incubation volume of 0.3–1 μl, which is necessary for a reliable fluorometric assay. In our hands seeding of about 100 000 cells in 4 ml per "mylar

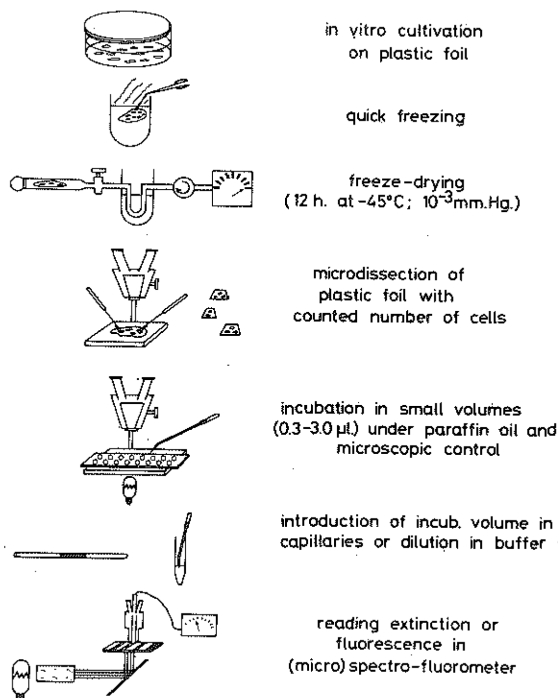

Fig. 4. Methods for microchemical analysis of small numbers of freeze-dried cells.

dish" and grown for 2–3 days usually gave the right cell density. In primary cultures of amniotic fluid cells as used in prenatal diagnosis the situation is more favorable as the first clones (after about 7–10 days cultivation) usual consist of closely packed epithelial like and/or fibroblast like cells, which are still sufficiently separated to allow accurate counting.

After freeze-drying the morphology of the cultured cells was found to be well preserved and under a stereomicroscope certain differences in structure, like different cell types within the same culture may well be detected. Free-hand microdissection of 10–15 groups of 100–200 cells can be carried out within an hour and during this period and even up to 2 days there are no changes in α-glucosidase activity in the lyophillized cultured cells.

To investigate if the lysosomal enzyme α-glucosidase is sufficiently liberated into solution and accessible for the substrate, when a piece of foil containing freeze-dried cells is directly incubated without previous homogenization, the following

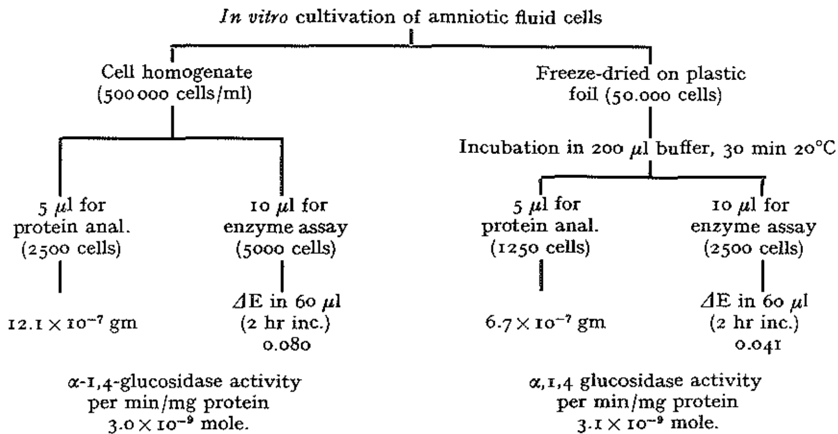

Fig. 5. Determination of α-1-4 glucosidase activity in small numbers of cultivated amniotic fluid cells.

experiment was designed (Fig. 5). Control amniotic fluid cells were cultivated on plastic foil (for this purpose a culture dish was used, designed by Dr. P. Hösli) and after freezing and freeze-drying a strip of foil containing a known number of cells was transferred into 200 μl acetate buffer. After 30 min at 20° a 10 μl aliquot of the buffer was used for an α-glucosidase assay (using maltose as a substrate) and a 5 μl sample for determination of the protein content. From the two values obtained the specific α-glucosidase activity in the buffer solution was found to be 3.1×10^{-9} moles/min/mg protein (Fig. 5). This value corresponds remarkably well with the α-glucosidase activity determined in a cell homogenate prepared from the same cell strain at the same time (3.0×10^{-9} moles/min/mg protein). These data indicate that the interaction between α-glucosidase and substrate after direct incubation of a piece of plastic foil containing freeze-dried cells is comparable to that in a conventional assay on cell homogenate.

TABLE III

α-GLUCOSIDASE ACTIVITY IN DISSECTED CLONES OF FREEZE-DRIED CULTURED FIBROBLASTS

Number of cells		*Fluorescence (% transm.-blank value*)*		*α-Glucosidase activity ($\times 10^{-14}$ mole M.U. h^{-1} per cell)*	
Control	*Pompe*	*Control*	*Pompe*	*Control*	*Pompe*
100	110	20	2	2.6	—
80	170	17	0	2.6	—
65	105	13	0	2.6	—
75	150	16	2	2.7	—
100	120	30	9	3.9	0.9
95	90	24	25	3.3	3.4
65	70	20	1	4.0	—
70	70	12	0	2.2	—
80	80	15	0	2.5	—
80	80	17	0	2.6	—
85	85	32	0	4.7	—
70	90	15	0	2.8	—
				$\bar{X}$ = 3.0	no detectable activity
				S.D. = 0.7	in >80% of the samples

* Fluorescence values of 10 empty pieces of plastic ranged from 2–5 with a mean of 3% transmission.

(b) α-Glucosidase activity in dissected freeze-dried cells and comparison with cell homogenates from controls, patients with Pompe's disease and heterozygote carriers. Fibroblasts from a normal control and a patient with Pompe's disease were cultivated on mylar dishes. The α-glucosidase activity was determined by microchemical analyses of 10–12 pieces of plastic foil containing a counted number (65–170) of cells using methylumbelliferyl-glucoside as a substrate. The results in Table III show that a clear distinction between these two categories of cells is possible using the micro-

TABLE IV

α-GLUCOSIDASE ACTIVITY IN CULTURED CELLS FROM CONTROLS, PATIENTS WITH POMPE'S DISEASE AND HETEROZYGOTES

	Dissected groups of freeze-dried cells from one cell strain (3 exp.) Mean activity $\times 10^{-14}$ mole·h^{-1} per cell*	*Cell homogenates from different cell strains***	
		Mean activity $\times 10^{-14}$ mole·h^{-1} per cell	*Activity range $\times 10^{-12}$ mole·min^{-1} per mg protein*
Control fibroblasts	4.0 ± 1.1 4.7 ± 2.0 3.0 ± 0.7	4.0	800–3400 (n = 5)
Control amniotic fluid cells	2.1 ± 0.9 2.8 ± 1.0 1.5 ± 0.6	2.0	390–1770 (n = 5)
Heterozygous fibroblasts	2.0 ± 1.6 1.0 ± 0.8 2.1 ± 1.8	2.2	414–2120 (n = 5)
Pompe fibroblasts	< 0.5 < 0.5 < 0.5	0.04	10– 50 (n = 4)

* From each cell strain 3 independent experiments were carried out, each consisting of α-glucosidase analysis in 8–18 pieces of plastic foil containing 70–260 cells each.
** The mean α-glucosidase activity has been determined from analyses in triplo on cell homogenates from 4–5 different individuals; the range of the values is presented in the right column.

techniques described. For the isolated groups of normal fibroblasts consistently higher fluorescence values than the blanks (empty pieces of plastic dissected adjacent to the cells) were observed and a mean activity of 3×10^{-14} mole·h^{-1} per cell was found. In 10 out of 12 groups of cells from the patient with Pompe's disease no α-glucosidase activity could be detected which means an activity of less than 0.5×10^{-14} mole·h^{-1} per cell.

In Table IV the α-glucosidase activity in dissected groups of freeze-dried cells is compared with that in cell homogenates from corresponding cell strains. From fibroblasts of controls, patients with Pompe's disease and heterozygote carriers and from control amniotic fluid cells one strain was used for microanalysis of freeze-dried cells in three independent experiments. The results were compared with analytical data from cell homogenates obtained from 4–5 individuals in each category. The results in the first column of Table IV show that there is quite a variation between the experiments within one cell strain and also between the α-glucosidase activity in different groups of cells. It is, however, possible to distinguish the enzyme activities in control fibroblasts and deficient cells whereas fibroblasts from a heterozygote give intermediate values comparable to those in normal amniotic fluid cells. Similar differences are observed in the studies on cell homogenates, shown in the last column of Table IV. For each category of cells, except those from patients with Pompe's disease there is a wide range in the enzyme activity between different individuals.

The analytical data obtained by microchemical analysis of small groups of freeze-dried cells correspond very well with those obtained on cell homogenates when the latter are expressed per cell. This latter value is calculated by supposing that 1 mg protein corresponds to $3 \cdot 10^{6}$ cells. This supposition is based on experiments where the cell number after trypsinization has been related to the protein content after homogenization and also on direct microinterferometric measurements of the dry mass of single cells[22]. The level of detection in the microchemical analysis of a few hundred freeze-dried cells under the experimental conditions of the experiment in Table IV only allows the conclusion that in cells from patients with Pompe's disease the α-glucosidase activity is less than $5 \cdot 10^{-15}$ mole·h^{-1} per cell. In studies on cell homogenates the exact value of the deficient activity was found to be in the order of $4 \cdot 10^{-16}$ mole·h^{-1} per cell.

The experimental data obtained indicate that the microchemical analysis of α-glucosidase activity in small groups of freeze-dried cultured cells gives reliable results compared with conventional analytical procedures. The method developed thus offers the possibility of rapid prenatal diagnosis of Pompe's disease because the small number of amniotic fluid cells now required for analysis will be available within 7–10 days after amniocentesis.

DISCUSSION

Methods for prenatal diagnosis of chromosomal aberrations and about 30 inborn errors of metabolism have been developed (see for reviews[23–25]). The application of these methods gains increasing interest of centers involved in the genetic counseling of families with a high genetic risk. It has been established that the most suitable period for transabdominal amniocentesis is between the 14th–16th week of pregnancy because at this stage sufficient amniotic fluid is available and the number of viable

fetal cells present in most cases allow a successful *in vitro* cultivation[24,26]. The prenatal diagnosis of metabolic disorders requires biochemical analysis of the specific enzyme defect or the detection of specific accumulated metabolites. Using conventional analytical techniques 10^6–10^8 cultured amniotic fluid cells corresponding to about 0.3–30 mg protein must be available. As a result the cultivation period and hence the waiting period for the parents involved has been 4–6 weeks for most prenatal diagnoses and in some instances, like in the analysis of cystinosis and mucopolysaccharidoses even up to 8–10 weeks[27,28]. Prenatal diagnosis of glycogenosis type II (Pompe's disease) required about 5 weeks[5,6]. Although parents with a high risk for an affected child are usually strongly motivated to have a prenatal diagnosis, in our opinion it is advantageous to accomplish the analysis before the mother has felt child movements, *i.e.* before the 18th week of pregnancy.

By adapting preparation procedures and microchemical analytical techniques which have mainly been developed for histochemical investigation on tissue sections (refs. 11, 12, 14, 20), a considerable reduction in the number of cultured amniotic fluid cells required for prenatal analysis can be obtained[8,15,16].

The use of freeze-dried cultured cells for direct microchemical analysis was found to give results comparable to those from conventional studies on cell homogenates (Fig. 5 and Table IV). As a consequence a protein determination can be avoided in the prenatal diagnosis of those enzyme defects which can be analyzed in a few hundreds of cultured cells. To obtain such sensitivity both in spectrophotometric and fluorometric reactions a reduction of the incubation volume is required. In spectrophotometry also the final volume has to be reduced and depending on the enzyme activity and the assay used, extinction measurements in volumes of less than 20–50 μl may be required. In such instances microcuvettes adapted to commercial spectrophotometers can no longer be used and a microscope spectrophotometer design has to be applied. Our analyses of *p*-nitrophenol using microspectrophotometry have shown that a 30–60 fold increase in sensitivity can be reached compared with a 60 μl volume using normal spectrophotometry when measurements are carried out in 1 μl (Fig. 2 and Table I). In this way a sensitivity could be reached which is comparable to that of a fluorometric assay using a methylumbelliferyl substrate.

Generally fluorometric procedures will be preferred because in many instances the final measurement can be performed in a normal fluorometer. As far as the use of methylumbelliferyl substrates is concerned the minimum amount of fluorescent product detectable with a normal fluorometer is in the order of 10^{-11}–10^{-12} moles, provided that small incubation volumes (0.5–1 μl) are used (see Table II). If the activity of the enzyme to be analyzed is in the order of 10^{-12} to 10^{-14} mole $\cdot$ h^{-1} per cell a direct analysis of a few hundred isolated freeze-dried cells according to the procedure described can easily be performed. This was so far found to be the case for about 6 lysosomal storage diseases which can be analysed with methylumbelliferyl substrates[8]. For these diseases and for some defects that can only be detected by a (micro)spectrophotometric assay prenatal diagnosis can be accomplished within 7–20 days, depending on the enzyme defect.

However, one of the main problems in prenatal diagnosis of metabolic disorders with an autosomal recessive inheritance is the difficulty of a clear distinction between cultured amniotic fluid cells with an enzyme deficiency and those with a decreased activity from a heterozygous carrier. The few examples presented for Pompe's disease

(Table IV) show that clear distinction between these two categories of cells is possible when the mean activities are compared. But within the wide range of activities for each cell type, very low values may be encountered in cells from a heterozygous carrier. The sensitivity of the assay must therefore be sufficient to detect such low values to avoid erroneous interpretations. The results of the model experiments using microfluorometry (Table II) indicate a possibility to obtain further increase in sensitivity of the detection of methylumbelliferone.

By using these microfluorometric techniques, certain lysosomal enzyme activities can even be measured quantitatively in one single cultured cell. This will provide new possibilities for more fundamental studies on metabolic cooperation and genetic complementation at the single cell level[29–31].

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REFERENCES

1 H. G. Hers, *Biochem. J.*, 86 (1963) 11.
2 H. L. Smith, L. D. Amick and J. B. Sidbury Jr., *Am. J. Dis. Childh.*, 111 (1969) 475.
3 K. Steinitz and A. Rutenberg, *Is. J. Med. Sci.*, 3 (1967) 411.
4 H. M. Nitowsky and A. Grunfeld, *J. Lab. Clin. Med.*, 69 (1967) 472.
5 H. L. Nadler and A. M. Messina, *Lancet*, ii (1969) 1277.
6 R. P. Cox, G. Douglas, J. Hutzler, J. Lynfield and J. Dancis, *Lancet*, i (1970) 893.
7 J. S. Salafsky and H. L. Nadler, *J. Pediat.*, 79 (1971) 794.
8 H. Galjaard, J. Fernandes, M. Jahodova, J. F. Koster and M. F. Niermeijer, *Bull. Eur. Soc. Hum. Genet.*, Nov. 1972, p. 79.
9 A. L. Fluharty, M. L. Scott, M. T. Porter, H. Kihara, M. G. Wilson and J. W. Towner, *Biochem. Med.*, 7 (1973) 39.
10 H. L. Nadler, *Biochemie*, 54 (1972) 677.
11 O. H. Lowry, *J. Histochem. Cytochem.*, 1 (1953) 420.
12 O. H. Lowry and J. V. Passonneau, *A flexible system of enzymatic analysis*, Academic Press, New York, 1972.
13 D. Glick, *Quantitative Chemical Techniques in Histo- and Cytochemistry*, Interscience Publ. New York, Vol. I (1961) and Vol. II (1963).
14 D. Glick, in U. C. Dubach and U. Schmidt (Eds.), *Recent advances in quantitative histo- and cytochemistry*, Hans Huber, Bern, 1971, p. 35.
15 H. Galjaard, M. Jahodova and M. F. Niermeijer, *Bruxelles Medical*, 52 (1972) 109.
16 H. Galjaard, *Pediat. Res.*, (1973) 56.
17 M. F. Niermeijer, J. Fernandes, M. Jahodova, J. F. Koster and H. Galjaard, *Pediat. Res.*, in press (1973).
18 M. F. Niermeijer, D. Halley, E. Sachs, C. Tichelaar-Klepper and K. L. Garver, *Humangenetik*, (in press).
19 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
20 F. Matschinsky, in U. C. Dubach and U. Schmidt (Eds.), *Recent advances in quantitative histo- and cytochemistry*, Hans Huber, Bern, 1971, p. 143.
21 J. S. Ploem, *Leitz. Mitt. Wiss. Techn.* VI (1969) 25.
22 J. E. De Josselin De Jong, W. Boender, L. Carlson and H. Galjaard, *Histochemie*, 35 (1973) 127.
23 A. Milunsky, J. W. Littlefield, J. N. Kanfer, E. H. Kolodny, V. E. Shih and L. Atkins, *New Engl. J. Med.*, 282 (1970) 1370, 1441 and 1498.
24 H. L. Nadler, in *Birth Defects, Symposium on Intrauterine Diagnosis*, p. 5, Nat. Found. March of Dimes, April 1971.
25 A. Milunsky and J. W. Littlefield, *Ann. Rev. Med.*, 23 (1972) 57.
26 M. N. Macintyre, in *Birth Defects, Symposium on Intrauterine Diagnosis*, p. 10, Nat. Found. March of Dimes, April 1971.
27 J. D. Schulman, W. Y. Fujimoto, K. H. Bradly and J. E. Seegmiller, *J. Pediat.*, 77 (1970) 468.
28 I. C. Fratantoni, E. F. Neufeld, W. Uhlendorf and C. B. Jacobson, *New Engl. J. Med.*, 280 (1969) 686.
29 S. Hickman and E. F. Neufeld, *Biochem. Biophys. Res. Commun.*, 49 (1972) 992.
30 E. A. De Weerd-Kastelein, W. Keijzer and D. Bootsma, *Nature (New Biol.)*, 238 (1972) 80.
31 H. Galjaard, in *Histo- and Cytochemistry, Proc. 4th Int. Congress*, Kyoto, August, 1972, p. 185 (abstr.).

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PAPER IV

GENETIC HETEROGENEITY AND VARIATION OF LYSOSOMAL ENZYME ACTIVITIES IN CULTURED HUMAN CELLS

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1. Summary

A better understanding of the variation in lysosomal enzyme activities (2 - 7 fold differences) in cultured cells from controls, heterozygotes and affected patients, is important both in (prenatal) diagnosis of metabolic diseases and in fundamental studies on somatic cell genetics and metabolic interaction. 5 - 10 fold variations in the activity of acid α-1.4-glucosidase and β-galactosidase were observed in relation to the growth curve of the culture (2 - 30 days after last subculture). Considerable differences in lysosomal enzyme activity were also found between various cell groups and between individual cells, using microchemical techniques. These differences are probably related to proliferative activity and phase in cell cycle. Experiments on co-cultivation (2 - 12 days) of normal and enzyme deficient cells showed no indication of metabolic coöperation for α-glucosidase and β-galactosidase. In the analysis of two cell populations within the same culture it must be realized that selection at the advantage of both normal and enzyme deficient cells may occur as was shown by model experiments on G6PD activity in single cells from subcultures of a heterozygote for the X-linked G6PD deficiency. Finally, the role of genetic heterogeneity was studied in fibroblasts from patients with various types of GM1-gangliosidosis using complementation analysis in individual binuclear heterokaryons after cell hybridization.

2. Introduction

During the last few years the genetic defect for an increasing number of lysosomal storage diseases has been demonstrated in cultured skin fibroblasts. Cultured cells have become an important tool in the postnatal and prenatal diagnosis of patients with inborn errors of metabolism and in the detection of heterozygous carriers[1]. Furthermore they provide a useful model in basic studies on the capacity of uptake of specific enzymes by

mutant cells[2-7]. Also, the use of cell hybridization of cultured fibroblasts from patients with an apparent similar enzyme defect may contribute to a better understanding of the genetic heterogeneity in metabolic diseases[8-10].

For a correct interpretation of the analytical data both in (prenatal) diagnosis and in fundamental studies, exact knowledge is required about the background of the variations observed in most enzyme assays on cultured human cells[11-19]. The present communication deals with studies on the variation of lysosomal enzyme activities in cultured human cells under different experimental conditions. The effect of different in vitro growth conditions was studied and microchemical techniques[20-22] enabled us to investigate at the single cell level, the effect of co-cultivation of normal and enzyme deficient cells and the behaviour of two cell populations within the same culture from a heterozygous carrier for an X-linked metabolic disease. When combined with somatic cell hybridization techniques the microchemical assay procedures also allowed complementation studies on individual binuclear heterokaryons, which provides more information about the genetic heterogeneity for a particular enzyme defect[23, 24]. The experimental data to be presented here will mainly be restricted to the lysosomal enzymes acid α-1.4-glucosidase and β-galactosidase.

3. Materials and Methods

Fibroblasts derived from skin biopsies from controls, heterozygous carriers for glycogenosis II (Pompe's disease) and glucose-6-phosphate dehydrogenase (G6PD) deficiency and from patients with Pompe's disease and patients with GM1-gangliosidosis type 1 and 2 were cultured according to procedures described earlier[19, 21, 22]. Amniotic fluid cell cultures from normal pregnancies and pregnancies at risk for Pompe's disease were initiated from 5 - 10 ml. samples of amniotic fluid obtained by transabdominal amniocentesis at the 14 - 16th week of pregnancy[19, 21].

Studies on the effect of different in vitro growth conditions on the activity of lysosomal enzymes were performed by seeding aliquots of about 10^5 human fibroblasts from early passages into 35 mm Falcon petri dishes and cultivation in Ham's F10 medium, supplemented with 15% fetal calf serum which was replaced three times a week. After 2, 4, 7, 10, 14, 22 and 30 days replicate cultures were harvested and the protein content and the lysosomal enzyme activities were determined. The procedures of cell homogenate preparation and of the microchemical assays for protein and for α-1.4-glucosidase and β-galactosidase activity using methylumbelliferyl substrates have been

described previously [21, 22, 24]. The activity of the same enzymes has been determined at various time intervals (16 - 55 days) after primary culturing of normal amniotic fluid cells.

The variation of α-1.4-glucosidase and β-galactosidase activity in individual fibroblasts within the same culture dish has been analyzed by microdissection of single freeze-dried cells which had been grown on the thin plastic foil which formed the bottom of the culture dish. Details of this isolation procedure, the incubation of single cells into microdroplets of substrate under paraffin oil and the subsequent measurement of the fluorescence intensity of the methylumbelliferone formed, by using microcapillaries and a microspectrofluorometer design have been described elsewhere[20 - 22].

As a model for the behaviour of two cell populations (with normal and deficient enzyme activity) within the same fibroblast culture of a heterozygous carrier of an X-linked disease, the distribution of G6PD activity in single cells was analyzed in successive subcultures of a cell strain from a heterozygote for G6PD deficiency. About $6 \cdot 10^4$ cells from each subculture were seeded into a "5 cm ø mylar dish" with a thin plastic bottom and after two days in vitro growth the G6PD activity in randomly dissected individual cells was analyzed using a NADP(H) cycling method[23, 24, 25].

The occurrence of metabolic coöperation between normal and enzyme deficient cells was studied by co-cultivation of equal numbers of control fibroblasts with fibroblasts from patients with Pompe's disease (α-glucosidase deficient) and with type 1 GM-1 gangliosidosis. Co-cultivation of 8.10^5 cells from a subculture of both cell strains was carried out in Falcon flasks during 2 - 12 days. At various time intervals cells were harvested and the protein content and the activity of β-galactosidase and α-glucosidase were determined. About 3 - 6. 10^4 cells of the mixed population after different time intervals of co-cultivation were seeded into a "mylar dish" and the α-glucosidase or β-galactosidase activity was analyzed in randomly isolated freeze-dried cells after two days further in vitro growth. The enzyme activities of cells in the mixed cell population were compared with those of both original cell strains.

The genetic heterogeneity of β-galactosidase deficiency in patients with different clinical forms of GM1 ganglisosidosis was tested by complementation studies in individual binuclear heterokaryons. Cell hybridization with Sendai virus was carried out with 10^6 cells of both parental cell strains according to procedures described earlier[9, 24]. Subcultures of β-galactosidase deficient fibroblasts from patients with GM1-gangliosidosis type 1 and 2 were fused and 3 - 4. 10^4 cells of the hybridized population were seeded into a "mylar dish" and grown for two days. Subsequently the medium was washed off the dishes were

freeze-dried and 40 - 80 binuclear heterokaryons were randomly dissected. Their β-galactosidase activity was determined with a microassay using 1 mM methylumbelliferyl-β-D-galactopyranoside at pH 4.2 as a substrate[22, 24]. The activity per cell was compared with that of mononuclear and binuclear cells from normal and hybridized cells of control cell strains.

4. Results

a. Variation in lysosomal enzyme activity in homogenates of fibroblast and amniotic fluid cell cultures from controls, heterozygotes and affected patients

In table 1 the results are presented of a large number of assays for acid α-1.4 glucosidase activity in fibroblasts from various controls, heterozygotes and patients with glycogenosis II and in cultured amniotic fluid cells. Cell cultivation was carried out under identical conditions but they were harvested and analyzed at different time intervals after the last subculture.

Table 1

α-1.4-Glucosidase activity in cultured amniotic fluid cells and in fibroblasts from controls heterozygotes and patients with glycogenosis II (Pompe's disease)*

	α-Glucosidase activity ($\times 10^{-12}$ mole/min/mg.protein)					
Control fibroblasts	<u>3900</u>	2645	<u>956</u>	1440		
Heterozygous fibroblasts	353	480	<u>1654</u>	<u>169</u>	215	158
Pompe's fibroblasts	25	18	26	<u>38</u>	<u>10</u>	
Control amniotic fluid cells, later subculture	<u>603</u>	1227	642	714	<u>1770</u>	
Control amniotic fluid cells, primary culture	261	479	414	<u>121</u>	<u>754</u>	

*Each value represents the mean enzyme activity obtained from 2 - 5 analyses of one cell strain. Activities for different cell strains within each category are presented. The lowest and highest activities for each category of cells are underlined.

The data show that for each category of cells there is considerable variation among the mean enzyme activities in cell strains from different individuals. The extreme values within each category may differ 300 - 500%. The greatest variation seems to exist in cells obtained from various heterozygotes (not belonging to the same family). However, in table 1 the data for each cell strain represent the mean of 2 - 5 independent analyses and as is shown in table 2 there may also be considerable variation among analyses on

different cultures of the same cell strain.

Table 2

Variation in α-glucosidase activity in various analyses on the same cell strains

	α-Glucosidase activity* ($x10^{-12}$ mole/min/mg.prot.)			
Control amniotic fluid cells	370 - 1049;	659 - 765;	548 - 763	
Control fibroblasts	2240 - 5326;	1010 - 1598;	2010 - 3280	
Heterozygous fibroblasts	142 - 475;	131 - 291;	420 - 541	
Pompe's fibroblasts	5 - 29;	22 - 29;	21 - 29;	3 - 24

*Figures are the extremes 2 - 5 analyses on the same cell strain; results for 3 - 4 cell strains in each category are presented.

As a consequence, correct interpretation in (prenatal) diagnosis may be difficult if this must be based on comparison of single enzyme assays on one cell strain from each category.

This is expecially the case for the detection of heterozygous carriers because the enzyme activities in this group may overlap control values (table 1). In prenatal diagnosis the distinction between heterozygous carriers and affected patients may cause problems. As is shown in table 1 the α-glucosidase activity in later subcultures of control amniotic fluid cells is about three times lower than in control fibroblasts. In primary cultures of amniotic fluid cells the enzyme activity may again be 2 - 3 fold lower than in later subcultures and if the same ratio's hold for cells from heterozygotes the enzyme activity in primary cultures of amniotic fluid cells may be very low (10 - 15% of fibroblast values) when the fetus is a heterozygous carrier.

The variation in enzyme activity as described above has also been observed for a number of other lysosomal enzymes. The effect of the cell population growth curve on the activity of β-N-acetylglucosaminidase, arylsulphatase A, α-galactosidase, β-glucosidase, β-galactosidase and α-glucosidase has been investigated by one of us (M.H.-D). Detailed results will be published elsewhere but as the data obtained were quite similar for all lysosomal enzymes investigated we will only present those for α-glucosidase and β-galactosidase. Fig. 1 shows the results for the specific β-galactosidase activity in a normal fibroblast strain. The protein content per dish as well as the specific β-galactosidase activity continue to increase up to the end (30 days) of the observation period. There is an 8 fold! increase in enzyme activity between day 2 and 30 after the last subculture. In other experiments with a higher initial cell density the increase in protein content came to a stop earlier but the β-galactosidase specific activity continued to increase. Primary

amniotic fluid cell cultures do not show a correlation between specific lysosomal enzyme activity and culture time.

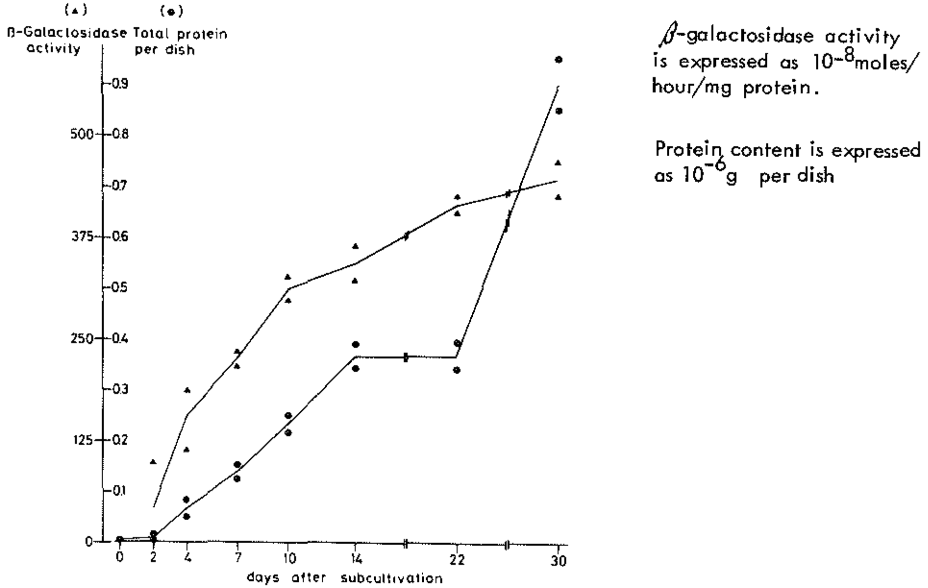


Fig. 1 Increase in β-galactosidase activity during the growth of normal fibroblasts.

Fig. 2 shows similar results for the activity of acid α-1.4-glucosidase which more or less linearly increases with a factor 10 from 2 to 30 days after subcultivation. Also in cells from heterozygous carriers there is an increase in enzyme activity during the growth curve, although when investigated for several cell strains, the increase seems to be less steep than in controls. The α-glucosidase activity in fibroblasts from patients with Pompe's disease remains low during the whole observation period. From these results it may be concluded that in comparative studies of lysosomal enzyme activities standardization of the cell density and the time between seeding and harvesting of a culture is very important.

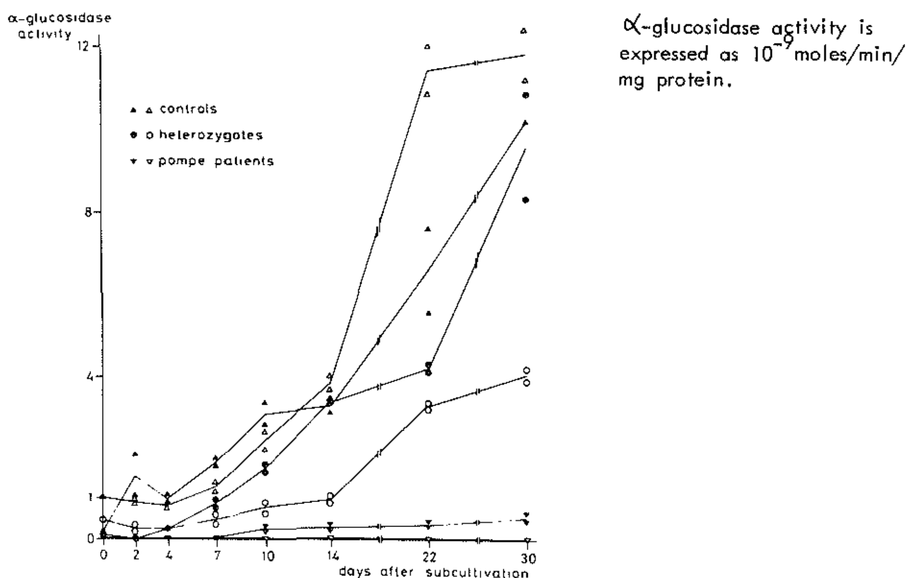


Fig. 2. Increase in acid α-1.4-glucosidase activity during the growth of fibroblasts derived from controls, heterozygous carriers and patients with glycogenosis II.

b. Variation in lysosomal enzyme activity in single and small numbers of cultured cells.

Small groups (100 - 300) of freeze-dried cultured cells can be isolated and analyzed and the enzyme activity can be expressed per cell without the need of parallel protein assays. This method allows enzyme assays on various groups cells within the same culture dish. An example of such analysis for acid α-1.4-glucosidase activity in groups of 110-260 cells from a primary culture of amniotic fluid in the same dish is illustrated in table 3.

Table 3

Acid α-1.4-glucosidase activity* in small groups (100 - 300) of freeze-dried amniotic fluid cells isolated from one culture dish

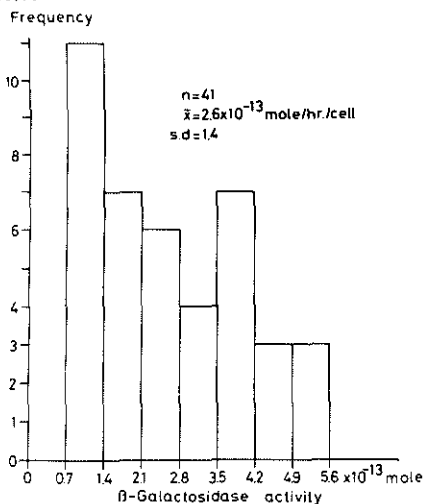
Number of cells per isolated group	170	190	110	170	160	140	260	170	160
α-1.4-Glucosidase activity per cell (x 10^{-14} mole/hour)	3.2	2.4	4.2	2.1	1.5	2.4	1.1	1.7	1.6

*Enzyme activity has been determined by incubation during 1 hour at 37°C in 3 μl 2.2 mM 4-Methylumbelliferyl-α-D-glucopyranoside pH4.0.

The results indicate that even within one culture dish there is considerable variation in

α-glucosidase activity when different cell groups are compared. Similar observations have been made for other lysosomal enzymes (α-galactosidase, β-galactosidase). The errors involved in the microchemical assay have been determined to be less than 10%[22] and the error in cell counting is 10 - 15%. Apart from these factors the variation in enzyme activity might be related to local differences in proliferative activity and unbalanced growth within one culture dish.

The activity of a number of lysosomal enzymes can also be determined in individual cultured cells. With the microtechniques mentioned this applies to enzymes which activity per cell exceeds 10^{-14} moles methylumbelliferone formed per hour. Fig. 3 and 4 illustrate respectively the β-galactosidase and α-1.4-glucosidase activities in single cells randomly dissected from one dish at two days after seeding of about 4.10^4 cells of an early passage of normal human fibroblasts. The results indicate that also among individual cells there is considerable variation in enzyme activity. Both for α-1.4-glucosidase and β-galactosidase the difference between the lowest and highest activity is a factor of 5 - 7 and in other (non-lysosomal) enzymes tested we have even observed greater variation. These differences might be related in part to differences in the phase of the cell cycle of the analyzed cells. Within one culture dish there exists also a considerable variation in the total cellular dry mass of individual cells[22, 26] and the differences in enzyme activity might be less if they were expressed on the basis of the cellular dry mass of each particular cell.



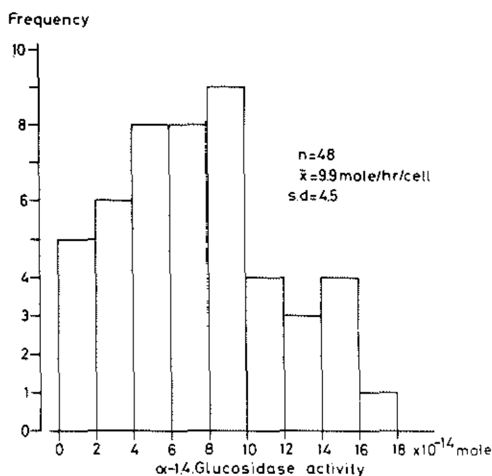


Fig. 3. Variation in β-galactosidase activity of single cells from a normal fibroblast culture.

Fig. 4. Variation in acid α-1.4-glucosidase activity of single cells from a normal fibroblast culture.

c. Behaviour of two cell populations with different enzyme activities within one culture.

The study of the behaviour of two cell populations within one culture is of practical importance in (prenatal) diagnosis of heterozygotes for X-linked metabolic diseases. Also in more fundamental studies of metabolic coöperation and genetic complementation, the analysis of enzyme activities in individual cultured cells from two populations is of interest. Examples of each aspect will be presented.

Equal numbers of subcultures of fibroblasts from a patient with Pompe's disease and from a normal control were co-cultivated during 2 - 12 days. Similar experiments were carried out with normal fibroblasts and β-galactosidase deficient cells from a patient with GM1-gangliosidosis. As is shown in table 4 neither for the α-glucosidase nor for the β-galactosidase deficient cell strains any residual activity could be detected. After various periods of co-cultivation with equal numbers of normal fibroblasts no indication was obtained for metabolic coöperation. The specific activity of both lysosomal enzymes in the mixed population was found to be about half of that of the normal control fibroblasts grown under similar conditions. However, in this type of experiment differences in protein content of both co-cultivated cell strains might complicate a correct interpretation of the data on enzyme activity.

Table 4

Specific activity of lysosomal enzymes in cell homogenates after co-cultivation of normal and enzyme deficient human fibroblasts

	β-Galactosidase activity (x 10^{-9} mole/hour/mg. protein)	α-Glucosidase activity
Normal fibroblasts	270**	44, 248*
Enzyme deficient fibrobl.	not detectable	not detectable
After 2-4 days co-cultivation	102 (41%)	19 (43%)
After 7-9 days co-cultivation	141 (52%)	80
After 12 days co-cultivation	-	116 (47%)

*Data obtained 2 and 12 days after subcultivation of the same cell strain.
**Grown for 4 days.

Similar experiments on co-cultivation were carried out but followed by enzyme analyses on individual cells which were randomly selected from the mixed cell population. In the case of β-galactosidase such assays were performed two days after seeding of equal numbers of normal and enzyme deficient cells in a "mylar dish". The results in

Fig. 5 illustrate the presence of a considerable number of enzyme deficient cells between cells with a normal β-galactosidase activity. The distribution of enzyme activity per cell in the latter resembles that in a normal cell strain. In the case of α-glucosidase analysis co-cultivation of equal numbers of normal and enzyme deficient cells was carried out in a Falcon flask and after 4 and 12 days cells were trypsinized and about 4.10^4 cells of the mixed cell population were seeded on a "mylar dish". After two days further cultivation, individual freeze-dried cells were randomly dissected and their α-glucosidase activity was determined. The results in Fig. 6 indicate that even after 12 days co-cultivation a population of enzyme deficient cells remained present among normal fibroblasts.

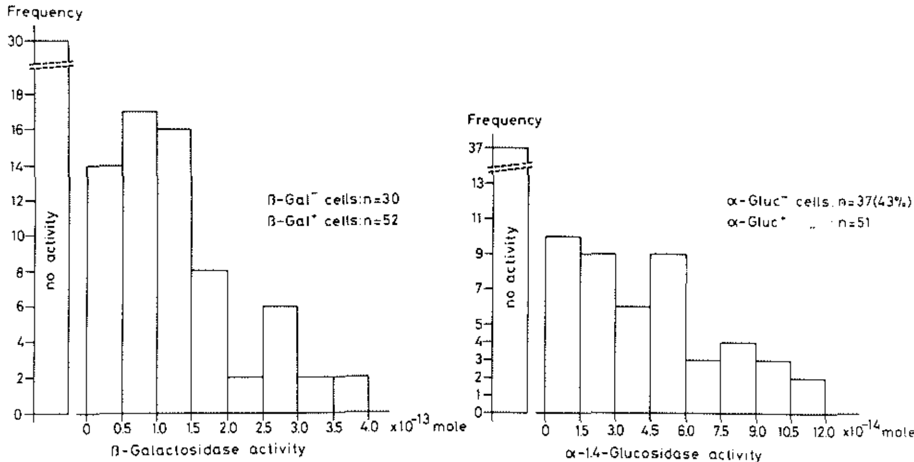


Fig. 5. β-Galactosidase activity of single cells isolated from a mixed culture after 2 days co-cultivation of enzyme deficient fibroblasts from a patient with GM1-gangliosidosis (type I) and of normal fibroblasts.

Fig. 6. Acid α-1.4-glucosidase activity of single cells isolated from a mixed culture after 12 days co-cultivation of enzyme deficient fibroblasts from a patient with glycogenosis II and of normal fibroblasts.

Another situation where two cell populations are present within the same culture dish is in a fibroblast culture from a heterozygous carrier for an X-linked enzyme deficiency. As a result of random inactivation of one of the X-chromosomes one may expect 50% of cells with a normal activity of the particular enzyme and 50% of enzyme deficient cells. We have tested the distribution of normal and enzyme deficient cells in successive subculture fibroblasts from a heterozygous carrier for G6PD deficiency. In table 5 the percentages of cells with normal G6PD activity and of enzyme deficient cells are illustrated in successive subcultures of the same cell strain. For each subculture about 4.10^4 cells were seeded in a "mylar dish" and the G6PD activity was analyzed in randomly dissected cells

from the same dish after two days cultivation.

Table 5

Single cell analysis of G6PD activity in cultured fibroblasts from a heterozygous carrier for G6PD deficiency

G6PD activity (x 10^{-12} moles/hour/cell)					
Experiment I			Experiment II		
Subculture	$G6PD^+$ cells	$G6PD^-$ cells	Subculture	$G6PD^+$ cells	$G6PD^-$ cells
10th	$\overline{X}$= 4.0 n= 28 (37%)	n= 47 (63%)	6th (A)	$\overline{X}$= 5.6 n= 36 (75%)	n= 12 (25%)
12th	$\overline{X}$= 4.8 n= 24 (32%)	n= 53 (68%)	9th (A)	$\overline{X}$= 4.6 n= 30 (91%)	n= 3 (9%)
13th	$\overline{X}$= 2.0 n= 20 (25%)	n= 60 (75%)	6th (B)	$\overline{X}$= 3.1 n= 36 (75%)	n= 11 (25%)
17th	$\overline{X}$= 2.1 n= 9 (18%)	n= 41 (82%)	9th (B)	$\overline{X}$= 3.6 n= 53 (98%)	n= 1 (2%)

*A and B are cells from different frozen subcultures from the same cell strain. $\overline{X}$= mean enzyme activity of $G6PD^+$ cells. n= number of cells analyzed.

The results in the left column show that in successive subcultures (from 10th to 17th) the percentage of G6PD deficient cells increases at the expense of cells with normal enzyme activity. The results of an independent experiment on the same cell strain show that during successive subculture also the percentage of G6PD deficient cells may decrease. Selection during in vitro cultivation may thus occur at the advantage of both enzyme deficient cells and cells with normal activity. This fact is of importance in the interpretation of enzyme activity in cultured cells from heterozygotes for X-linked diseases.

d. Genetic heterogeneity studied by complementation analysis in single binuclear hybrid cells.

Apart from the variations in lysosomal enzyme activity resulting from the factors mentioned there are genetic factors which determine the clinical and pathological manifestations arising from a specific enzyme deficiency and also the (residual) activity of the defective enzyme. This genetic background of clinical and enzyme heterogeneity can be investigated by complementation analysis in hybridized cultures of fibroblasts from two patients with "the same" enzyme deficiency but different clinical manifestations. The following experiments illustrate the feasibility of microchemical enzyme assays in individual binuclear hybrid cells randomly selected from a culture, two days after fusion of sub-

cultures of fibroblasts derived from patients with type 1 and type 2 GM1-gangliosidosis.

In cell homogenates and in individual freeze-dried cultured fibroblasts from both types of patients no activity of β-galactosidase could be demonstrated with methylumbelliferyl substrate, compared with an activity of 1 - 2.10^{-13} mole/hour/cell in normal cultured fibroblasts. Cell hybridization with Sendai virus was carried out between fibroblasts from a normal control, between cells from the same patient and between cells from the two different patients. Two days after cultivation of the hybridized cell population in a "mylar dish" individual binuclear heterokaryons were analyzed for β-galactosidase activity and the results were compared with those of normal mononuclear fibroblasts. In table 6 and Fig. 7 and 8 the experimental data are summarized. The β-galactosidase activity in binuclear heterokaryons after fusion of cells from a normal fibroblast culture was found to be higher than in mononuclear cells from the same culture (Fig. 7). The latter values correspond to those found in a normal, non-hybridized culture. After fusion of β-galactosidase deficient cells with normal fibroblasts types of binuclear heterokaryons were observed which is in agreement with the theoretical expectation (Fig. 8).

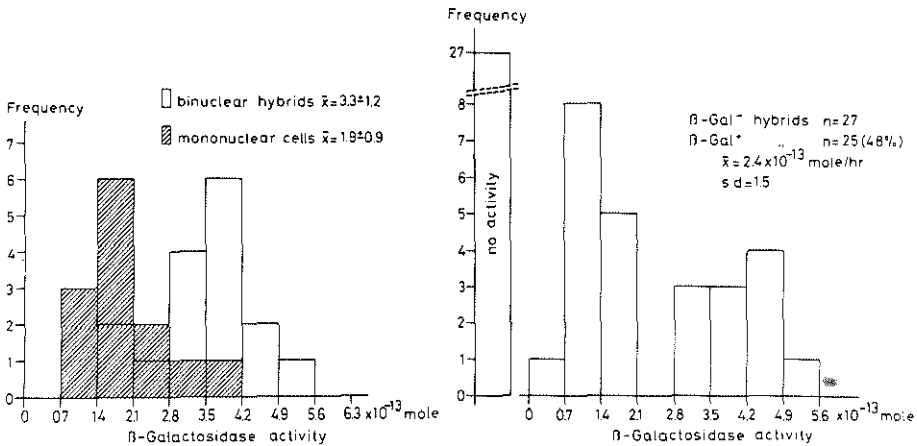


Fig. 7. Gene dossage effect for β-galactosidase activity demonstrated by comparing enzyme assays on single mononuclear cells and binuclear heterokaryons after cell hybridization of normal fibroblasts.

Fig. 8. Distinction of single binuclear heterokaryons with different levels of β-galactosidase activity as demonstrated after cell hybridization of normal fibroblasts with fibroblasts from a patient with GM1-gangliosidosis type 1.

A number of hybrid cells had no β-galactosidase activity, other binuclear cells showed an enzyme activity comparable to that in normal mononuclear cells (probably fusions of

normal and deficient cells) and the remaining cells had a high activity (probably the result of fusion of two normal cells). These results clearly indicate the possibility of detecting within one cell population binuclear heterokaryons with different levels of lysosomal enzyme activity.

Table 6

Genetic complementation studies in individual binuclear hybrids after fusion of β-galactosidase deficient fibroblasts from patients with GM1-gangliosidosis type 1 and type 2

Type of cell fusion	β-Galatosidase activity ($x10^{-13}$ mole/hour cell)	
	β-Gal^{-} cells	β-Gal^{+} cells
Control x control	-	n= 67 $\overline{X}$= 3.3 s.d. 1.3
GM1 gangliosidosis		
Type 1 (GM) x type 1 (GM)	n= 21	-
Type 2 (Garr) x type 2 (Garr)	n= 42	-
Type 1 (GM) x type 2 (Garr)	n= 53	-

X= mean enzyme activity in α-gal^{+} cells and s.d. is the standard deviation.
n= number or cells with either a deficient or a normal β-galactosidase activity.

Complementation studies carried out between fibroblasts from patients with different types of GM1-gangliosidosis have so far not provided any indication for genetic complementation. In table 6 an example of such experiments illustrate that after fusion of two different enzyme deficient cell strains only binuclear heterokaryons were observed with a deficient β-galactosidase activity.

5. Discussion

The large variation observed for α-1.4-glucosidase activity in different cell strains of the same category (controls, heterozygotes or affected patients) is in agreement with data on other lysosomal enzymes in cultured fibroblasts and amniotic fluid cells[13, 14, 17, 18]. Part of these variations may be explained by the marked effect of the cultivation period and the degree of confluency on the activity of several enzymes[11, 12, 15, 16], but even when cells are cultured under standardized conditions some of the variation remains[14]. This variation and the differences in lysosomal enzyme activity between cultured fibroblasts, later subcultures of amniotic fluid cells and primary cultures of amniotic fluid cells indicate the necessity of using control material grown under standardized conditions when reliable prenatal diagnosis of metabolic disorders is attempted. Microchemical assays on groups of 100 - 300 freeze-dried cultured amniotic fluid cells or

control fibroblasts show that even within the same culture dish there is an appreciable variation in lysosomal enzyme activity between various cell groups[19, 27]. Enzyme analyses on individual cells (Figs. 3 and 4) also showed considerable variations in activity of α-glucosidase and β-galactosidase. It seems that these variations in enzyme activity are related to differences in proliferative activity and in phase of the cell cycle[22, 28]. When the enzyme activities as measured in single cells will be related to the total cellular dry weight[22, 26] the variation at this level might be reduced.

In the interpretation of enzyme assays on cultured cells from a heterozygous carrier for an X-linked metabolic disease and also on studies of metabolic coöperation between two cell populations precise knowledge about the behaviour of both cell types during prolonged cultivation is required. In fibroblast cultures of heterozygotes for various X-linked metabolic diseases the presence of both enzyme deficient and normal cells have been demonstrated by cloning techniques[29 - 31]. The microchemical assay of enzyme activities in individual cells enabled us to study the behaviour of two cell populations within the same culture dish. The results obtained for successive subcultures of the same fibroblast strain from a heterozygote for G6PD deficiency revealed that selection may occur in the direction of both enzyme deficient cells and cells with normal activity (table 5). Similar experiments on heterozygotes for X-linked lysosomal storage diseases may provide further insight in this aspect and also in the occurence of metabolic coöperation between normal and enzyme deficient cells[27]. So far we have not been able to show metabolic coöperation for acid α-1.4-glucosidase and β-galactosidase after co-cultivation of normal fibroblasts with cells from patients with Pompe's disease and with GM1-gangliosidosis (table 4 and Figs. 5 and 6). These results are in contrast with the experimental evidence for metabolic coöperation between cell strains from patients with various types of mucopolysaccharidosis[32]. The finding that several lysosomal enzymes are readily taken up from the culture medium by enzyme deficient cells[2 - 6] indicates that the absence of metabolic coöperation should be explained by differences in enzyme secretion rather than by different behaviour in enzyme uptake. Further information on the possibility of a different behaviour in intercellular transport of different lysosomal enzymes [4, 7] should be obtained. Attention should also be given to differences in the amounts of enzyme added in model experiments and the amounts of lysosomal enzymes secreted by normal cultured cells.

Part of the variation in lysosomal enzyme activity as well as in the clinical and pathological manifestations resulting for "the same" enzyme deficiency seems to be genetically determined. For a number of metabolic disorders the use of cell hybridization

techniques followed by complementation analysis for the particular metabolic defect have provided information about genetic heterogeneity[8-10]. For most enzyme deficient human somatic cell hybrids there is no selective system and complementation studies must therefore be performed on isolated hybrid cells. The applicability of enzyme assays on individual binuclear heterokaryons isolated after fusion of two human cell strains with "a similar" enzyme deficiency is illustrated by complementation studies on β-galactosidase activity after hybridization of fibroblasts from patient with different types of GM1-gangliosidosis [33 - 36]. So far no genetic complementation has been observed in binuclear hybrid cells after fusion of cells from patients with GM1-gangliosidosis type 1 and type 2. If different mutations were present in the same polypeptide chain of the deficient enzyme complementation will not easily be detected. Another unanswered question is whether complementation for lysosomal enzymes can be demonstrated in binuclear heterokaryons since we do not exactly know how the synthesis and coupling of various subunits of a lysosomal enzyme occurs. For a better understanding of the genetic heterogeneity in lysosomal storage diseases more information about these fundamental processes is required and also about the molecular properties and functions of the normal and deficient (iso)enzymes in different organs[33-37].

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References

1. Milunsky, A. and Littlefield, J.W., 1972, Ann. Rev. Med. 23, 57.
2. Bach, G., Friedman, R., Weissmann, B. and Neufeld, E.F., 1972, Proc. Natl. Acad. Sci., 69, 2048.
3. Wiesmann, U.N., Rossi, E.E. and Herschkowitz, N.H., 1972, Acta Pediat. Scand. 61, 296.
4. Hickman, S. and Neufeld, E.F., 1972, Biochem. Biophys. Res. Comm., 49, 992.
5. O'Brien, J.S., Miller, A.L., Loverde, A.W. and Veath, M.L., 1973, Science, 181, 753.
6. Lagunoff, D., Nicol, D.M. and Pritzl, P., 1973, Lab. Invest. 29, 449.
7. Schneck, L., Amsterdam, D., Brooks, S.E., Rosenthal, A.L. and Volk, B.W., 1973, Pediatrics, 52, 227.
8. Nadler, H.L., Chacko, E.M. and Rackmeler, M., 1970, Proc. Natl. Acad. Sci., 67, 976.
9. Weerd-Kastelein, E.A. de, Keijzer, W. and Bootsma, D., 1972, Nature New Biology, 238, 80.
10. Lyons, L.B., Cox, R.P., Dancis, J., 1973, Nature, 243, 533.
11. DeMars, R., 1964, Nat. Cancer Inst. Monogr., 13, 181.
12. Okada, S., Veath, M.L., Leroy, J. and O'Brien, J.S., 1971, Amer. J. Human Gen. 23, 55.
13. Kaback, M.M., Leonard, E.O. and Parmiley, T.H., 1971, Pediat. Res. 5, 366.
14. Milunsky, A., Spielvogel, C. and Kanfer, J., 1972, Life Sciences, 11, 1101.
15. Shanfield, B. and Pinsky, L., 1972, Can. J. Genet. Cytol., 14, 871.
16. Ryan, C.A., Lee, S.Y. and Nadler, H.L., 1972, Exptl. Cell Res., 71, 388.
17. Butterworth, J., Sutherland, G.R., Broadhead, D.M. and Bain, A.D., 1973, Life Sciences, 13, 713.
18. Butterworth, J., Sutherland, G.R., Broadhead, D.M. and Bain, A.D., 1973, Clin. Chim. Acta, 44, 295.
19. Niermeijer, M.F., Koster, J.F., Jahodova, M., Fernandes, J. and Galjaard, H. Ped. Res., submitted.
20. Galjaard, H., Fernandes, J., Jahodova, M., Koster, J.F. and Niermeijer, M.F., 1972, Bull. Eur. Soc. Human Genetics, 79.
21. Galjaard, H., Mekes, M., Josselin de Jong, J.E. and Niermeijer, M.F., 1973, Clin. Chim. Acta, 49, 361.
22. Galjaard, H., Hoogstraaten J.J. van, Josselin de Jong, J.E. de and Mulder, M.P., 1974, Histochem. J., 6, 409.
23. Galjaard, H., 1972, Proc. 4th Int. Congr. Histo- & Cytochem. T. Takeuchi, K. Ogawa and S. Fujita eds. Kyoto, pp. 185.
24. Galjaard, H. Hoogeveen, A., Keijzer, W., Wit-Verbeek, E. de, and Vlek-Noot, C. Histochem. J., in press.

25. Lowry, O.H. and Passonneau, J.V., A flexible system of enzymatic analysis. Acad. Press, N. York, 1972.

26. Josselin de Jong, J.E. de, Boender, W., Carlson, L. and Galjaard, H., 1973, Histochemie, 35, 1973.

27. Galjaard, H., Niermeijer, M.F., Hahnemann, N., Mohr, J. Sørensen, S.A., Clin. Genetics, in press.

28. Mitchison, J.M., 1969, Science, 165, 657.

29. Danes, S.B. and Bearn, A.G., 1967, J. Exp. Med., 126, 509.

30. Migeon, B.R., Kaloustian, V.M. der, Nyhan, W.L., Young, W.J. and Childs, B., 1968, Science, 160, 425.

31. Romeo, G. and Migeon, B.R., 1970, Science, 170, 181.

32. Neufeld, E.F. and Barton, R.W., 1972, Biology of Brain Dysfunction. Vol. 1 G.E. Gaull ed. Plenum Press, pp. 1-30.

33. Okada, S. and O'Brien, J.S., 1968, Science, 160, 1002.

34. O'Brien, J.S., 1972, Advances in human genetics. Vol. 3, H. Harris and K. Hirschhorn eds. Plenum Press, N. York, pp. 39-98.

35. Wolfe, L.S., Callahan, J., Fawcett, J.S., Andermann, F. and Scriver, C.R., 1970, Neurology, 20, 23.

36. Pinsky, L. and Powell, E., 1970, Nature, 228, 1093.

37. Ho, M.W. and O'Brien, J.S., 1969, Science, 165, 611.

38. Kint, J.A., Dacremont, G., Carton, D., Cruye, E. and Hooft, C., 1973, Science, 181, 352.

PAPER V

PRENATAL DIAGNOSIS OF TYPE II GLYCOGENOSIS (POMPE'S DISEASE) USING MICROCHEMICAL ANALYSES

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EXTRACT

The number of cultured amniotic fluid cells required for the prenatal diagnosis of metabolic defects can be reduced by using microchemical techniques. Six cases are presented of prenatal monitoring for glycogenosis type II (Pompe's disease), in which α-glucosidase assays were performed in (sub)microliter volumes with 4-methylumbelliferyl- α-D-glucopyranoside as a substrate. Using cell homogenates, the period of time between amniocentesis (performed in the 14th-15th week of pregnancy) and the diagnosis varied from 12-22 days. This could further be reduced to about 10 days when microchemical enzyme analyses were performed on dissected groups of small numbers (100-300) of freeze-dried cultured cells. The α-glucosidase activities (expressed per cell) obtained by these microtechniques were found to be in good agreement with data from conventional biochemical assays. Furthermore, it was observed that the acid α-glucosidase activity is much lower in primary cultures of amniotic fluid cells, approximately 100-200 pmol/min/mg protein than in cells from later subcultures, which had activities up to 700-1.600 pmol/min/mg 2-3 weeks after the last subcultivation. Primary cultures of normal amniotic fluid cells should therefore be used as a control in prenatal diagnosis; this is especially important when microtechniques are used to enable an early prenatal diagnosis.

SPECULATION

In prenatal diagnosis of at least seven lysosomal storage diseases the time interval between amniocentesis and the diagnosis can be reduced by using microchemical assays on dissected groups of 100 - 300 freeze-dried cultured amniotic fluid cells. Correct interpretation of the analytical data requires the use of primary cultures of normal amniotic fluid cells as controls. If enzymatic activities in various strains of fibroblasts and amniotic fluid cells are to be compared, cell cultivation conditions should be strictly controlled.

INTRODUCTION

The presence of lysosomal α-1,4-glucosidase activity in leucocytes (18) has facilitated the diagnosis of glycogenosis type II (Pompe's disease). The finding that acid α-1,4-glucosidase activity was also deficient in cultured skin fibroblasts from patients with Pompe's disease (34) suggested the possibility of prenatal detection (6, 30, 31). When parents at risk for an affected child ask for prenatal monitoring the time interval between amniocentesis and the diagnosis should be kept as short as possible. Unfortunately a direct assay of enzymatic activities in uncultured amniotic fluid cells or in amniotic fluid supernatant is unreliable (6, 30). However, when cultured amniotic fluid cells were used for biochemical analysis, 4 - 6 weeks were required to obtain sufficient cell material (6, 24, 30, 31) and even longer periods (2, 10) have been described for other metabolic diseases.

The number of cells required and the time needed for the diagnosis can be reduced if microchemical techniques are used. These methods were originally developed for histochemical studies on tissue sections (16, 25, 26, 28) and have been adapted for the analysis of small numbers of cultured amniotic fluid cells (12, 13). The first examples of their application for prenatal diagnosis have been reported (11, 14).

The present paper describes six prenatal analyses for Pompe's disease using microchemical techniques. Some problems in the interpretation of the analytical data are discussed in relation to the clinical and biochemical diagnosis of affected siblings, the use of proper control material, and the influence of cell culture conditions on enzymatic activity.

MATERIAL AND METHODS

Acid and neutral α-1,4-glucosidase assays on tissues and leucocytes of affected patients and heterozygous carriers were performed as described previously (21). In pregnancies at risk for Pompe's disease 10 - 20 ml of amniotic fluid was obtained by transabdominal amniocentesis in the 14 - 16th week of pregnancy (calculated from the last menstrual period) after ultrasound localization of the placenta.

Preparation of cell material

The amniotic fluid sample is centrifuged in siliconized glass tubes for 5 min. at 80 x g and the supernatant is centrifuged for another 10 min. at 1,500 x g and stored at -70°C until analysis. The cell pellet is resuspended in Ham's F10 medium (44) supplemented with 20% fetal calf serum (45), penicillin (100 U/ml), and streptomycin (50 μg/ml). Cell cultivation is carried out in a fully humidified atmosphere with 5% CO_2 in air. For microbiochemical analyses on cell homogenates cell cultivation is performed in 35 mm plastic Petri dishes (46) and cell counting and homogenization is carried out as described earlier (13). When microchemical assays are to be performed on dissected groups of freeze-dried cells an aliquot of the cell suspension is cultured on a "mylar dish" consisting of a glass ring of 5 cm outside diameter with a bottom of thin plastic film (47). When sufficient cell growth has occurred, the dish is quickly frozen and freeze-dried overnight at -45°C *in vacuo*. Subsequently a measured number of cells (100-300) are dissected under a stereomicroscope as described earlier (13). In each prenatal diagnosis confluent subcultures of amniotic

fluid cells from a normal pregnancy (14-17th week), fibroblasts from a previous affected sibling, or a nonrelated patient with Pompe's disease, and fibroblasts from the heterozygous parents and from normal individuals were used as controls. Cells were grown in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics. In one case (V Co. 2) a primary culture of normal amniotic fluid cells was used as a control (48).

Microchemical analysis

Either 0.3 - 1 µl of cell homogenate or dissected pieces of plastic film containing a small number of lyophilized cells were incubated under microscopic control in small volumes (0.6 - 2 µl) of substrate covered by paraffin oil, using the "oil well technique" (28).
Incubation in 2.2 mM 4-methylumbelliferyl- α-D-glucopyranoside (49) in 0.2 M acetate buffer at pH 4.0 was carried out for 1-2 hr at 37°. The reaction mixture was then diluted with 500 µl 0.5 M carbonate buffer (pH 10.7) and the fluorescense was read in a fluorometer (50)(excitation 365 nm; emission 448 nm). The enzymatic activity was expressed per cell when dissected groups of freeze-dried cells were analysed. In the analysis of cell homogenates the protein content was determined according to the method of Lowry (27) in a final volume of 60 µl. The enzymatic activity was expressed as moles of methylumbelliferone liberated per minute per milligram of protein. For comparative studies with the assays on dissected groups of cells, the analytical data on cell homogenates were also expressed per cell as calculated from the cell counts after trypsinization. The enzymatic analyses of amniotic fluid supernatant for acid and neutral α-1,4-glucosidase were performed as described previously (21) and expressed per milligram of protein. Neutral α-glucosidase assays were performed as a reference enzyme; assay conditions were the same as for acid glucosidase except that incubation was carried out in 0.1 M fumarate buffer at pH 6.5.

RESULTS

1. PRENATAL DIAGNOSIS USING MICROCHEMICAL ANALYSIS OF CELL HOMOGENATES

Family 1 Bi had one healthy boy and two children who died at 6 and 5 months of cardiopulmonary failure; in the youngest child (1 Bi 3) Pompe's disease was diagnosed by the demonstration of a deficient acid a-1,4-glucosidase activity in leucocytes and muscle (Table 1). In the fourth pregnancy amniocentesis had to be repeated because of insufficient amniotic fluid cell growth. The results of enzyme assays on cultured cells from the second sample (obtained in the 19th week) are given in Table 2 (1 Bi 4). The acid a-glucosidase activity was found to be about 1% of the value in control fibroblasts, and it was less than 10% of the values found in fibroblast cultures from the heterozygous parents.

TABLE 1

ACID AND NEUTRAL a-1,4-GLUCOSIDASE ACTIVITIES* OF VARIOUS CELL TYPES IN PATIENTS WITH GLYCOGENOSIS II

Patient Code	Leucocytes			Muscle		
	acid	neutral	ratio	acid	neutral	ratio
I Bi 3	4.2	7.1	0.60	1.26	2.54	0.46
II No1				0.17	0.65	0.26
III Nie 1						
IV Vo 3	6.0	11.0	0.54			
Controls (n=34)	5.6-16.3	5.0-17.9	0.8-1.3	4.1-21.4	1.4-2.7	3.4-7.9(n=9)
V Co 1**	0.0	0.19	0			
Controls**(n=16)	0.39-0.89	0.21-0.69	1.1-1.8			

* Enzyme activities were measured with maltose as substrate at pH 4.0 and 6.5 and expressed in 10^{-9} mol glucose formed/min/mg protein.

** In these analyses enzyme activities were measured with glycogen as substrate at pH 4.0 and 6.5 and expressed in the same units.

TABLE 2

PRENATAL DIAGNOSIS OF POMPE'S DISEASE USING HOMOGENATES OF CULTURED AMNIOTIC FLUID CELLS

a-1,4-GLUCOSIDASE ACTIVITY* (x 10^{-12} mole/min/mg. protein)

Patient code and cultivation period	Pregnancy at risk**	Control amniotic fluid cells°	Control fibroblasts	Heterozygous fibroblasts	Pompe's fibroblasts
I Bi 4 (20 days)	26	381	2760 3280	353 ♀ 480 ♂	28
II No 4 (17 ")	501	1049	4130	1654 ♀	59
III Nie 3 (22 ")	680	1227 715	1598 5326	2120 ♀ 822 ♂	21
IV Vo 4 (14 ")	112	614	1010	475 ♀	27
V Co 2 (12 ")	104** and 391***	261**		372 ♀	25

* Enzyme activity was determined with methylumbelliferyl substrate and in microvolumes.

** Analyses in primary cultures and *** after two subcultivations of the same cell strain.

° All control samples were later passages except for ** which was a primary culture.

Assays on neutral glucosidase as a reference showed activities in the control range. The parents were informed of the presence of an affected fetus and they requested interruption of the pregnancy. Analyses on fetal tissues after induced abortion with hypertonic saline showed a deficiency of acid glucosidase activity (Table 3) which confirmed the prenatal diagnosis of Pompe's disease.

In family II No the first child (II No 1) died at the age of 2 months with symptoms of generalized muscle hypotonia, cardiorespiratory insufficiency and hepatosplenomegaly. A deficient acid α-glucosidase activity was found in a muscle biopsy. In a second pregnancy in 1969 amniocentesis was performed but enzyme assays could only be performed on the amniotic fluid supernatant; a normal value was observed and fortunately a healthy girl was born (Table 3, II No 2). In the third pregnancy amniocentesis was carried out in the 15th week but the mother came into spontaneous labour in the 19th week. A deficient acid α-glucosidase activity was observed in various fetal tissues (II No 3, Table 3). In the fourth pregnancy enzyme assays in cultured amniotic fluid cells were performed 17 days after amniocentesis (Table 2, II No 4). The activity was about half of the value in control amniotic fluid cells and about 30% of that in fibroblasts from the heterozygous mother. As the activity was about 10 fold higher than that in fibroblasts from an affected sibling (Table 2) we decided that the fetus could be a carrier or normal. After the birth of a healthy girl enzyme analyses on leucocytes of umbilical cord blood showed a ratio of acid to neutral α-glucosidase activity which was intermediate between the heterozygote and control range when maltose was used as a substrate (Table 3). With glycogen as substrate enzyme levels were found in the range of heterozygotes (22).

In family III Nie the first male child died at 6 months of shock and cardiopulmonary failure. Microscopic examination at autopsy showed vacuolization and glycogen accumulation in heart muscle fibers; no abnormalities were found

TABLE 3

CONFIRMATION OF DIAGNOSIS AFTER PRENATAL MONITORING

Patient material	Acid α-1,4-Glucosidase activity			Ratio $\frac{acid}{neutral}$ Glucosidase*	Diagnosis in neonate or in abortion material
	Muscle	Heart	Liver	in Leucocytes	
I Bi 4 abortion	1.2	0	0.4		M. Pompe
II No 2 neonate				0.78	Heterozygote or normal
II No 3 abortion	0.9				M. Pompe
II No 4 neonate				0.93	Heterozygote or normal
IV Vo 4 abortion	9.32	6.2	23.8		Heterozygote or normal
V Co 2 neonate				0.98	Heterozygote or normal
Control subject	4.2 - 21.4 (n=9)		21.7 - 38.8 (n=4)	0.8 - 1.3 (n=34)	
Heterozygotes				0.63 - 0.97 (n=12)	
M. Pompe	0 - 1.6 (n=16)	0	0.1 - 0.5 (n=3)	0.25 - 0.71 (n=11)	

* α-1,4-glucosidase activities were assayed with maltose as the substrate at pH 4.0 and pH 6.5 and expressed as nmoles glucose formed/min/mg protein.

in other organs. No biochemical studies were performed and a tentative diagnosis of Pompe's disease was made. The second child was a healthy girl. In the third pregnancy prenatal monitoring was requested. Amniocentesis was performed and the acid glucosidase activity in the cultured amniotic fluid cells (III Nie 3, Table 2) was comparable with that in a control sample and the fetus seemed not affected. The acid glucosidase activity in fibroblasts from both parents showed quite high values compared with most other values in obligate heterozygotes (Table 2). Later α-glucosidase assays in the leucocytes of the parents were carried out using glycogen as substrate. Activities in the control range were observed which made it unlikely that the first affected sibling had died from Pompe's disease. Unfortunately, no cell material of the affected sibling was available for enzyme studies. If biochemical assays had been performed or cultured cells had been stored the amniocentesis could have been avoided. The third pregnancy resulted in a healthy girl who had a normal ratio of acid to neutral α-glucosidase in her leucocytes using glycogen as substrate.

In family IV Vo the first child died at the age of three months and the diagnosis of Pompe's disease was based on morphologic studies at autopsy. A second child was healthy. In the third pregnancy amniocentesis was carried out in the 14th week but *in vitro* growth failed and enzyme assays could be performed only on the amniotic fluid supernatant. A normal value was found but the parents were informed about the limitations of this assay. They decided to continue the pregnancy, but an affected child (IV Vo 3) was born with cheilopalatoschizis, muscular hypotonia, cardiomegaly, and a deficient acid α-glucosidase activity in the leucocytes (Table 1). In the fourth pregnancy amniocentesis was performed in the 15th week and the results of enzyme assays on cultured amniotic fluid cells were available two weeks later (Table 2, IV Vo 4). The acid α-glucosidase activity was quite low compared with the control sample and with prenatal analyses performed earlier. The

activity was about 25% of that of the fibroblasts from the heterozygous mother and about four times higher than in fibroblasts from the affected sibling. The fetus could have been a Pompe patient with a relatively high residual enzyme activity or a heterozygote with a very low activity. The parents were informed that the fetus was likely to be affected and the pregnancy was terminated at their request in the 17th week. No deficiency of acid α-glucosidase activity could be demonstrated in various fetal organs. We then speculated that the mistake in diagnosis was due to the fact that short term primary cultures of amniotic fluid cells from the pregnancy at risk had been compared with later passages from control amniotic fluid cell strains. The acid α-glucosidase activities in various amniotic fluid cell samples were then measured at different periods of time in primary culture and were compared with the enzyme activities in subcultured cells at different intervals after the last subculture (Fig. 1).

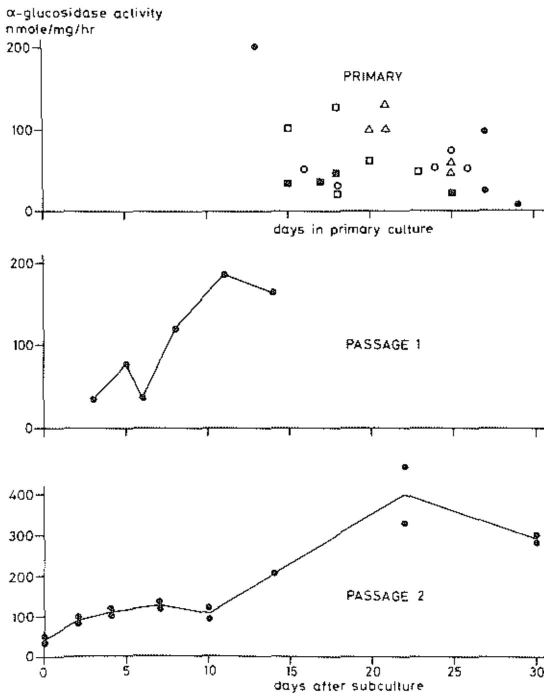

FIGURE 1

Relations between acid α-1,4-glucosidase activity and _in vitro_ cultivation conditions of amniotic fluid cells. Different symbols represent cultures from different amniotic fluid samples. ● = cells from the same amniotic fluid sample at different time intervals in primary culture and after subcultivation.

Acid α-glucosidase activity showed no obvious correlation with time in culture whereas the specific activity in subcultured cells showed a definite increase with time after subculture.

In the fifth pregnancy amniocentesis was performed and the enzyme activity in a 12-day culture was compared with that of a primary culture of the same duration from a control sample. The acid α-glucosidase activity in cells from the pregnancy at risk showed normal values and the pregnancy ended at term with the birth of a healthy child.

In family V Co the first child died at 13 months of age with the classical symptoms of Pompe's disease. Biochemical assays on leucocytes revealed an acid α-glucosidase deficiency (Table 1). In the second pregnancy enzyme assays on cultured amniotic fluid cells could be carried out 10 and 14 days after the second amniocentesis in the 17th week. The results in Table 2 (V Co 2) show that the acid α-glucosidase activity in cells from the pregnancy at risk was 104 picomole/min/mg protein, whereas a value of 391 was found in the second subculture of these cells. In control amniotic cells an activity of 261 was found at 21 days after initiation of a primary culture. From these results it was concluded that the fetus was not affected with Pompe's disease. The pregnancy was continued and a boy was born at term; the acid α-glucosidase activity in his leucocytes was in the range between heterozygotes and controls with maltose as substrate (Table 3).

The results of these analyses indicate that for a reliable prenatal diagnosis, α-glucosidase activities in short term (10 - 20 days) amniotic fluid cell cultures from pregnancies at risk should be compared with primary cultures of control amniotic fluid samples that have been grown under similar conditions.

TABLE 4

MICROCHEMICAL ANALYSIS OF α-1,4-GLUCOSIDASE IN DISSECTED GROUPS OF FREEZE-DRIED AMNIOTIC CELLS FROM A PREGNANCY AT RISK FOR POMPE'S DISEASE (III Nie 3)

Number of dissected cells	160	160	100	120	115	165	200	110	95	130
Fluorescence intensity* (relative units)	11	12	9	15	12	12	20	10	9	10
α-1,4-Glucosidase Activity ($\times 10^{-14}$ mol/hr/cell)	1.0	1.0	1.1	1.8	1.4	1.0	1.5	1.2	1.0	1.0

Mean activity in pregnancy at risk : 1,2 (cell homogenate: 1,3 $\times 10^{-14}$ mol/hr/cell)

Mean activity control amniotic fluid cells : 2,2

Mean activity fibroblasts Pompe : no detectable activity ($\leq 0,3 \times 10^{-14}$ mol/hr/cell)

*Blank values of 3% (range 2-5%) consisted of 10 empty pieces of plastic foil dissected adjacent to cell groups

2. PRENATAL DIAGNOSIS USING MICROANALYSIS OF ISOLATED CELL GROUPS

In the last three pregnancies at risk (III Nie 3, IV Vo 4, V Co 2) part of the amniotic fluid sample was used for microchemical analysis of isolated groups of freeze-dried cultured cells. This technique does not require parallel protein assays and harvesting can be carried out as soon as small cell clones appear. As a consequence prenatal diagnosis can be completed 10 - 14 days after amniocentesis during the 14th - 15th week of pregnancy.

Table 4 shows the acid α-1,4-glucosidase activities determined by this technique in 10 dissected groups of amniotic fluid cells from a pregnancy at risk (III Nie 3), in control amniotic fluid cells and in fibroblasts from a patient with Pompe's disease. The fluorescence intensities are well above the blank values for all groups of cells from the pregnancy at risk and the mean α-glucosidase activity was found to be about half the value of that of control amniotic fluid cells. No acid α-1,4-glucosidase activity was detected in dissected groups of fibroblasts from a patient with Pompe's disease. The data obtained indicated either a normal fetus or a heterozygous carrier. The mean glucosidase activity found in dissected cells from the pregnancy at risk are in good agreement with the enzymatic activity determined in cell homogenates from the same sample (Table 4).
To test the validity of microchemical enzyme analysis on dissected groups of cells, the results of such assays on various cell strains have been compared with data obtained by analysis on cell homogenates (Table 5). In the latter the enzyme activity is expressed as both per milligram of protein and per cell as calculated from the cell number of the same before homogenization and from data on the protein content of single cultured cells ($2 - 3 \times 10^{-10}$g) as determined by interference microscopy. These comparative studies indicate a good agreement between the results of the two methods for primary cultures of

TABLE 5

COMPARISON OF MICROANALYSIS OF α-1,4-GLUCOSIDASE ACTIVITY USING DISSECTED GROUPS OF FREEZE-DRIED CELLS AND CELL HOMOGENATES

TYPE OF CELL STRAIN		DISSECTED CELL GROUPS*	ENZYME ACTIVITY CELL HOMOGENATE	
			per cell $\times 10^{-14}$ mol/hour	per mg protein $\times 10^{-12}$ mol/min
Pregnancies at risk - III Nie 3		1.2	1.3	680
- IV Vo 4		0.3	0.2	123
- V Co 2**		0.4	0.2 and 0.8	104 and 391**
Control Amniotic Fluid Cells				
- primary culture***		0.3	0.5	261***
- later subcultures	(n = 2)	1.2; 2.1	1.1; 1.5	548; 763
Control Fibroblasts	(n = 4)	3.1-5.1	2.9-5.3	1440-2645
Heterozygous Fibroblasts		1.5	1.0	500
Pompe's Fibroblasts	(n = 3)	0.3	0.03 - 0.05	18-26

* Data are the mean of values from 10-26 groups of cells.

** Freeze-dried cells have been analyzed after 10 days growth, but cell homogenate studies were performed after 14 days in primary culture and after the 3rd subculture.

*** Analysis of freeze-dried control cells after 14 days primary culture, studies on cell homogenate after 21 days primary culture.

amniotic fluid cells and for later subcultures of normal amniotic fluid cells, fibroblasts from control subjects, patients with Pompe's disease and heterozygous carriers.

3. ANALYSIS OF α-1,4-GLUCOSIDASE IN AMNIOTIC FLUID SUPERNATANT

For five pregnancies at risk for Pompe's disease, where enzymatic analysis had been carried out on cultured amniotic fluid cells, the activities of acid and neutral α-glucosidase have been determined in the amniotic fluid supernatant. The results in Table 6 show that there was no correlation between the acid glucosidase activity in amniotic fluid supernatant and the status of the fetus. Furthermore, it is apparent that the ratio of acid to neutral glucosidase was rather constant in all cases tested.

TABLE 6

α-1,4-GLUCOSIDASE ACTIVITIES IN AMNIOTIC FLUID SUPERNATANT IN PREGNANCIES AT RISK FOR POMPE'S DISEASE

Patient code and time of amniocentesis	α-1,4-Glucosidase activity* ($x10^{-12}$ mol/min/mg protein) pH 4.0	pH 6.5	Ratio $\frac{acid}{neutral}$	Outcome of Pregnancy
I Bi 4 (14 wk)	421	1850	0.22	Heterozygote
II No 4 (17 wk)	759	3498	0.21	M. Pompe
II No 4 (19 wk)	216	1059	0.20	
III Nie 4 (15 wk)	196	823	0.23	Heterozygote or normal
IV Vo 3 (15 wk)	445	1899	0.23	Normal
V Co 2 (17 wk)	359	1465	0.24	Heterozygote or normal
Control (16 wk)	227	833	0.27	Normal

* In all analyses methylumbelliferyl-α-D-glucopyranoside was used as the substrate.

"It is difficult, to conceive of a diagnostic approach which must be made under greater "blind" conditions: an opportunity does not exist to check the laboratory diagnosis with the physical examination and clinical course of the patient".

Kirkman, 1972. (20)

DISCUSSION

For the prenatal diagnosis of Pompe's disease three potential sources of diagnostic material have been proposed: uncultured amniotic fluid cells (31), amniotic fluid supernatant (31), and cultured amniotic fluid cells (6, 30, 31). At present it is clear that only cultured fetal cells permit reliable prenatal monitoring. The analysis of uncultured amniotic fluid cells is complicated by contamination with maternal white blood cells (6), the large proportion of dead cells which lose part of their enzymatic activity (7, 32), and contamination with enzymes which may be present in the amniotic fluid supernatant (41). The absence of acid α-1,4-glucosidase activity in the amniotic fluid supernatant was reported in a pregnancy of a fetus with Pompe's disease (31) but later studies (30, 37) could not substantiate a relation between the enzyme activity in the fluid supernatant and that in the fetal cells. The results in the present paper (Table 6) are in agreement with this last finding. Neither acid α-1,4-glucosidase activity nor the ratio of acid to neutral glucosidase activity of the supernatant gave any conclusive information. This finding contradicts the assumption that acid α-1,4-glucosidase activity in the supernatant is mainly derived from fetal cell organelles (38). Another suggested source for acid α-1,4-glucosidase activity is an enzyme of renal origin with properties differing from the enzyme found in other tissues (37). The function

of this enzyme and its rapid disappearance from the amniotic fluid supernatant after the 20th week of pregnancy (9, 42, 43) needs further clarification. Since fetal urine contributes to amniotic fluid it is difficult to understand why Pompe's disease cannot be detected prenatally using amniotic fluid supernatant (9) if urine from patients can be used for enzymatic diagnosis after birth (39).

The only reliable material for prenatal diagnosis of Pompe's disease is cultured amniotic fluid cells. Our results (Table 2) showed that microchemical analysis of α-glucosidase activity (13) in cell homogenates permits prenatal diagnosis 14 - 20 days after amniocentesis during the 15th week of pregnancy. When microanalyses were carried out on small groups of dissected freeze-dried cells (Table 4 and 5) a further reduction to 10 - 12 days was realized. Comparable procedures have been developed for the prenatal detection of a number of other lysosomal diseases (11,12, 14). The advantage of these micro-methods is that a prenatal diagnosis can be completed before the mother experiences fetal movements and that multiple analyses including karyotyping can be performed on a small amount of cell material. In addition assays of other, non-affected enzymes in the same culture can be performed to test enzyme preservation in the sample used.

Although the results of α-1,4-glucosidase analyses on small groups of dissected freeze-dried cells correspond very well with assays on cell homogenates, the former method has some limitationsif a conventional fluorometer is used. Groups of 100 - 300 freeze-dried cells can be accurately analyzed with methylumbelliferyl substrates only if the enzyme activity is higher than 0.3 - 1x 10^{-14} mol/hr/cell (which corresponds to about 150 pmol/min/mg protein). In order to distinguish cells from heterozygous carriers from cells from affected patients enzymatic activities higher than this level should be present. This is the case for enzymes with relatively high activities in cultured cells such as those involved in GM_1 and GM_2 gangliosidoses. In some instances of Fabry's disease (14) and Pompe's disease (see Table 5) the enzymatic activity

in cells from heterozygous fetuses may be below the level of detection and erroneous interpretation is possible. Mistakes can be avoided either by microanalysis on cell homogenates in the case that more cell material is available or by the use of more sensitive microfluorometric methods (12, 13).

A prerequisite for prenatal monitoring should be that the diagnosis of the metabolic disease in the affected sibling is based on the demonstration of the enzyme defect in tissues or cultured cells. In two of the presently described cases such data were not available and the only possibility remaining is carrier detection in both parents. For this purpose the use of glycogen as substrate (22) has given the best results so far, although in larger series there is some overlap between control and heterozygote values.

For a reliable distinction between normal, heterozygous, and enzyme-deficient cells the use of appropriate controls is required. The residual activity of acid α-glucosidase shows considerable variations in fibroblasts from different affected patients and in different heterozygous carriers (see Table 2). Part of this variation might be due to differences in cell cultivation conditions (3-5, 8, 23, 35, 36, 40), but also the residual enzyme activity may in part be genetically determined. Therefore, in prenatal diagnosis the enzymatic assays of cells from a pregnancy at risk should be compared with control amniotic fluid cells and with cultured fibroblasts from heterozygotes and an affected patient in that particular family. For this purpose parents of a child affected with a metabolic disease which can be detected *in utero* (29) should be informed in time about this possibility and skin biopsies should be taken so that cells can be stored until required for prenatal monitoring in a subsequent pregnancy.

Ideally, control amniotic fluid cell cultures should be from the same gestational period and grown for the same period of time as those from the pregnancy at risk. The use of later subcultures of control samples may lead to erroneous interpretations such as described for patient IV Vo 3 (see Table 2).

The activity of acid α-glucosidase(Fig. 1) and also of several other lysosomal enzymes is usually low in primary cultures and unpredictable variations have been observed at different time intervals after initiation of a primary culture. In this respect possible differences in enzyme activity between epitheloid and fibroblast-like amniotic fluid cells (15, 19) should be further investigated. In later subcultures of amniotic fluid cells the activity of acid α-glucosidase increases during prolonged cultivation (2 - 3 weeks) after the last subculture. A similar effect has been reported by others and in fibroblasts the activity of several enzymes was found to increase considerably during growth in confluency (3, 4, 8, 35, 40). The use of primary cultures of control amniotic fluid cells has been facilitated by methods for storing samples of uncultured amniotic fluid cells with maintenance of cell viability (33).

Of course, therapy of Pompe's disease by enzyme substitution would be preferable to preventive control by prenatal diagnosis followed by selective abortion, but preliminary results of therapeutic approaches (1, 17) warrant the conclusion that at present genetic counseling and prenatal diagnosis are the only practical means for prevention of this disease.

SUMMARY

1. In order to reduce the time interval between amniocentesis and prenatal diagnosis of Pompe's disease microchemical techniques were used for assay of acid α-1,4-glucosidase activities in cultured amniotic fluid cells.

2. Microtechniques used on homogenates of cultured amniotic fluid cells enabled the waiting period to be reduced to 2 - 3 weeks.

3. When dissected lyophilized groups of 200 - 300 cultured cells were analyzed, a prenatal diagnosis was possible at about 10 days after amniocentesis.

4. The acid α-1,4-glucosidase activity in the amniotic fluid supernatant is not informative in prenatal diagnosis of Pompe's disease.

5. Conditions of cell cultivation such as length of time in culture were found to influence markedly the acid α-1,4-glucosidase activity in cultured amniotic fluid cells.

6. For a reliable prenatal diagnosis of metabolic disorders primary cultures of control amniotic fluid cells should be used and the analytical results from the pregnancy at risk should be compared with primary cultures of control amniotic fluid cells and with those in cultured fibroblasts from heterozygous carriers, and an affected sibling from the particular family.

REFERENCES AND NOTES

1. Barsy, T. de, Jaiquemin, P., Van Hoof, F., and Hers, H.G.: Enzyme replacement in Pompe's disease: an attempt with purified human acid α-glucosidase. Birth Defects: Original Article Series, vol IX, no 2, 184 (1973).
2. Brady, R.O., Uhlendorf, B.W., and Jacobson, C.B.: Fabry's disease: antenatal detection. Science, 172: 174 (1971).
3. Butterworth, J., Sutherland, G.R., Broadhead, D.M., and Bain, A.D.:Lysosomal enzymes of cultured amniotic fluid cells. Clin.Chim.Acta, 44: 295 (1973).
4. Butterworth, J., Sutherland, G.R., Broadhead, D.M., and Bain, A.D.: Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. Life Sci., 13: 713 (1973).
5. Ceccarini, C., and Eagle, H.: pH as a determinant of cellular growth and contact inhibition. Proc. Nat. Acad. Sci. U.S.A., 68: 229 (1971).
6. Cox, R.P., Douglas, G., Hutzler, J., Lynfield, J., and Dancis, J.: In-utero detection of Pompe's disease. Lancet, i: 893 (1970).
7. Davidson, R.G., and Rattazzi, M.C.: Prenatal diagnosis of genetic disorders: trials and tribulations. Clin. Chem., 18: 179 (1972).
8. DeMars, R.: Some studies of enzymes in cultivated human cells. Natl. Cancer Inst. Monogr., 13: 181 (1964).
9. Fluharty, A.L., Scott, M.L., Porter, M.T., Kihara, H., Wilson, M.G., and Towner, J.W.: Acid α-glucosidase in amniotic fluid. Biochem. Med., 7: 39 (1973).
10. Fratantoni, J.C., Neufeld, E.F., Uhlendorf, B.W., and Jacobson, C.B.: Intrauterine diagnosis of the Hurler and Hunter syndromes. New Engl. J. Med. 280: 686 (1969).
11. Galjaard, H.: Techniques for rapid prenatal diagnosis of some inborn errors of metabolism. (Abstract) Pediat. Res. 7: 56 (1973).
12. Galjaard, H., Hoogstraten, J.J. van, Josselin de Jong, J.E. de, and Mulder, M.P.: Methodology of the quantitative cytochemical analysis of single or small numbers of cultured cells. Histochem. J. 6: 409 (1974).
13. Galjaard, H., Mekes, M., de Josselin de Jong, J., and Niermeijer, M.F.: A method for rapid prenatal diagnosis of glycogenosis II (Pompe's disease). Clin. Chim. Acta 49: 361 (1973).
14. Galjaard, H., Niermeijer, M.F., Hahnemann, N., Mohr, J., and Sørensen, S.A.: An example of rapid prenatal diagnosis of Fabry's disease using microtechniques. Clin. Gen. 5 : 368 (1974).
15. Gerbie, A.B., Melancon, S.B., Ryan, C., and Nadler, H.L.: Cultivated epithelial- like cells and fibroblasts from amniotic fluid: Their relationship to enzymatic and cytologic analysis. Amer. J. Obstet. Gynec. 114: 314 (1972).

16. Glick, D.: Quantitative chemical techniques in histo- and cytochemistry. Vols. I & II, (Interscience Publishing Co., New York, 1961, 1963).

17. Hug, G., Schubert, W.K., and Soukup, S.: Treatment related observations in solid tissues, fibroblast cultures and amniotic fluid cells of type II glycogenosis, Hurler disease and metachromatic leucodystrophy. Birth Defects: Original Article Series, Vol IX, No 2, p. 160 (1973).

18. Huijing, F., van Creveld, S., and Losekoot, G.: Diagnosis of generalized glycogen storage disease (Pompe's disease). J. Pediat., 63: 984 (1963).

19. Kaback, M.M., Leonard, C.O., and Parmley, T.H.: Intrauterine diagnosis: Comparative enzymology of cells cultivated from maternal skin, fetal skin, and amniotic fluid cells. Pediat. Res., 5: 366 (1971).

20. Kirkman, H.: Enzyme defects. Progr. Med. Genet., 8: 125 (1972).

21. Koster, J.F., Slee, R.G., and Hülsmann, W.C.: The use of leucocytes as an aid in the diagnosis of a variant of glycogen storage disease type II (Pompe's disease). Eur. J. Clin. Invest., 2: 467 (1972).

22. Koster, J.F., Slee, R.G., and Hülsmann, W.C.: The use of leucocytes as an aid in the diagnosis of glycogen storage disease type II (Pompe's disease). Clin. Chim. Acta, 51: 319 (1974).

23. Lie, S.O., Schofield, B.H., Taylor, H.A., Jr., and Doty, S.B.: Structure and function of the lysosomes of human fibroblasts in culture: dependence on medium pH. Pediat. Res., 7: 13 (1973).

24. Littlefield, J.W.: Problems in the use of cultured amniotic fluid cells for biochemical diagnosis. Birth Defects: Original Article Series, Vol VII, No 5, p. 15 (1971).

25. Lowry, O.H.: The quantitative histochemistry of the brain: histological sampling. J. Histochem. Cytochem., 1: 420 (1953).

26. Lowry, O.H., and Passonneau, J.V.: A flexible system of enzymatic analysis. (Academic Press, New York, 1972).

27. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265 (1951).

28. Matschinsky, F.: Quantitative histochemistry of glucose metabolism in the islets of Langerhans. In: U.C. Dubach and U. Schmidt: Recent advances in quantitative histo- and cytochemistry (Hans Huber, Bern, 1971).

29. Milunsky, A., and Littlefield, J.W.: The prenatal diagnosis of inborn errors of metabolism. Ann. Rev. Med., 23: 57 (1972).

30. Nadler, H.L., Bigley, R.H., and Hug. G.: Prenatal detection of Pompe's disease. Lancet, ii: 369 (1970).

31. Nadler, H.L., and Messina, A.M.: In-utero detection of type -II glycogenosis (Pompe's disease). Lancet, ii: 1277 (1969).

32. Navon, R., and Padeh, B.: Prenatal diagnosis of Tay-Sachs genotypes. Brit. Med. J., 4, 17 (1971).

33. Niermeijer, M.F., Halley, D., Sachs, E., Tichelaar-Klepper, C., and Garver, K.L.: Transport and storage of amniotic fluid samples for prenatal diagnosis of metabolic diseases. Humangenetik, 20: 175 (1973).

34. Nitowsky, H.M., and Grunfeld, A.: Lysosomal a-glucosidase in type II glycogenosis; activity in leucocytes and cell cultures in relation to genotype. J. Lab. Clin. Med., 69: 472 (1967).

35. Okada, S., Veath, M.L., Leroy, J., and O'Brien, J.S.: Ganglioside GM_2 storage disease: hexosaminidase deficiencies in cultured fibroblasts. Am.J.Hum.Genet., 23: 55 (1971).

36. Ryan, C.A., Lee, S.Y., and Nadler, H.L.: Effect of culture conditions on enzyme activities in cultivated human fibroblasts. Exp. Cell Res., 71: 388 (1972).

37. Salafsky, I.S., and Nadler, H.L.: Alpha-1,4-glucosidase activity in Pompe's disease. J. Pediat., 79: 794 (1971).

38. Salafsky, I.S., and Nadler, H.L.:Intracellular organelles and enzymes in cell-free amniotic fluid. Amer. J. Obstet. Gynecol., 111: 1046 (1971).

39. Salafsky, I.S., and Nadler, H.L.: Deficiency of acid alpha glucosidase in the urine of patients with Pompe's disease. J. Pediat., 82: 294 (1973).

40. Shanfield, B., and Pinsky, L.: Effect of population density on the specific activity of various acid hydrolases in human skin fibroblast monolayers. Can. J. Genet. Cytol., 14: 871 (1972).

41. Sutcliffe, R.G., and Brock, D.J.H.: Enzymes in uncultured amniotic fluid cells. Clin. Chim. Acta, 31: 363 (1971).

42. Sutcliffe, R.G., Brock, D.J.H., Robertson, J.G., Scrimgeour, J.B., and Monaghan, J.M.: Enzymes in amniotic fluid: A study of specific activity patterns during pregnancy. J. Obstet. Gynaec. Brit. Commonw., 79: 895 (1972).

43. Sutcliffe, R.G., and Brock, D.J.H.: Observations on the origin of amniotic fluid enzymes. J. Obstet. Gynaec. Brit. Commonw., 79: 902 (1972).

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PAPER VI

An example of rapid prenatal diagnosis of Fabry's disease using microtechniques

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In a pregnancy at risk for Fabry's disease, a prenatal diagnosis could be established 11 days after amniocentesis in the 15th week of pregnancy. This was possible by microchemical analysis of the α-galactosidase activity in isolated groups of 100 – 200 freeze-dried, cultured amniotic fluid cells. The results of this microassay were confirmed by (micro)biochemical analysis in cell homogenates from replicate cultures performed in two independent laboratories. Some problems in prenatal diagnosis of heterozygotes are discussed, as well as the possibilities of microchemical techniques for the more rapid prenatal diagnosis of other lysosomal storage diseases.

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During the last few years it has been shown that the specific metabolic defect in more than 30 inborn errors of metabolism is expressed in cultured skin fibroblasts and in amniotic fluid cells, thus enabling their prenatal detection (for reviews see Milunsky et al. 1970, Nadler 1971, Milunsky & Littlefield 1972). In most instances so far reported, amniocentesis has been performed between the 14th and 20th week of pregnancy, and a period of amniotic fluid cell culture varying from 4 to 10 weeks was necessary to obtain sufficient cell material for biochemical analysis (Fratantoni et al. 1969, Schulman et al. 1970, Milunsky et al. 1970, Nadler 1972). A long waiting period implies not only psychological stress for the parents but termination of a pregnancy at a later stage may also involve obstetrical problems (Olsen et al. 1970).

The application of microchemical techniques, enabling biochemical assays on very little cell material, offers the possibility of reducing the waiting period. The principles of such methods for the purpose of histochemical studies on tissue sections have been described mainly by Lowry (1953), Matschinsky (1971), Lowry & Passoneau (1972) and Glick (1961, 1963). The adaptations of some of these methods for the analysis of small numbers of cells, or even single cultured cells, have already been briefly described (Mulder & Galjard 1972, Galjaard et al. 1972a, Galjaard 1972) and the first examples of the application of these methods in prenatal diagnosis have been reported (Galjaard et al. 1972b, Galjaard 1973, Galjaard et al. 1973).

The present paper deals with the application of such microtechniques in the sec-

ond reported case of prenatal monitoring for Fabry's disease. After the identification of the primary biochemical defect of this disease (Brady et al. 1969), the recognition by Kint (1970) that artificial substrates could be used for the determination of an α-galactosidase deficiency in leucocytes greatly simplified the diagnosis of Fabry's disease. Romeo & Migeon (1970) demonstrated the presence of α-galactosidase activity in normal cultured fibroblasts, which suggested that prenatal detection would be feasible. The first example of prenatal diagnosis of Fabry's disease was described by Brady et al. (1971), who analyzed α-galactosidase activity with p-nitrophenyl substrate in cultured amniotic fluid cells 4 weeks after amniocentesis at the 17th week of pregnancy.

The prenatal diagnosis to be described here was established 11 days after amniocentesis in the 15th week of pregnancy, using microassays on isolated groups of cultured amniotic fluid cells. The data obtained were compared with those of conventional analyses on cell homogenates. These results will be discussed together with some problems concerning the prenatal detection of female heterozygotes.

Materials and Methods

At transabdominal amniocentesis in the 15th week of pregnancy (as counted from the first day of the last menstruation) approximately 20 ml of amniotic fluid was obtained. The fluid was not visibly sanguine, although some erythrocytes were observed microscopically. The sample was divided into two, one aliquot being examined at the University Institute of Medical Genetics, Copenhagen, the second being sent to the Department of Cell Biology and Genetics, Erasmus University, Rotterdam, to be examined by micro-methods. In addition, this procedure gained the additional reliability given by making diagnostic tests in two independent laboratories.

The first aliquot was cultured and examined at the University Institute of Medical Genetics as follows: centrifugation for 10 min at 1000 rev/min; resuspension of cell pellet in 4 ml Eagle's medium supplemented with fetal calf serum (20%); culturing in two Leighton tubes and subsequent transfer to Falcon flasks, 11 changes of medium in 25 d of culturing; for the purpose of sex determination by karyotyping, a cell sample had been taken out earlier, which showed male sex at 12 d after amniocentesis. For the purpose of α-galactosidase assay, the cells were scraped from the bottom of the Falcon flasks, washed three times with physiological saline, resuspended in 0.7 ml 0.25 mol/l acetate buffer, pH 4.5, disintegrated by sonic vibration and centrifuged for 15 min at 15000 rev/min at 4°C.

The α-galactosidase activity was assayed according to Kint (1970) and Brady et al. (1971). 200μl of substrate (0.05 mol/l p-nitrophenyl-α-D-galactoside in 0.25 mol/l acetate buffer, pH 4) was added to 200 μl of amniotic fluid cell homogenate and the mixture incubated at 37°C for 4 h. After incubation, 400μl of 1 mol/l tris, pH 10.5 was added and the extinction was measured at 400nm in a Beckman DB spectrophotometer. The protein content of the reaction mixture was measured according to Lowry et al. (1951). Three samples of cultured amniotic fluid cells from normal pregnancies served as controls (Table 3). Additionally, the enzymatic activities were determined (3 h incubation) in various fibroblast cultures: from the consultant, "L", from a normal man, "S", from two heterozygotes, "M" and "O", and from a male patient, "P".

Enzymatic activities were determined in leucocytes for the consultant, "L", her normal half brother, "S", her carrier aunt, "O", as well as her Fabry cousin, "P".

The procedure was: collection of a 20 ml blood sample in heparin, separation of leucocytes, washing three times in physiological saline, resuspension of cell pellet, and assay as described above for cultured cells.

The second aliquot of the amniotic fluid sample was examined as follows (Dept of Cell Biology and Genetics, Erasmus University, Rotterdam):

a. Microchemical analysis of α-galactosidase activity in small clones of freeze-dried cells
After centrifugation (5 min at 1000 rev/min) of the 10 ml amniotic fluid sample (8700 cells per ml) the cell pellet was resuspended in 3 ml Ham's F10 medium, supplemented with 20% fetal calf serum. The sample was divided into three parts and cultured in two 35 mm plastic Falcon petri dishes and in a "mylar dish" consisting of a glass ring (5 cm diam.) and a base of thin plastic ("Melinex" polyester film type 0, ICI). After 10 d cultivation in the mylar dish, a number of clones, mainly consisting of epithelial-like cells, were observed. The dish was quickly frozen in a mixture of CO_2 and acetone (−70°C) and subsequently freeze-dried overnight in vacuo (10^{-3} mmHg) at −45°C using a WKF freeze-drying apparatus (Wetzlar instr. B.V.). After raising the temperature to 20°C the vacuum was released, and small pieces of the plastic base of the dish, containing a counted number of freeze-dried cells, were dissected out under a stereomicroscope. About 10–15 pieces of plastic, each with 100–250 cells (see Table 1) were used for microchemical analysis.

The dissected pieces of film, containing small numbers of cells from various cell strains, were incubated in 0.3 – 0.6 μl of 5 mmol/l methylumbelliferyl-α-D-galactoside in 0.1 mol/l acetate buffer pH 4.5 for 2 h at 37°C. Incubation was carried out using the "oil well technique" (Matschinsky 1971) in which small volumes of substrate are introduced into wells in a teflon rack and covered with a mixture of hexadecane and paraffin oil (40%:60% v/v) to prevent evaporation. The microvolumes are transferred under microscopic control with specially constructed constriction micropipettes (Lowry & Passonneau 1972). After incubation, the reaction volume was pipetted into microcuvettes containing 500 μl 0.5 mol/l carbonate buffer pH 10.7. The fluorescence of the methylumbelliferone formed was read in a Perkin Elmer fluorometer (extinction 365 nm and fluorescence 448 nm). The data were compared with those from methylumbelliferone standards ($1 - 8.10^{-12}$ mol dissolved in substrate) and pieces of plastic film without any cells were used as a blank. The α-galactosidase activities were expressed per cell by dividing the fluorescence values by the counted number of cells on each incubated piece of film.

During the same period, control amniotic fluid cells from the same stage of a normal pregnancy, and fibroblasts from a control individual, from a patient with Fabry's disease (the latter cells obtained from, and diagnosis established by Prof. H. Loeb and Dr. E. Vamos, Dept. of Pædiatrics, Hospital St. Pierre, Bruxelles), and from a heterozygous carrier were cultured and prepared under the same conditions.

b. Microchemical analysis of α-galactosidase activity in cell homogenates
Parallel amniotic fluid cell cultures and control cells were prepared for conventional assays on cell homogenates. Cells were grown on Falcon dishes in Ham's F10 medium, supplemented with 15% fetal calf serum for the fibroblast cultures and with 20% for the amniotic fluid cells. After 14 d, sufficient cells were available for harvesting for microassays for protein and α-galactosidase activity. After trypsinization (0.25% trypsin), the cell number was counted in a haemocytometer. After washing and suspension in 0.01% bovine albumine solution (to a concentration of about 10^6

cells per ml) homogenization was carried out by sonic vibration. Subsequently, 1 μl samples of cell homogenate were incubated with 2 μl methylumbelliferyl substrate using the same "oil well technique" and identical conditions as mentioned above. After 2 h incubation at 37°C the reagent was transferred to 500 μl carbonate buffer and the fluorescence was read.

From each cell homogenate 5 μl samples were used for a protein assay according to Lowry (1953) adapted to a final volume of 60 μl. The extinctions were read in 20 μl microcuvettes which fitted a commercial Zeiss spectrophotometer. The α-galactosidase activities were expressed per mg protein and also per cell, based on the cell counting after trypsinization.

Case Report and Kindred

The consultant, "L", born 11th March, 1951, sought genetic counselling in 1969, at the University Institute of Medical Genetics, Copenhagen. Her father, "K" and four other male relatives, "I", "P", "A", and "B" had been diagnosed as having Fabry's disease (Fig. 1), according to Christensen Lou (1972). The consultant appeared to be clinically healthy, but was

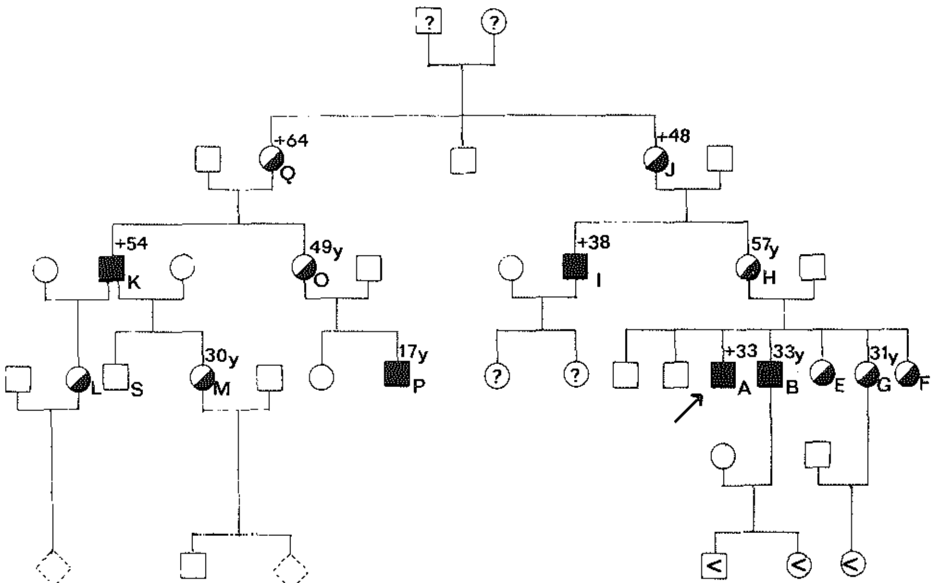

Fig. 1. Kindred of the consultant (L)

- Fabry's hemizygote
- Fabry's heterozygote
- Embryo
- Information inadequate
- Proband
- Relatives less than 10 years old, so far no symptoms or signs of Fabry's disease
- +38 Dead at the age of 38 years
- 30y 30 years old at the last examination

an obligate heterozygote, since the disease is X-linked and her father had been affected. This was confirmed by a slit lamp examination performed at Rigshospital, Copenhagen, 1969, which revealed the characteristic corneal opacities. The consultant gave a history of a few instances of buzzing paraesthesias in the fingers, accompanied by a temperature rise to about 38°C, as well as diffuse headache since the age of 5 years, and loss of consciousness for a few seconds on a single occasion (Christensen Lou 1972).

The consultant's father, born 1914, had died in 1968 at the age of 54 years. From the age of 5 years he showed episodes of fever, or pain and/or paraesthesias of the extremities, provoked by fever or overheating; from the age of 15 years a slowly progressive rash of dust- or grain-sized, elevated eruptions on the trunk and genitals; from the age of 25 years proteinuria and low back pain; from the age of 35 years episodes of paraesthesias spreading to most areas of the arms and to the face in the course of about 10 min, followed by throbbing, often one-sided, frontal headache. From the age of 44 years symptoms of functional loss in the central nervous system occurred, followed by deteriorating sight and death at 54 years from a cardiac insufficiency which had progressed gradually since the age of 39 years. The clinical features of the four remaining male relatives of the consultant were largely in agreement with this picture, "A" and "I" had died at the ages of 33 and 38 years, respectively, while "P" and "B" at the time of the examination (1969) were 17 and 33 years old, respectively, and seriously affected (Christensen Lou 1972).

The consultant had eight female carrier relatives, "Q", "J", "O", "H", "M", "E", "G" and "F" (Fig. 1), of whom "J" and "Q" had died at ages 48 and 64 years, from cardiac failure and mammary carcinoma, respectively. The remaining six carrier relatives, "O", "H", "M", "G" and "F" showed, at the time of the last clinical examination (at ages 49, 57, 30, 31 and 29 years, respectively), some symptoms of the disease such as episodes of pain in the extremities (all six), "Fabry rash" (four), corneal opacities (three), proteinuria (three), and cardiomegaly (two).

These findings confirm observations from other families that the Fabry carrier state may not be considered as clinically harmless, although the symptoms generally occur later, and are milder than in male patients (Christensen Lou 1972, Sweeley et al. 1972).

At the first counselling in 1969, only prenatal sex determination could be offered to prevent the birth of affected males. In consideration of the rapid development in this field, the consultant was informed that a technique for biochemical prenatal diagnosis might become available in the near future. When she became pregnant in 1972, a prenatal diagnosis was possible assaying the α-galactosidase activity in cultured amniotic fluid cells (Kint 1970, Brady et al. 1971). In the meantime, microtechniques had been adapted for the analysis of cultured fluid cells (Mulder & Galjaard 1972, Galjaard et al. 1972a, b) which enable a prenatal diagnosis at an earlier stage of pregnancy than is possible with conventional techniques.

Results

The first results of α-galactosidase activity in cultured amniotic fluid cells from the pregnancy at risk were obtained by the microassays performed in the Dept. of Cell Biology and Genetics at Rotterdam. Analyses on dissected groups of small numbers of freeze-dried cells (100 – 300 cells) (see Table 1) showed that the fluorescence intensities were well above the blank values.

Table 1

Microchemical analysis of α-galactosidase in clones of freeze-dried amniotic fluid cells from a pregnancy at risk for Fabry's disease

	Cell number per sample									
	260	240	190	150	250	200	120	215	145	110
Fluorescence intensity[1] (relative units)	31	42	31	30	23	31	23	33	19	16
α-Galactosidase activity (x10^{-14} mol /h/cell)	1.16	1.71	1.60	1.91	0.90	1.51	1.87	1.50	1.29	1.43

	Exp. I[3]	Exp. II[3]
Pregnancy at risk	X = 1.49 ± 0.31 (n = 10)	X = 1.88 (n = 6)
Control amniotic fluid cells	X = 2.76 ± 0.46 (n = 16)	X = 2.19 (n = 8)
Control fibroblasts	X = 2.60 ± 0.60 (n = 20)	X = 2.20 (n = 15)
Fabry's fibroblasts	X = 0.70[2] (n = 21)	X = 0.70 (n = 15)

[1] The blank values were 2–4% (empty pieces of plastic film dissected adjacent to cell groups).
[2] In a number of clones (10) no activity could be detected, hence calculation of the standard deviation did not seem informative.
[3] Mean activities ± s.d.

The enzyme activity, expressed per cell, showed a considerable variation between the various cell groups dissected. The same was found for all other cell strains tested, as is indicated by the standard deviations. The mean α-galactosidase activity for the pregnancy at risk was found to be 1.49 × 10^{-14} mol/h/cell. An independent experiment (exp. II in Table 1) performed the next day on cells from the same culture dish showed a somewhat higher activity (1.88 × 10^{-14} mol/h/cell). These values are very close to those found for control amniotic fluid cells and for control fibroblasts. The enzymatic activity in fibroblasts from a patient with Fabry's disease was much lower, although a residual activity of about 30% was present.

Microchemical analyses of α-galactosidase activity in cell homogenates, performed a few days later (Table 2), also showed similar values for the pregnancy at risk and for control cell strains. The activity in fibroblasts from a heterozygous carrier was somewhat lower, and that in fibroblasts from a patient with Fabry's disease again showed a residual activity of about 30%. When these data on cell homogenates

Table 2

Microchemical analysis of α-galactosidase in homogenates from various amniotic fluid cell and fibroblast strains

	Protein content (x10^{-6} g/ml)	α-galactosidase activity (mol/h) per mg	protein per cell*
Amniotic fluid cells pregnancy at risk	325	62 × 10^{-9}	1.9 × 10^{-14}
Control amniotic fluid cells	330	65 × 10^{-9}	1.7 × 10^{-14}
Control fibroblasts	340	52 × 10^{-9}	1.6 × 10^{-14}
Heterozygote Fabry fibroblasts	270	34 × 10^{-9}	1.3 × 10^{-14}
Fabry fibroblasts	360	18 × 10^{-9}	0.6 × 10^{-14}

* Enzyme activities per cell are based on counts of cultured cells after harvesting (these counts involve an error of 10–15%).

were expressed per cell (based on cell counting after trypsinization) a remarkable agreement exists with the analytical data as obtained by microassays on isolated groups of freeze-dried cells (compare last column Table 2 with Table 1).

A chromosome preparation from amniotic fluid cells grown in a Falcon dish at the tenth day after amniocentesis showed male sex, thus excluding the possibility of a heterozygous carrier. Hence, it could be concluded that the expected child would be a boy free from Fabry's disease. This diagnosis was available the eleventh day after amniocentesis.

The α-galactosidase activities of the cultured amniotic fluid cells from the pregnancy at risk, from control samples and of fibroblasts from the consultant, relatives and control individuals, as determined at the University Institute of Medical Genetics, Copenhagen, are shown in Table 3. The α-galactosidase activity in cells from the pregnancy at risk appeared to be the same as those in the three control samples. It could be concluded on the 25th day after amniocentesis i.e. in the 18th – 19th week of pregnancy, that the fetus was a male not suffering from Fabry's disease, which was in agreement with the analytical results obtained earlier. Using p-nitrophenyl substrate again, the α-galactosidase activity in normal amniotic fluid cells was comparable to that in control fibroblasts. The activities in the three heterozygotes, although showing considerable differences, were all lower than the controls. In the patient with Fabry's disease the α-galactosidase in fibroblasts showed a residual activity of 15 – 20% whereas in leucocytes no activity was found at all. The range of α-galactosidase activities in the different cell strains using p-nitrophenyl substrate was about two times lower than that with methylumbelliferyl substrate.

A male child was born after an uneventful pregnancy and delivery. Assay of α-galactosidase activity in a leucocyte preparation from peripheral blood showed a normal value (130.0×10^{-9} mol/hour/mg protein) confirming the prenatal diagnosis.

Table 3

α-galactosidase activities as determined by conventional techniques using p-nitrophenyl substrate

Cell type	Individuals (see Fig. 1)	α-galactosidase activity* $\times 10^{-9}$ mol/h/mg protein
Cultured amniotic fluid cells	Pregnancy at risk	30.6
	Control no. 476	29.8
	Control no. 474	21.7
	Control "Gøteborg"	22.3
Fibroblasts	"L" consultant	18.0
	"S" normal male	31.4
	"M" heterozygote	15.1
	"O" heterozygote	09.9
	"P" male Fabry patient	04.6
Leucocytes	"L" consultant	37.4
	"S" normal male	149.6
	"P" Fabry male	00.0
	"O" heterozygote	28.4

* The quantity of available material was sufficient for only one reading, therefore, standard deviations could not be assessed.

Discussion

Microtechniques compared with conventional methods

The results illustrate the feasibility of using microchemical analysis for a more rapid prenatal diagnosis than is possible with conventional analytical methods. In the present case, analysis could be carried out 11 d after amniocentesis using microtechniques, the prenatal diagnosis being available before the 17th week of pregnancy, while with conventional techniques it was available 2 weeks later. Confirmation of the prenatal diagnosis was obtained by α-galactosidase activity studies in leucocytes of the male child after birth.

The same principles may be applied to the monitoring of other metabolic defects when these involve spectrophotometric or fluorometric assays (Galjaard et al. 1972a, b, Galjaard 1973). Although in enzyme studies the activity for artificial substrates is usually less than for natural substrates, the simplicity of assays using artificial substrates gives an important advantage in microchemical analysis. In both spectrophotometry and fluorometry, less cell material is required for analysis when incubation and final volumes are reduced (Galjaard et al. 1972b, 1973). The extent to which the reaction volumes should be reduced depends on the activity of the enzyme to be analyzed and on the kind of assay. It may be deduced from the results for α-galactosidase shown in Table 1, that for enzyme activities in the order of 10^{-14} mol/h/cell, incubation volumes between 0.5 – 1 μl result in fluorescence values which are well above the blank values.

Reliability of micromethods

The values for α-galactosidase activities in various cell strains, as measured in cell homogenates (Table 2), correspond remarkably well with data obtained by other workers using the same methylumbelliferyl substrate (Wood & Nadler 1972, Ho et al. 1972). When expressed per cell, the data are also in fair agreement with the enzyme analyses on isolated freeze-dried groups of amniotic fluid cells, although the latter values are somewhat higher. This difference might be due to errors in cell counting and/or the fact that the accessibility of the enzyme for the substrate is generally better when freeze-dried material is incubated, as compared with other precedures (Bonting & Rosenthal 1960).

The variation in α-galactosidase activity per cell between the various dissected cell groups (Table 1) cannot yet be completely explained. The experimental errors in cell counting and the micro-assay procedure are relatively small (Galjaard et al. 1973), and it is likely that the cell density and the proportion of proliferative versus non-proliferative cells are mainly responsible for the variation observed. In comparing enzymatic activities of different cell strains, these factors, as well as the phase in the growth curve of the culture, should be considered. In normal fibroblast cultures the activity of α-galactosidase has been found to increase by a factor of 10 from 2 to 30 d after subculture (Heukels-Dully et al. to be published). The considerable variation between individuals shown in Table 3, may also be partly due to these factors.

Distinction between hemizygotes, heterozygotes, and normal homozygotes

Prenatal diagnosis of Fabry's disease and some other lysosomal storage disorders now seems possible within a relatively short period after amniocentesis, using the micromethods described. However, the reliability and usefulness of prenatal diagnosis of metabolic defects, both by means of microchemical assays and conventional analysis on cell homogenates, depend decisively on the ability to distinguish clearly between

cells from a homozygous (or hemizygous) patient, a heterozygous carrier and a normal control. In the case of Fabry's disease this may be difficult for three reasons. Firstly, a relatively high residual α-galactosidase activity (10 – 30%) is present in cells from Fabry's patients (Romeo & Migeon 1970, Brady et al. 1971, Romeo et al. 1972, Ho et al. 1972). Secondly, there may be genetic heterogeneity within the group of Fabry's patients (Clarke et al. 1971, Wood & Nadler 1972, Romeo et al. 1972). Thirdly, within the same culture of amniotic fluid cells from a heterozygous female fetus, two cell populations (deficient and normal cells) are present (Romeo & Migeon 1970), and a difference in growth between these two cell types might lead to erroneous interpretations. Such growth differences have been observed in fibroblast cultures from a heterozygous carrier for another X-linked disease, glucose-6-phosphate dehydrogenase deficiency (Galjaard 1972). However, in prenatal diagnosis the problem of distinction between hemizygotes and heterozygous carriers can be avoided by combining the biochemical assay af α-galactosidase with karyotyping of the amniotic fluid cells. When a female karyotype is found, microchemical analysis of single cells, or different clones of cultured amniotic fluid cells, should enable prenatal diagnosis of the heterozygous state (Galjaard 1972).

When sufficient knowledge is available about the residual enzyme activity in cells from patients from the particular family involved, and when proper controls, (i.e. primary cultures of control amniotic fluid cells at the same stage of pregnancy) are cultured under standardized conditions, reliable prenatal diagnosis of Fabry's disease is possible. When microtechniques are being used the diagnosis can be completed 10 – 14 d after amniocentesis at the 14th to 15th week of pregnancy.

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References

Bonting, S. L. & J. M. Rosenthal (1960). Effects of the method of tissue preparation on the assay of tissue enzyme activities. *Nature (Lond.)* **185**, 616–687.

Brady, R. O., B. W. Uhlendorf & C. B. Jacobsen (1971). Fabry's disease: Antenatal detection. *Science* **172**, 174–175.

Brady, R. O., A. E. Gal, R. M. Bradley, E. Mårtensson, A. L. Warshaw & L. Larter (1967). Enzymatic defect in Fabry's disease: ceramide trihexosidase deficiency. *New Engl. J. Med.* **276**, 1163–1167.

Christensen Lou, H. O. (1972). Angiokeratoma Corporis Diffusum (Fabry's Sygdom). Copenhagen, Polyteknisk Forlag.

Clarke, J. T. R., J. Knaack, J. C. Crawhall & L. S. Wolfe (1971). Ceramide trihexosidoses (Fabry's disease) without skin lesions. *New Engl. J. Med.* **284**, 233–235.

Fratantoni, J. C., E. F. Neufeld, W. Uhlendorf & C. B. Jacobson (1969). Intrauterine diagnosis of the Hurler and Hunter syndromes. *New Engl. J. Med.* **280**, 686–688.

Galjaard, H. (1972). Possibilities of quantitative histochemical analyses in prenatal detection and genetic classification of hereditary metabolic disorders. *Proc. 4th Int. Congr. Histo- Cytochemistry*. ed. Takeuchi, T., K. Ogawa & S. Fujita. Kyoto, Japan. p. 185–186.

Galjaard, H. (1973). Techniques for rapid prenatal diagnosis of some inborn errors of metabolism. *Pediat. Res.* **7**, 56.

Galjaard, H., M. Jahodova & M. F. Niermeijer (1972a). Possibilités et limitations dans le diagnostic prénatal de maladies génétiques. *Brux.–méd.* **52**, 109–121.

Galjaard, H., J. Fernandes, M. Jahodova, J. F. Koster & M. F. Niermeijer (1972b). Prenatal diagnosis of genetic disease. *Bull. Europ. Soc. Hum. Genetics* pp. 79–91.

Galjaard, H., M. Mekes, J. de Josselin de Jong & M. F. Niermeijer (1973). A method for rapid prenatal diagnosis of glycogenosis II (Pompe's disease). *Clin Chim. Acta* **49**, 361–375.

Glick, D. (1961 and 1963). *Quantitative chemical techniques in histo- and cytochemistry* Vol. I & II. New York, Interscience.

Ho, M. W., S. Beutler, L. Tennant & J. S. O'Brien (1972). Fabry's disease: evidence for a physically altered α-galactosidase. *Amer. J. hum. Genet.* **24**, 256–266.

Kint, J. A. (1970). Fabry's disease: α-galactosidase deficiency. *Science* **167**, 1268–1269.

Lowry, O. H. (1953). The quantitative histochemistry of the brain, histological sampling. *J. Histochem. Cytochem.* **1**, 420.

Lowry, O. H. & J. V. Passonneau (1972). *A flexible system of enzymatic analyses.* New York, Academic Press.

Lowry, O. H., N. J. Rosebrough, A. L. Farr & R. J. Randall (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

Matschinsky, F. (1971). Quantitative histochemistry of glucose metabolism in the islets of Langerhans. *Recent advances in quantitative histo- and cytochemistry*. ed. Dubach, U. C. & U. Schmidt pp. 143–180. Bern, Huber.

Milunsky, A. & J. W. Littlefield (1972). The prenatal diagnosis of inborn errors of metabolism. *Ann. Rev. Med.* **23**, 57–76.

Milunsky, A., J. W. Littlefield, J. N. Kanfer, E. H. Kolodny, V. E. Shih & L. Atkins (1970). Prenatal diagnosis. *New Engl. J. Med.* **283**, 1370–1381, 1441–1447. 1498–1504.

Mulder, M. P. & H. Galjaard (1972). Determination of enzyme activities in studies on gene dose effects in cultivated cells. *Acta histochem. (Jena)* Suppl. **12**, 289–290.

Nadler, H. L. (1971). Indications for amniocentesis in the early prenatal detection of genetic disorders. *Birth Defects: Original Articles Series* VII, No. 5, 5–9.

Nadler, H. L. (1972). Tissue culture and antenatal detection of molecular diseases. *Biochemie* **54**, 677–681.

Olsen, C. E., H. B. Nielsen & E. Østergaard (1970). Complications to therapeutic abortions. *Int. J. Gynec. Obstet.*, **8**, 823–829.

Romeo, G., B. Childs & B. R. Migeon (1972). Genetic heterogeneity of α-galactosidase in Fabry's disease. *FEBS Letters* **27**, 161–165.

Romeo, G. & B. R. Migeon (1970). Genetic inactivation of the α-galactosidase locus in carriers of Fabry's disease. *Science* **170**, 180–181.

Schulman, J. D., W. Y. Fujimoto, K. H. Bradley & J. E. Seegmiller (1970) Identification of heterozygous genotype for cystinosis in utero by a pulselabelling technique. *J. Pediat.* **77**, 468–470.

Sweeley, C. C., B. Klionsky, W. Krivit & R. J. Desnick (1972). Fabry's disease Glycospingolipid lipidosis. *The Metabolic Basis of Inherited Disease*. ed. Stanbury, J. B., J. B. Wijngaarden & D. S. Fiedrickson. New York, McGraw-Hill. pp. 668–687.

Wood, S. & H. L. Nadler (1972). Fabry's disease: absence of an α-galactosidase isoenzyme. *Amer. J. hum. Gen.* **24**, 250–255.

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PRENATAL DIAGNOSIS OF SOME LYSOSOMAL STORAGE DISEASES

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1. Summary

The similarity between cultured amniotic fluid cells and cultured fibroblasts indicates that prenatal diagnosis of those lysosomal storage diseases is possible when the enzymatic or metabolic defect is expressed in in vitro cultured cells from patients affected with the lysosomal disease involved. The application of microtechniques in the study of cultured amniotic fluid cells enabled the establishing of a diagnosis in 12-22 days after amniocentesis in five pregnancies at risk for Pompe's disease, in 16-18 days in two pregnancies at risk for metachromatic leukodystrophy and 11 days in a case for Fabry's disease. Hunter's disease could be excluded within 4 weeks after amniocentesis. Contribution of microtechniques to a more rapid prenatal diagnosis are discussed. Control of conditions of cell culture and knowledge of possible variations in enzymatic activity of cultured amniotic fluid cells is essential for the interpretation of data in prenatal diagnosis.

2. Introduction

Amniocentesis in early pregnancy and the subsequent cultivation and biochemical analysis of amniotic fluid cells is a new and important tool in the precise genetic counseling of families at risk for a lysosomal storage disease[1]. Potentially all lysosomal diseases where the metabolic defect is expressed in cultured fibroblasts from affected patients, are amenable to prenatal diagnosis.
A number of lysosomal storage diaseases have already been detected in early pregnancy using cultured amniotic fluid cells[2-10]. However, the cultivation period between amniocentesis and the diagnostic assay by conventional biochemical procedures required 4-8 weeks. Such long waiting periods could theoretically be avoided by the use of uncultured amniotic fluid cells. This is complicated by contaminating maternal white blood cells[11], the large proportion of dead fetal cells which may loose part of their enzymatic activity [12, 13] and the possible admixture with enzymes from the amniotic fluid supernatant[14].

The use of the cell free amniotic fluid supernatant seems another alternative. However, this approach proved to be unreliable for glycogenosis type II and mucopolysaccharidoses[15-19]. Hexosaminidase A activity in amniotic fluid seems to be related with the fetal status for GM_2 gangliosidosis type[6, 20, 21], but contamination of the sample with blood may limit its diagnostic use[21]. For GM_1 gangliosidosis type 1[22], I cell disease[23] and metachromatic leukodystrophy[24] analysis of amniotic fluid indicated the fetal condition for these diseases. More data are needed before the value of this approach can be established.

Since cultured amniotic fluid cells are preferentially used in prenatal diagnosis of lysosomal storage diseases, we have tried to reduce the number of cells required for enzyme assays by the application of microchemical techniques[25, 26]. This approach in principle enables the prenatal diagnosis of various lysosomal storage diseases within 10-12 days after amniocentesis in the 14-16th week of pregnancy, i.e. before the mother experiences fetal movements[27, 28]. In this paper first experiences in prenatal diagnosis of Pompe's disease, metachromatic leukodystrophy, Hunter's syndrome and Fabry's disease are described.

3. Materials and Methods

After transabdominal amniocentesis in the 14th-16th week of pregnancy amniotic fluid cell cultures were performed as described[25, 26]. Microchemical assay techniques for acid α-1.4-glucosidase and α-galactosidase were performed with methylumbelliferyl substrates according to methods described[25-27]. Homogenates of cultured amniotic fluid cells or groups of a counted number of lyophillized cells grown on a transparent thin plastic film were incubated in small volumes of substrate, using the oilwell technique[29]. For the assay of arylsuphatase A activity 5 µl of cell homogenate was incubated with 25 µl of substrate, consisting of 0.01 M p. nitrocatecholsulphate, 0.0005 M sodium pyrophosphate and 1.7 M sodiumchloride in 0.5 M sodiumacetate buffer at pH 5.0. The reaction was stopped after 1 hour incubation at 37°C with 25 µl 1 N sodiumhydroxide. Extinction was read in microcuvettes in a Zeiss PM Q II spectrophotometer. The assay for stored mucopolysaccharides was carried out according to Fratantoni et al[30]. Cells were grown to confluency in 35 mm Falcon Petri dishes in Eagle's MEM medium (Difco) supplemented with 10% fetal calf serum and 5 m M sodium bicarbonate and organic buffers[31]. Cultures were kept during 5 days in medium containing 2,5 µ Ci/ml $H_2{}^{35}SO_4$(Amersham) (spec. activity 4 mCi/mmol); on day 5 cultures were washed and trypsinized; half of the cell pellet was used for the study of uptake of labelled sulphate, the other half was cultured for 2 days in cold medium for a chase experiment. The cell pellets were extracted in 80% ethanol, dissolved in 1 ml 1N NaOH and processed for scintillation counting and a

protein assay according to Lowry[32]. Karyotyping of cultured amniotic fluid cells in pregnancies at risk for Hunter's syndrome and Fabry's disease was performed on a coverslip culture of an aliquot of amniotic fluid cells using atebrine staining.

4. Results

Five pregnancies at risk were monitored for Pompe's disease. Detailed results will be published elsewhere[28]. The cultivation period ranged from 12 to 22 days, one affected fetus (Table 1, pregnancy I) was detected and analysis of acid α-1.4-glucosidase in fetal organs obtained from the saline induced abortion confirmed the prenatal diagnosis.

Table 1

Prenatal diagnosis in five pregnancies at risk for Pompe's disease

Pregnancy	cultivation period (days)	acid α-1.4-glucosidase activity ($\times 10^{-12}$ mole/min/mg.protein)			outcome of pregnancy
		cells from pregnancy at risk	control amniotic fluid cells	fibroblasts Pompe's disease	
I	20	26	381	28	Pompe's disease (AB)
II	17	501	1049	59	unaffected
III	22	680	715	-	unaffected
IV	14	112	614	27	unaffected (AB)
V	12	104	261*	25	unaffected

AB = saline induced abortion after prenatal prediction of Pompe's disease.
*Primary culture of control amniotic fluid cells, all others were later confluent subcultures of control amniotic fluid cells.

In pregnancy IV a low enzymatic activity in an amniotic fluid cell culture analyzed 2 weeks after amniocentesis was erroneously interpreted as indicative for Pompe's disease. Analysis of fetal organs obtained at abortion showed normal acid α-1.4-glucosidase activities. Later it was found that primary cultures of amniotic fluid cells may show lower enzymatic activities compared to later subcultures of amniotic fluid cells that have been grown into confluency. Comparison of cultured cells from pregnancy V with a primary culture of control amniotic fluid cells showed little difference in enzymatic activity. In this case it was concluded that the fetus was unaffected and the pregnancy resulted in a child with normal acid α-1.4-glucosidase activity in its leukocytes.

Analysis of acid α-1.4-glucosidase activity in the amniotic fluid supernatant in these 5 pregnancies showed no relation with the fetal condition.

Table 2

Pregnancies at risk for metachromatic leukodystrophy (MLD)

	arylsulphatase A activity ($\times 10^{-9}$ mole/PNC/min/mg protein)				
	amniotic fluid cells		fibroblasts		
	pregnancy at risk*	control**	MLD	heterozygotes	control
I	1.0 (16 days) 1.6 (27 days)	2.2 - 5.0	0.2	3.4 4.2	10.8
II	1.8 (18 days)	2.7 - 3.5[4]	0.0	2.7	-

*Duration of cell culture after amniocentesis is indicated between brackets.
**Data are the extreme values of assays on 2 - 5 cell strains. In case I later subcultures were used as control and in case II primary cultures.

Table 2 presents results on two prenatal diagnosis in pregnancies at risks for metachromatic leukodystrophy. In family I a previous child showed a deficient arylsulphatase A activity in cultured fibroblasts (0.2). Cultured amniotic fluid cells from pregnancy I were analyzed 16 and 27 days after amniocentesis and on both occasions a clear arylsulphatase A activity was found, the second culture showing a higher specific activity. This pregnancy resulted in a female child with arylsulphatase A activity in the leukocytes in the range between controls and heterozygotes. In pregnancy II the previous affected child died before enzymatic confirmation of the diagnosis could be made and the diagnosis was based on the histology of the brain. Fibroblasts of an unrelated patient with MLD had to be used as a control. Cultured amniotic fluid cells analyzed 18 days after amniocentesis showed an arylsulphatase A activity slightly lower than two control cultures grown for the same period. This pregnancy is continuing.

Prenatal analyses in 2 pregnancies at risk for Hunter's syndrome as presented in table 3 indicated that in pregnancy I, where a male sex was detected, the cultured amniotic fluid cells incorporated nearly twice as much labelled sulphate as control fibroblasts. However, the residual radiactivity after 2 days of chase was normal and in agreement with that obtained by Dr. Dorfman in Chicago. In pregnancy II (where a female sex was detected on karyotyping of cultured amniotic fluid cells) the uptake of $^{35}SO_4$ was found to be in the control range and a normal rate of degradation was observed. These studies were performed 22 days after amniocentesis. Analysis of acid-mucopolysaccharides in the

amniotic fluid supernatant in pregnancy II showed a normal pattern for the period of gestation (Dr. M.A.H. Giesberts, Leiden).

Table 3

Results of two prenatal analyses for Hunter's disease

	$^{35}SO_4$ incorporation after 5 days C.p.m./mg. protein	% residual radioactivity after 2 days "chase"
pregnancy I ("F") (sex: ♂)	1448	0
Hunter fibroblasts	3380	100
control fibroblast 1	393	16
" " 2	668	21
pregnancy II ("V") (sex: ♀)	470	0
Hunter fibroblasts	4159	61
control fibroblast 1	662	8
" " 2	245	18

In a pregnancy at risk for Fabry's disease (table 4) prenatal diagnosis was performed by microchemical assay of the α-galactosidase activity in small groups (50-200) of lyophilized cultured cells isolated from the amniotic fluid cell culture 11 days after amniocentesis. Results as described in detail elsewhere[27], were compared with studies on a cell homogenate prepared from a parallel culture and enzyme activities expressed per cell showed a good corrleation between both techniques. A male karyotype was found on atebrine staining of a chromosome preparation of cultured amniotic fluid cells. A boy was born at term, showing normal α-galactosidase activity in a leukocyte preparation.

Table 4

Prenatal diagnosis of Fabry's disease using microchemical techniques

	α-galactosidase activity ($\times 10^{-14}$ mole/hr/cell)	
	microanalysis on groups of 50 - 200 freeze-dried cells	analysis on cell homogenate and enzyme activity expressed per cell
control amniotic fluid cells	2.76 ± 0.46*	1.70
pregnancy at risk (male sex)	1.49 ± 0.31	1.96

*The mean activity of 10-16 groups of 50 -200 cells. s.d. = standard deviation.

5. Discussion

Our first experiences with microchemical techniques in the prenatal diagnosis of a number of lysosomal enzyme deficiencies showed that enzyme assays on small numbers of cultured amniotic fluid cells could be established within 11-22 days after amniocentesis in the 14-16th week of pregnancy. The period required for obtaining sufficient cultured cells for biochemical analysis, which ranged from 4 - 8 weeks[3, 9, 33-35], is shortened and such a reduction in the waiting time is not only of psychological advantage for the parents but also an interruption of pregnancy is likely to cause less problems than after the 5-7th month of pregnancy.

The use of primary cultures of control amniotic fluid cells from the same gestational age is required for a correct interpretation of the analytical data on cells from a pregnancy at risk. As was noted in this study (Table 1) and in other reports[28, 36, 37] the specific activity of some lysosomal enzymes may be considerably lower in primary cultures than in later subcultures of the same amniotic fluid sample. If later subcultures of normal amniotic fluid cells are used as a control erroneous interpretations can be made especially if low enzyme activities are observed in cells from a pregnancy at risk (see Table 1, case IV). In this respect it should be noted that for acid α-1.4-glycoside the mean activity in primary cultures of normal amniotic fluid cells was found to be less than 10 percent of that in normal fibroblasts. The enzyme activities in cells from heterozygotes will show even lower values. Since amniotic fluid samples can be stored with the maintenance of cell viability[38] primary cultures of control samples can be available for prenatal diagnoses at any desired time.

The examples of prenatal diagnosis given in the present paper all involve incubation in microliter volumes followed by measurements in final volumes between 50 and 500 μl using microcuvettes adapted to commercially available spectrofluorometers. For five cases of prenatal diagnosis of Pompe's disease this enabled an enzyme assay after 12 - 22 days of in vitro growth of the amniotic fluid cells, for Fabry's disease after 11 days and for metachromatic leucodystrophy after 16 - 18 days. A further reduction in the number of cells required and hence in the waiting time can be accomplished by performing the extinction or fluorescence measurements in microdroplets (1-5 μl) using a microspectrofluorometer design. Such methods can be used for the assay of about ten lysosomal enzyme deficiencies where artificial substrates can be used[39, 40].

If biochemical assays are to be performed with radioactive labelled substrates there

is less scope for increasing the sensitivity of the measurement except by using radioisotopes with high specific activity. In our examples of prenatal diagnosis for Hunter's disease liquid scintillation counting of $^{35}SO_4$ incorporation could be carried out within 4 weeks after amniocentesis, and the use of adapted culture techniques[41] might enable some additional reduction in this period. In the near future analysis of the specific enzyme defects in mucopolysaccharidoses are likely to replace the assay for the stored products. So far the number of cells required for the assay of one of these enzymes (α -iduronidase) has been reported to be the same as for $^{35}SO_4$ incorporation studies[42]. However, by simply scaling doen of the incubation and final volumes also for this enzyme assay the number of cells might be reduced 100 - 1000 fold[26, 39, 40].

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References

1. Milunsky, A. and Littlefield, J.W. 1972, Ann. Rev. Med. 23, 57.
2. Nadler, H.L. and Messina, A.M., 1969, Lancet ii: 1277.
3. Fratantoni, J.C., Neufeld, E.F., Uhlendorf, B.W. and Jacobson, C.B., 1969, New Engl. J. Med., 280, 686.
4. Nadler, H.L. and Egan, T.J., 1970, New Engl. J. Med , 282, 302.
5. Brady, R.O., Uhlendorf, B.W. and Jacobson, C.B., 1971, Science, 172, 174.
6. O'Brien, J.S., Okada, S., Fillerup, D.L., Veath, M.L., Adornato, B., Brenner, P.H. and Leroy, J.G., 1971, Science, 172, 61.
7. Suzuki, K., Schneider, E.L. and Epstein, C.J., 1971, Biochem. Biophys,. Res. Comm., 45, 1363
8. Epstein, C.J., Brady, R.O., Schneider, E.L., Bradley, R.M., and Shapiro, D., 1971, Am. J. Hum. Genet., 23, 533.
9. Schneider, E.L., Ellis, W.G., Brady, R.O., McCulloch, J.R. and Epstein, C.J., 1972, J. Pediat., 81, 1134.
10. Kaback, M.M., Sloan, H.R., Sonneborn, M., Herndon, R.M. and Percy, A.K., 1973 J. Pediat., 82, 1037.
11. Cox, R.P., Douglas, G., Hutzler, J., Lynfield, J. and Dancis, J., 1970, Lancet, i 893.
12. Davidson, R.G. and Rattazzi, M.C., 1972, Clin. Chem., 18, 179.
13. Navon, R. and Padeh, B., 1971, Brit. med. J., 4, 17.
14. Sutcliffe, R.G. and Brock, D.J.H., 1971, Clin. Chim. Acta, 31, 363.
15. Nadler, H.L., Bigley, R.H. and Hug, G., 1970, Lancet, ii, 369.
16. Fluharty, A.L., Scott, M.L., Porter, M.T., Kihara, H. , Wilson, M.G. and Towner, J.W., 1973, Biochem. Med., 7, 39.
17. Matalon, R., Dorfman, A., Nadler, H.L. and Jacobson, C., 1970, Lancet, i, 83.
18. Ferguson-Smith, M.A., Duncan, D.M., Logan, R.W., Hall, F., Wusteman, F.S. and Harper, P.S., 1973, Lancet, ii, 46.
19. Brock, D.J.H., Gordon, H., Seligman, S. and Lobo, E. de H., 1971, Lancet, ii, 1324.
20. Friedland, J., Perle, G., Saifer, A., Schneck, L. and Volk, B.W., 1971, Proc. Soc. Exptl. Biol. Med., 136, 1297.
21. Ellis, R.B., Ikonne, J.U., Patrick, A.D., Stephens, R. and Willcox, P., 1973, Lancet, ii, 1144.
22. Lowden, J.A., Cutz, E., Conen, P.E., Rudd, N. and Dorran, T.A., 1973, New Engl. J. Med., 288, 225.
23. Huijing, F., Warren, R.J. and McLeod, A.G.W., 1973, Clin. Chim. Acta, 44, 453.
24. Børessen, A.L. and van der Hagen, C.B., 1973, Clin. Genet., 4, 442.
25. Galjaard, H., Fernandes, J., Jahodova, M., Koster, J.F. and Niermeijer, M.F., 1972, Bull. Europ. Soc. Hum. Gen., Nov., 79.

26. Galjaard, H., Mekes, M., de Josselin de Jong, J.E. and Niermeijer, M.F., 1973, Clin. Chim. Acta, 49, 361.

27. Galjaard, H., Niermeijer, M.F., Hahnemann, N., Mohr, J., and Sørensen, S.A., Clin. Genetics, in press.

28. Niermeijer, M.F., Koster, J.F., Jahodova, M., Fernandes, J. and Galjaard, H., manuscript submitted for publication.

29. Lowry, O.H. and Passoneau, J.V., 1972, A flexible system of enzymatic analysis, Acad. Press, New York.

30. Fratantoni, J.C., Hall, C.W. and Neufeld, E.F., 1968, Proc. Natl. Acad. Sci. (USA), 60, 699.

31. Eagle, H., 1971, Science, 174, 500.

32. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., 1951, J. Biol. Chem., 193, 265.

33. Littlefield, J.W., 1971, Birth Defects: Original Article Series, VII, no. 5, 15.

34. Nadler, H.L., 1972, Biochimie, 54, 677.

35. Sutherland, G.R. and Bain, A.D., 1973, Humangenetik, 20, 251.

36. Butterworth, J., Sutherland, G.R., Broadhead, D.M. and Bain, A.D., 1973, Life Sci., 13, 713.

37. Heukels-Dully, M.J., et al., manuscript in preparation.

38. Niermeijer, M.F., Halley, D., Sachs, E.S. and Tichelaar-Klepper, C., 1973, Humangenetik, 20, 175.

39. Galjaard, H., van Hoogstraten, J.J., de Josselin de Jong, J. and Mulder, M.P., 1974, Histochem. J., in press.

40. Galjaard, H., Hoogeveen, A., Keijzer, W., de Wit-Verbeek, E. and Vlek-van der Noot, C., 1974, Histochem. J., in press.

41. Wendel, U., Rüdiger, H.W. and Passarge, E., 1974, Monatschr. Kinderheilk., 122, 23.

42. Hall, C.W., and Neufeld, E.F., 1973, Arch. Bioch. Biophys., 158, 817.

PRENATAL DIAGNOSIS OF GENETIC DISORDERS IN 350 PREGNANCIES

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SUMMARY

350 Pregnancies were monitored by transabdominal amniocentesis in the 14th - 16th week of gestation followed by karyotyping or biochemical assays of cultured amniotic fluid cells and analysis of alpha-fetoprotein in the amniotic fluid supernatant. The pregnancy was interrupted in 36 cases (10%) either because of a fetal abnormality or the presence of a male fetus in pregnancies at risk for an X-linked disease.

Four chromosomal aberrations were found in 87 pregnancies tested because of advanced maternal age. In 101 pregnancies with a recurrence risk of Down's syndrome, 2 fetuses with an abnormal karyotype were detected. In 11 cases, in which one parent was a carrier of a balanced translocation, 2 unbalanced fetal karyotypes were found. Fetal chromosome studies in 43 pregnancies at risk for an X-linked disease indicated the presence of a male fetus in 21 cases.

Prenatal diagnosis of 11 different metabolic diseases was performed in a total of 34 cases. Microchemical techniques were used to allow completion of the diagnosis of seven different enzyme deficiencies within 9 - 22 days after amniocentesis.

Alpha-fetoprotein assay in the amniotic fluid supernatant of 47 pregnancies at risk for an open neural tube defect resulted in the detection of 3 anencephalic fetuses during the second half of pregnancy.

The safety and reliability of amniocentesis and the possible effects on the outcome of pregnancy are evaluated. Prenatal diagnosis offers a promising alter-

native for parents who are at risk of having a child with a genetic disease which can be detected in amniotic fluid or in cultured amniotic fluid cells.

INTRODUCTION

Since the first reports of fetal karyotyping (Steele & Breg, 1966; Jacobson & Barter 1967) and biochemical assays (Nadler, 1968; 1969) after amniocentesis in early pregnancy, an increasing number of centers have gained experience on prenatal diagnosis of genetic disease. Recent reviews (Milunsky, 1973; Hsu & Hirschhorn, 1974) demonstrate how the application of prenatal diagnosis in genetic counseling has already enabled many parents at risk for genetic disease to have non-affected children. Prenatal chromosome analysis has become routine and the number of inborn errors of metabolism which can be detected in cultured amniotic fluid cells has steadily increased (Milunsky & Littlefield, 1972; Burton et al., 1974). Although theoretically over 40 metabolic disorders are now detectable in cultured amniotic fluid cells, only limited experience with each of these diseases has been gained. A few small series have been accumulated: for GM_2 gangliosidosis type 1 (Tay-Sachs' disease) (O'Brien et al., 1970; Navon & Padeh, 1971; Ellis et al., 1973), glycogenosis type II (Pompe's disease) and the mucopolysaccharidoses (Milunsky, 1973; Niermeijer et al., .1974). Conventional biochemical analysis requires relatively large cell numbers, which sometimes resulted in rather long waiting periods for the parents (4 - 8 weeks). The development of microchemical techniques enabled enzyme assays on small numbers of cultured amniotic fluid cells and a reduction in the time required for prenatal diagnosis (Galjaard et al., 1973; 1974[a]; 1974[b]; Wendel et al., 1973).
A recent and important contribution to the field of prenatal diagnosis was the finding by Brock & Sutcliffe (1972), that the alpha-fetoprotein level in amniotic fluid is increased when the fetus has an open defect of the neural tube. When relatively new techniques like those of prenatal diagnosis are used full

assesment of the scope and risks of the various procedures is possible only when sufficiently large series of diagnoses for different groups of genetic diseases can be analysed, including follow-up studies on children born after amniocentesis. The present report describes 350 prenatal diagnoses performed in pregnancies at risk for a chromosomal aberration, X-linked disease, neural tube defect or one of 11 different metabolic diseases.

METHODS AND MATERIALS

1. Clinical methods

The actual genetic risk was estimated in each woman referred for prenatal diagnosis by taking the family history. Additional diagnostic tests on previous affected children, parents or relatives (karyotyping, enzymatic studies, etc.) were performed when necessary in order to make sure that amniocentesis was justified. A risk higher than 1% for a disease amenable to prenatal diagnosis was taken as an indication for amniocentesis (table I). The indication for advanced maternal age was accordingly limited to women 38 years and older. The procedure of amniocentesis and its related risks were explained to the patients.

Transabdominal amniocentesis (Queenan, 1970) was performed under local anesthesia in the 14th - 16th week after the last menstrual period. Ultrasound examination was carried out to localize the placenta, measure the fetal biparietal diameter, estimate the volume of amniotic fluid and to exclude the presence of twins. 10 - 15 ml of amniotic fluid was aspirated into a siliconised glass syringe.

Selective abortion was performed when indicated by intraamniotic injection of hypertonic saline or by intra- or extraovular infusion of prostaglandin F2-alpha.

Follow-up studies on children born after amniocentesis were performed using a schedule modified from the British Medical Research Council "Working Party on Amniocentesis".

The vast majority of amniocenteses and different analyses were performed by our own group. Samples referred by other centers were transported in siliconised glass flasks. Amniotic fluid samples from normal pregnancies were kindly provided by Dr. K.L. Garver, Magee Womens Hospital, Pittsburgh (U.S.A.) and stored until use as controls in biochemical studies according to a procedure described earlier (Niermeijer et al., 1973).

2. Cell culture methods for amniotic fluid cells

After counting the cells with a hemocytometer the amniotic fluid was centrifuged for 5 min. at 80 x g. and the supernatant was centrifuged for another 10 min. at 1500 x g. and stored at -70°C until needed. The collected cells were cultured according to different techniques, especially during the initial phase of this study. The procedure used in the last 300 cases is described here.

The cell pellets were resuspended in Ham's F10 medium supplemented with 25% v/v of fetal calf serum (Flow Company), penicillin (50 U/ml) and streptomycin (50 µg/ml). For chromosome analysis cells were seeded on 22 x 22 mm coverslips in different 35 mm Falcon plastic Petri dishes. Aliquots of 0.75 ml, containing 50,000 - 100,000 cells, were gently pipetted on the top of the coverslips and left overnight in a fully humidified incubator with 5% CO_2 in air as the gaseous phase, before additional medium was added up to 2 ml. The medium was changed after 5 days and subsequently every second day until sufficient mitotic cells for harvesting were present. Some bloody samples of amniotic fluid were processed according to Lee et al., (1970); since this did not give significant improvement in cell growth, the method was omitted. Culture methods for biochemical analysis were similar. Cells cultured in the Petri dishes were used to provide cell homogenates. In addition cells were seeded in Mylar dishes with a thin plastic bottom to enable microchemical studies on groups of 100 - 300 dissected cells (Galjaard et al., 1973; 1974[b]).

For each prenatal diagnosis amniotic fluid cell samples from a comparable gestational period cultivated under similar conditions (medium, pH, cell number seeded, period of cell cultivation after subculture) were used as controls. Amniotic fluid cell cultures received from outside centers were grown in Ham's F10 medium for at least 24 hours before harvesting.

3. Chromosome analysis of cultured amniotic fluid cells

When sufficient mitotic cells were observed in a culture, colchicine (0.02 µg/ml) was added to the medium for 3.5 - 4 hours. A procedure adapted from Hösli (personal communication) was used for *in situ* spreading and gradual fixation to avoid loss of mitotic cells. Q banding was performed with a 0.5% solution of atebrin (Caspersson et al., 1971). Metaphases were studied with a Leitz fluorescence microscope equipped with a vertical illuminator and filter combinations as described by Van der Ploeg and Ploem (1973).
A minimum of 16 metaphases was analysed from at least two different cultures and a minimum of 3 metaphases was karyotyped (Paris Conference, 1971). Polyploid metaphases were not included. A complete fetal chromosome analysis was used for prenatal sex determination in pregnancies at risk for an X-linked disease.

4. Microchemical analysis of cultured amniotic fluid cells

Cell homogenates or dissected groups of 100 - 300 freeze-dried cultured cells were prepared as described (Galjaard et al., 1973; 1974[b]). The procedures for the microchemical assay of α-galactosidase (Galjaard et al., 1974[a]), acid α-1,4-glucosidase (Galjaard et al., 1973), acid β-galactosidase (Galjaard et al., 1974[c]), β-D-N-acetylglucosaminidase (Galjaard et al., 1974[d]) and arylsulfatase A (Niermeijer et al., 1974) have been described. α-L-Iduronidase was measured according to Hall & Neufeld (1973) using a final volume of 30 µl. β-Glucosidase was assayed using a procedure modified from

Beutler et al. (1971).

Electrophoresis of β-D-N-acetylglucosaminidase was performed as described (Galjaard et al., 1974[d]) and heat inactivation studies of β-D-N-acetylglucosaminidase isoenzymes in amniotic fluid supernatant were performed according to O'Brien et al.(1970).

The results of assays on cell homogenates were expressed per mg protein as determined according to Lowry et al. (1951) using a final volume of 60 μl. When possible the activity of a second, non-related enzyme localized in the same subcellular compartment was measured as a test for the preservation of enzymatic activity during preparation of the sample.

The enzymatic activities were expressed per cell in microchemical assays on dissected groups of freeze dried cells.

Cultured cells from control amniotic fluids and fibroblasts from a previous affected child and heterozygous carriers were analyzed at the same time as the cells for diagnosis, whenever these specific fibroblast strains were available.

Studies on the uptake and release of $^{35}SO_4$-sulphate in cultured amniotic fluid cells from pregnancies at risk for mucopolysaccharidoses type I and II were performed according to Fratantoni et al.(1969) as modified by Neufeld (personal communication).

Branched chain ketoacid decarboxylase was assayed in cultured amniotic fluid cells using a microassay described by Wendel et al.(1973).

Cultured cells from a pregnancy at risk for Krabbe's disease were assayed for β-galactocerebrosidase activity using the natural substrate by Dr. A.D. Patrick, Institute of Child Health, London.

5. Determination of α-fetoprotein (AFP) in amniotic fluid

The single radial immunodiffusion technique using Partigen plates (Behringwerke) was used and duplicate samples from pregnancies with a recurrence risk for a neural tube defect were analysed by Dr. D.J. Brock, Dept. Human

Genetics, Edinburgh, with rocket immunoelectrophoresis. This test has only been used since January, 1974, and in addition to pregnancies at risk for a neural tube defect all other amniotic fluids were routinely tested. In pregnancies at risk for a neural tube defect a chromosome analysis was performed as well to exclude the presence of a fetus with a chromosome aberration.

6. Confirmational studies after selective abortion

Fetal autopsies were performed by Dr. J.L.J. Gaillard, Dept. Pathology. Cell cultures were initiated from fetal skin. Fetal organs or fetal cells were used for biochemical assays by Dr. J.F. Koster (Dept. of Biochemistry) in the case of metabolic diseases.

TABLE I

PREGNANCIES MONITORED BY AMNIOTIC FLUID (CELL-) ANALYSIS

INDICATION	N tested	N selective abortions	PRENATAL DIAGNOSIS
CHROMOSOMAL ABERRATIONS			
-recur. risk Down's syndrome	101	2	1 trisomy 21;1 7/21 transloc.
-advanced maternal age	87	4	3 trisomy 21;1 47 XXY
-Down's syndr. in relatives	8	-	
-inherited translocations	11	2	1 13/22 unbal.;1 G/G unbal.
-miscellaneous	19	2	1 trisomy 18;1 47 XYY
	226	10	
X-LINKED DISEASES			
-Duchenne's musc. dystrophy	18	10	11 ♂ fetuses (1 XO/XY mosaic)
-Haemophilia	16	7	7 ♂ fetuses
-others	9	3	3 ♂ fetuses
	43	20	
METABOLIC DISEASES			
-Glycogenosis type II	13	3	2 affected;1 misdiagnosed
-GM_2 gangliosidosis (Tay-Sachs)	3	-	
-GM_2 gangliosidosis (Sandhoff)	3	-	
-GM_1 gangliosidosis	2	-	
-Fabry's disease	1	-	
-Metachromatic leukodystrophy	3	-	
-Krabbe's disease*	1	-	
-Hurler's disease	3	-	
-Hunter's disease	3	-	
-Gaucher's disease	1	-	
-Maple syrup urine disease	1	-	
	34	3	
NEURAL TUBE DEFECTS			
-recur. risk neural tube defects	28	-	
-hydramnios after 20 weeks	19	3	3 anencephalics
	47	3	
	350	36	

* Assay performed by Dr. A.D. Patrick, London

RESULTS

1. PRENATAL CHROMOSOME STUDIES

The results of 350 pregnancies monitored for a variety of genetic risks are listed in table I. The period between amniocentesis and diagnosis in 269 prenatal chromosome analyses was 6 - 14 days in 47%, 15 - 21 days in 40% and 22 - 28 days in 13% of the cases. Most cultures had colonies with varying morphology (epitheloid, fibroblast-like and intermediate). No 46 XX/46 XY mosaicism was observed, which might indicate admixture with maternal cells, nor was a male child diagnosed as female.

In 101 pregnancies with a recurrence risk of Down's syndrome 60 mothers had a previous child with cytogenetic evidence of trisomy 21 and 2 had a child with a sporadic translocation. The diagnosis of Down's syndrome in the other children had been made because of the clinical features. In one case of fetal trisomy 21, the mother had had a previous child with the same aberration. Lymphocyte and fibroblast studies on the parents showed no evidence for mosaicism. A second abnormal karyotype found in the group with recurrence risk of Down's syndrome was a sporadic translocation 46 XY, t (7p-,21q+) (figure I). The previous child had had Down's syndrome caused by a 46 XY/ 47 XY + 21 mosaicism; the parents' karyotypes were normal (Dr. W.L. Gouw, Dept. Anthropogenetics, Groningen). The parents were informed about the possible consequences of this type of chromosomal aberration and they asked for interruption of the pregnancy. Chromosome studies on fetal cord blood gave similar results as the prenatal karyotype, however the fetus showed no macroscopical abnormalities.

In 87 cases tested because of advanced maternal age 4 non-disjunctions were detected (table I). These 4 cases occurred in 68 mothers of 40 years and older. The chromosome abnormalities were confirmed after elective abortion.

Eight patients were referred because one of the sibs of the parents had Down's syndrome. No abnormalities were detected.

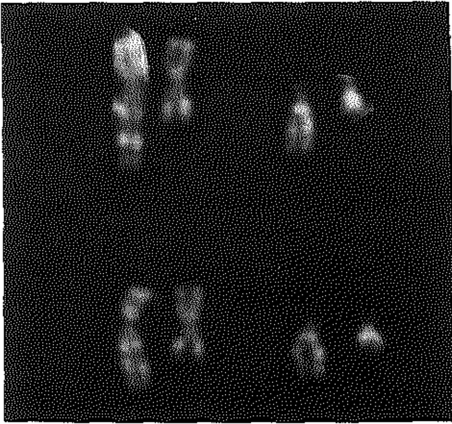
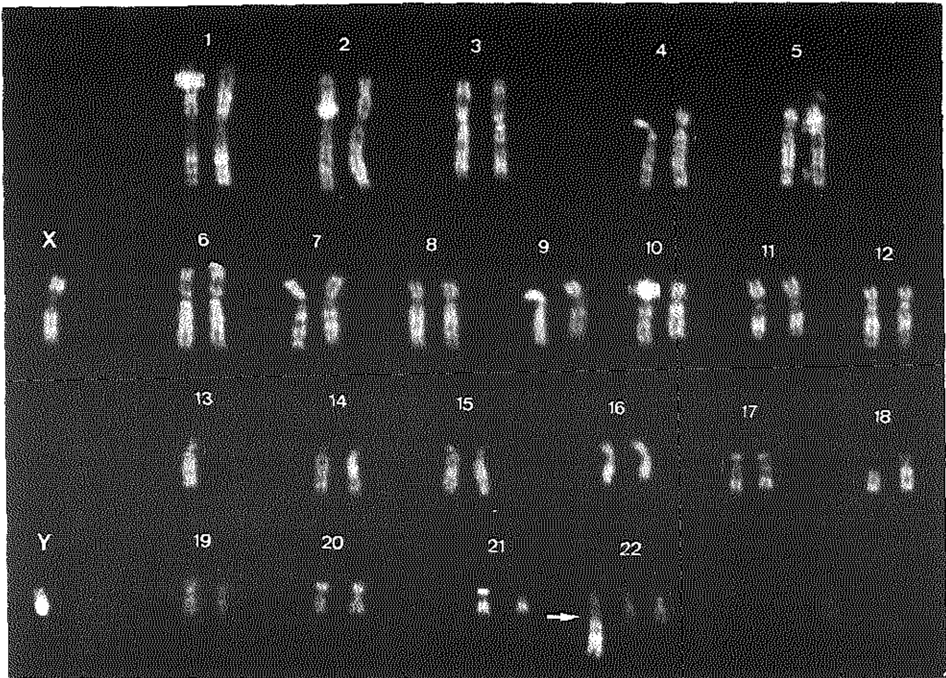


Figure 1.

Prenatal karyotype (Q-banding) 46 XY, t(7p-, 21q+) from a pregnancy with a recurrence risk of trisomy 21.

Figure 2.

Prenatal karyotype (Q-banding) 46 XY, D-, t(22-13)+ showing partial trisomy 22.



In 11 cases one of the parents carried a balanced chromosomal translocation. The type of translocation and the result of prenatal testing are listed in table II. Two unbalanced karyotypes were observed. After interruption of the pregnancy the prenatal diagnosis was confirmed in case 4. Case 2 was referred in the 23th week of pregnancy and the mother was found to be a 22/13 translocation carrier. A 46 XY,D-,t (22-13) + karyotype was found in the fetus (figure 2) in the 27th week of pregnancy. A male child weighing 1950 gm was born in the 37th week and died on the second day of life. The following malformations were observed: cleft palate, antimongoloid eye slant, bilateral iris colobomata, broad nasal bridge, dextrocardia, ventricular septal defect, transposition of the aorta, anal atresia with rectourethral fistula, hypospadia and undescended testes and a small cystic left kidney. This pattern of malformations resembled the cases of trisomy 22 as described by Punnett et al.(1973) and Hirschhorn et al. (1973).
In case 3 (table II) spontaneous abortion occurred 4 weeks after amniocentesis; there had been previous signs of imminent abortion. The amniotic fluid cells

TABLE II

PREGNANCIES AT RISK FOR AN INHERITED TRANSLOCATION

case	type of translocation	carrier-parent[1]	karyotype of amniotic fluid cells
1	14 - 21	M	46 XY
2	13 - 22	M	46 XY, t(13q;22q)
3	14 - 21	M	spont. abortion 4 weeks after amniocentesis
4	21 - 22	M	46 XY, t(21q;22q); selective abortion
5	14 - 21	M	45 XY, t(14q;21q)
6	14 - 21	M	45 XY, t(14q;21q)
7	14 - 21	F	45 XY, t(14q;21q)
8	6 - 13 (p23;q22-31)	M	46 XX
9	t(4;10)-(q21;q11)	M	46 XX, t(4;10)(q21;q11)
10	14 - 21	F	45 XX, t(14q;21q)

1. M = mother, F = father

TABLE III

METHODS OF DIAGNOSIS IN PREVIOUS AFFECTED CHILDREN IN PREGNANCIES AT RISK FOR METABOLIC DISEASES

	N pregnancies studied	diagnosis of index-patient by studies on [1]					
		leukocytes	fibroblasts[2]	urine	autopsy	clinical features	pedigree
GLYCOGENOSIS TYPE II	13	11	11		1		
GM_2 GANGLIOSIDOSIS (Tay-Sachs)	3	2	1				
GM_2 GANGLIOSIDOSIS (Sandhoff)	3	3					
GM_1 GANGLIOSIDOSIS	2		1		1		
FABRY'S DISEASE	1	1					
METACHROMATIC LEUKODYSTROPHY	3	1	1		1		
KRABBE'S DISEASE	1				1		
HURLER'S DISEASE	3		2	1			
HUNTER'S DISEASE	3					3 +	3
GAUCHER'S DISEASE	1	1					
MAPLE SYRUP URINE DISEASE	1			1			
	34	19	16	2	4	3 +	3

(1) Data as obtained from centers referring amniotic fluid or amniotic fluid cell cultures for prenatal diagnosis.

(2) Fibroblasts from index case studied simultaneously with amniotic fluid cells.

failed to grow. Unfortunately, the abortion material was not available for chromosome analysis. Miscellaneous indications for cytogenetic studies in 19 cases included the recurrence risk of the 5p- and 18q- syndromes, maternal mosaicism, multiple abortions and severe intrauterine growth retardation in the second half of pregnancy. A trisomy 18 was found in the 35th week of gestation in the last case. The child was delivered stillborn. A 47 XYY fetus was detected in a pregnancy from a mother who had two previous spontaneous abortions; a number of her relatives were mentally retarded. The parents requested interruption of the pregnancy.

In 34 of the 43 cases in the group at risk for X-linked recessive diseases the mother was a proven or possible carrier of either Duchenne's muscular dystrophy or hemophilia. Other indications for prenatal sex determination were risks for X-linked mental retardation, oculocerebrorenal (Lowe's) syndrome and X-linked agammaglobulinemia. These parents wanted to limit their offspring to non-affected females, knowing that a male would have a 50% chance of being affected. One couple at risk for Duchenne's muscular dystrophy choose to continue their pregnancy after the prenatal test showed that a male child would be born. In one culture obtained from the 15th week of a pregnancy at risk for Duchenne's muscular dystrophy a 45 XO/46 XY mosaicism was found. Cultures initiated from amniotic fluid obtained at termination of the pregnancy all showed 46 XY metaphases. The fetus had a male phenotype and male internal genitals; cells from fetal tissues could not be grown.

2. PRENATAL DIAGNOSIS OF METABOLIC DISEASES

34 Pregnancies were monitored for 11 different metabolic diseases (table I). Some of these results have been presented in detail elsewhere (Niermeijer et al.,1974; 1975; Galjaard et al., 1974[a]).

The diagnosis in a previous affected child was made according to the methods listed in table III and it is apparent that the enzyme defect had been identi-

fied in the majority of cases by studies on leukocytes and fibroblasts. When only autopsy studies were available, the parents were informed that prenatal diagnosis in their cases had certain restrictions.
In the first two pregnancies tested for Pompe's disease insufficient cell growth was obtained and the activity of acid α-1,4-glucosidase in the amniotic fluid supernatant was normal. At that time (1969) such a test was still considered as reliable (Nadler & Messina, 1969). Unfortunately, one of these children proved to be affected with Pompe's disease. In all 11 subsequent cases prenatal diagnosis was based on assays on cultured cells. In two cases very low activities of acid α-1,4-glucosidase were observed; after interruption of these pregnancies in the 18th week enzyme assays on fetal tissues (liver, muscle, heart, brain and kidney) confirmed the diagnosis. In one instance a low activity of acid α-1,4-glucosidase in an early primary amniotic fluid cell culture was erroneously interpreted as compatible with Pompe's disease in the fetus and the pregnancy was terminated. Later it was observed, that the enzymatic activities in later subcultures of control amniotic fluid cells might be considerably higher than those in early primary cultures. Since then we have used control cells which have been cultured under similar conditions and for the same period as those from the pregnancy at risk (Niermeijer et al., 1975). In 8 cases the activities of acid α-1,4-glucosidase were found to be in the range of controls or heterozygotes and all children born were found to be normal. These prenatal diagnoses were completed in 14 - 22 days after amniocentesis.
Three pregnancies were tested for Tay-Sachs' disease. In one case a normal hexosaminidase A activity was detected on electrophoresis of cultured amniotic fluid cells. A normal electrophoretic pattern in amniotic fluid supernatant was found in the other two cases; this result was confirmed by analysis of cultured cells in other laboratories.
Three amniotic fluid cell cultures were monitored for Sandhoff's disease and all showed the presence of both hexosaminidase A and B by electrophoresis and a normal total hexosaminidase activity. One of these children has been

TABLE IV

PRENATAL DIAGNOSIS FOR GM_1-GANGLIOSIDOSIS USING DIFFERENT MICROTECHNIQUES

A. STUDIES ON GROUPS OF 10-20 FREEZE-DRIED CELLS[1]

Amniotic fluid cells	β-galactosidase activity $\times 10^{-13}$ mol/h/cell
pregnancy at risk (Mrs. E)	2.5
control	3.6
Fibroblasts	
GM_1-gangliosidosis	0.08

B. STUDIES ON CELL HOMOGENATES[2]

Amniotic fluid cells	β galactosidase nmol/h/mg P	β-D-N-acetyl-glucosaminidase nmol/h/mg P
pregnancy at risk (Mrs. E)	690	5400
control 1	520	5100
control 2	710	4100
control 3	1500	11200
Fibroblasts		
control	780	11300
GM_1 patient E	63	12500

1. performed 9 days after amniocentesis.
2. performed 12 days after amniocentesis.

born and has serum hexosaminidase activity in the normal range.
Microchemical assays of β-galactosidase were performed on dissected groups of 10 - 20 freeze-dried cultured cells from two pregnancies at risk for GM_1-gangliosidosis. The diagnoses were completed 9 and 15 days after amniocentesis and showed normal values (table IV). β-galactosidase activities obtained from freeze-dried cells showed a good correspondance with values obtained in analyses on cell homogenates prepared from replicate cultures. Activities are expressed per mg protein in cell homogenates but can be calculated per cell by using the factor 1 mg protein=3.10^6 cells (Galjaard et al., 1974[b]).
Microchemical analyses were also done on freeze-dried cells cultured from a pregnancy at risk for Fabry's disease. The result was available 11 days after amniocentesis. Cell homogenates from replicate cultures were also analyzed, both in our laboratory and in the Institute of Medical Genetics, Copenhagen. Normal α-galactosidase activities and a normal male karyotype were found. A normal boy was born (Galjaard et al., 1974[a]).
In three cases at risk for Hurler's disease $^{35}SO_4$-incorporation and chase studies suggested normal mucopolysaccharide metabolism in the fetus. In one case however, α-L-iduronidase activity fell in the same range found in fibroblasts from Hurler patients. According to the suggestion by Hall & Neufeld (1973), who observed a similar phenomenon, we relied upon the results of $^{35}SO_4$-studies and the pregnancy was continued.
In three pregnancies at risk for Hunter's syndrome chromosome analyses were performed along with $^{35}SO_4$ incorporation studies. Three female fetuses were detected and all showed normal mucopolysaccharide degradation, as expected.
Microchemical assays of arylsulfatase A activity in three pregnancies at risk for metachromatic leukodystrophy were performed 16 and 18 days after amniocentesis and indicated the presence of unaffected fetuses.
In a pregnancy at risk for Krabbe's disease the assay of β-galactocerebrosidase (performed by Dr.A.D. Patrick, London) on a homogenate of cultured cells

showed a normal activity.

A β-glucosidase activity half of control amniotic fluid samples but clearly higher than that found in a previous pregnancy was found in cultured amniotic fluid cells from a pregnancy at risk for Gaucher's disease.

The assay of branched-chain ketoacid decarboxylase activity was completed 11 days after amniocentesis in a pregnancy at risk for Maple Syrup Urine Disease. A normal activity was found.

In children born after prenatal testing for metabolic defects biochemical assays on leukocytes or fibroblasts were performed whenever possible and in 25 cases confirmation of the prenatal diagnosis has been obtained. The other pregnancies were not completed at the moment of this report.

3. PRENATAL DIAGNOSIS OF NEURAL TUBE DEFECTS

a-Fetoprotein levels were elevated (90 µg/ml in week 27; 13 in week 29 and 90 in week 22, respectively) in 3 of 19 cases where hydramnios developed in the second half of pregnancy. Ultrasound examination showed anencephalic fetuses in these three cases. No abnormal elevated levels were found in 28 pregnancies tested for the recurrence risk of a neural tube defect.

In a pregnancy tested for the recurrence risk of trisomy 21 during the 16th week an AFP level of 70 µg/ml was found; this sample however showed admixture with fetal red blood cells and a second sample obtained at the 19th week of the pregnancy showed a normal AFP level.

4. COMPLICATIONS OF AMNIOCENTESIS

No serious maternal complications were observed. Two patients experienced a transient vaginal leakage of amniotic fluid for a few days after amniocentesis. Spontaneous abortions within one month after amniocentesis were observed in 3 pregnancies:

case 1: Spontaneous abortion within two weeks after transvaginal amniocentesis, which was performed because the placenta was localized over the complete anterior uterine wall. Fetal tissues showed signs of amnionitis and the transvaginal route has since never been repeated.

case 2: Pregnancy at risk for Pompe's disease; spontaneous abortion 3 weeks after amniocentesis. Analysis of fetal tissues showed a deficiency of acid α-1,4-glucosidase activity.

case: 3: Balanced carrier of a 14/21 translocation. Spontaneous abortion occurred 4 weeks after amniocentesis. Amniotic fluid cells failed to grow. Fetal tissues were not available for study.

5. FAILURES OF AMNIOCENTESIS AND AMNIOTIC FLUID CELL CULTIVATION

In 14 of 350 cases (4%) attempts to obtain amniotic fluid failed at the first puncture. In every case a repeat puncture was succesfully carried out 10-14 days later.
In the whole series of 350 pregnancies monitored "no result" (no prenatal diagnosis established) was obtained in 9 cases (2.5%); in 18 cases (5%) a repeat amniocentesis was indicated because of failure in cell cultivation. However, in the 198 consecutive cases studied in 1974 the figure for "no result" was 2/198 or 1%. In both failures (cases at risk for Down's syndrome) either the repeat amniocentesis failed to obtain sufficient amniotic fluid or cell growth was insufficient for karyotyping. The figure for failure in cell cultivation in 1974 dropped to 2.5%. The reasons for these failures were: transport failures (2 cases), insufficient amniotic fluid obtained at the first puncture (1) and insufficient growth in the first sample (2). In one of these latter cases multiple chromosomal breaks were observed in the first sample obtained; in the repeat culture a normal karyotype was found.
At present the failure rate due to insufficient growth of amniotic fluid cells may be estimated as 2/198 or 1%.

6. FOLLOW-UP OF CHILDREN BORN AFTER PRENATAL DIAGNOSIS

The outcome of pregnancy is known in ± 300 cases. No increase in complications of pregnancy and delivery was found. Children were seen for follow-up studies at different ages by one of the authors (M.J.). No serious damage or malformations were seen which could be related to amniocentesis. Detailed results on the follow-up studies will be presented elsewhere.

DISCUSSION

Evaluation of amniocentesis and amniotic fluid cell culture

Prenatal diagnosis may be evaluated according to several different criteria. The risk of amniocentesis to the mother may be estimated as negligible; the risk to the fetus as measured as the rate of spontaneous abortion after amniocentesis differs from one study to the other: 1 : 155 (Nadler & Gerbie,1970), 4 : 73 (Doran et al., 1974), 3 : 100 (Golbus et al., 1974), 1 : 50 (Prescott et al., 1973),10 : 128 (Robinson et al., 1973) and 7 : 477 (Milunsky & Atkins, 1974). These variations may be caused by differences in techniques and by different criteria during the follow-up period. For an abortion to be caused by amniocentesis, a time relationship between these two events should exist (Wahlström et al., 1974). For the period of one month after amniocentesis three spontaneous abortions occurred in our series of 350 pregnancies (about 1%); in one of these amniocentesis was clearly causal to the fetal loss. The average risk of about 1% is not significantly different from the risk of spontaneous abortion in the second trimester, which was estimated in the U.S.A. as 1.2% (Javert, 1957; Shapiro et al., 1962) and as 1 - 3% in Sweden (Petterson, 1968). 32% Of the total fetal loss occurred in the 12 - 19th weeks of gestation, and 12% at 20 weeks or more in a study on spontaneous abortion (Shapiro et al., 1962).

Follow-up studies on the children born after prenatal diagnosis failed to detect either an increase in congenital malformations or a specific malformation pattern associated with the puncture. Hsu & Hirschhorn (1974), Golbus et al. (1974) and Allen et al. (1974) observed similar results.

We failed to obtain amniotic fluid at the first amniocentesis in 4% of the cases in the present series; failure rates of 1% (Nadler & Gerbie, 1970), 6% (Golbus et al., 1974; Prescott et al., 1973; Robinson et al., 1973; Wahlström et al., 1974), 8.5% (Doran et al., 1974) and 10% (Milunsky & Atkins, 1974) have been reported.

The failure rate of amniotic fluid cell culture in the present study is 1%; others reported either similar data (Nadler & Gerbie, 1970) or higher figures: 10% (Milunsky & Atkins, 1974; Allen et al., 1974) and 30% (Robinson et al., 1973). The length of the waiting period between amniocentesis (in the 14th - 16th week) and the eventual diagnosis is another criterium for evaluating prenatal diagnosis. This period should not exceed 2 to 3 weeks so that if necessary abortion can be performed before the mother experiences fetal movements (Ferguson-Smith et al., 1971; Golbus et al., 1974). In the present study the result of prenatal chromosome analysis was available within 3 weeks in 87% of the cases (47% of the parents knew the result within 2 weeks). These data are similar to those from Nadler & Gerbie (1970), Therkelsen et al. (1972) and Milunsky & Atkins (1974); Prescott et al. (1973) and Golbus et al. (1974) used longer periods.

Indications and problems in prenatal chromosome analysis

Prenatal monitoring has become a routine for pregnancies at advanced maternal age and for cases with a recurrence risk of trisomy 21 (Down's syndrome). The recurrence risk of trisomy 21 was found to be 1% in a series of prenatal diagnoses collected by Milunsky (1973) and a similar risk was observed in the present series. In the group of pregnancies at advanced maternal age the risk

for chromosomal aberrations (determined from several series of prenatal diagnosis) is 1.5% in mothers between 35 and 39 years and about 3% in the group of 40 years and older (Hsu & Hirschhorn, 1974). In the present series 4 non-disjunctions were found in 68 pregnancies in mothers 40 years and older. These risk figures are higher than those generally given in genetic counseling to older mothers. The risk for a child with a chromosomal aberration is much higher when one of the parents is a carrier of a balanced translocation (table II). Following the detection of the familial nature of a translocation in a family with one child affected with Down's syndrome (table II, case 1), several relatives were karyotyped. In this way another high-risk pregnancy was identified in time to give the parents the possibility to request prenatal diagnosis (table II, case 5). Timely identification of translocation carriers is one of the important tasks of a genetic counseling service.

A number of the technical problems in chromosome analysis on cultured amniotic fluid cells has been discussed recently, including chromosomal mosaicism (Kardon et al., 1972; Hsu et al., 1973; Cox et al., 1974) and structural aberrations arising in vitro by contamination with mycoplasma (Schneider et al., 1974). One case of a 45 XO/46 XY mosaicism in the present study is comparable with the observation of Kardon et al. (1972) and this mosaicism probably arose in vitro; however, cytogenetic studies on the fetus were not possible. In one case inconsistent structural aberrations were detected; metaphases from a repeat culture (obtained after a second amniocentesis)were normal. The role of mycoplasma in this series is difficult to evaluate. Although all tests were negative, mycoplasma testing was performed only periodically. Nadler (1972) estimated that maternal cells would be growing in 0.5% of amniotic fluid cell cultures and this would accordingly lead to a number of false diagnoses. Philip et al. (1974) reported 2 false sex predictions in 93 cases and they advocate the comparative study of Q-banded maternal and fetal ka-

ryotypes, since in most cases differences in fluorescent markers might enable a distinction between mother and fetus. In the present series no sex chromosome mosaicism was observed and comparative studies of maternal and fetal cells were not done.

Hsu & Hirschhorn (1974) discuss the problem of counseling parents when an apparent balanced reciprocal translocation is found. This rearrangement may be an aneusomy by recombination, although true cases should be detectable by banding techniques (Boué & Boué, 1973). In sporadic balanced reciprocal translocations, which occur in about one of every 3000 newborns (Jacobs et al., 1974), small deletions, not discernable with present techniques, or a position effect may be present (Skovby & Niebuhr, 1974). In addition, _de novo_ balanced structural autosomal rearrangements might be associated or causal to severe mental retardation (Jacobs, 1974). Our finding of an apparently balanced _de novo_ 7 - 21 translocation is an example of this problem, when the prenatal karyptype leaves doubts over the eventual phenotype.

Another couseling problem arises upon the finding of a numerical abnormality of the sex chromosomes. An XYY karyotype may be expected in 0.09% of newborns, an XXY in 0.1% (Jacobs et al., 1974). Even after extensive studies on the clinical aspects of the XYY genotype (see for reviews: Hook, 1973; Borgaonkar & Shah, 1974) the number of unanswered questions warrants counseling of the parents to inform them that the definite risk is not known. The final decision rests with the parents.

Prenatal diagnosis of metabolic disease

The prenatal diagnosis of metabolic defects formerly required a period of 4-8 weeks after amniocentesis and parents had to be warned about waiting periods of up to 6 weeks, when prenatal diagnosis is discussed (Milunsky et al., 1972). Microtechniques facilitated the enzymatic analysis of fewer numbers of cells, either small groups of freeze-dried cultured amniotic fluid cells or homogenates

of a few thousands cells. Prenatal analysis for Pompe's disease was completed in 11 - 22 days (Niermeijer et al., 1975), for Fabry's disease in 11 days (Galjaard et al., 1974[a]) and for GM_1-gangliosidosis in 9 and 12 days. In previous reports on prenatal diagnosis of GM_1-gangliosidosis the result became available after 18 days (Lowden et al., 1973) and 4 weeks (Kaback et al., 1973; Booth et al., 1973). The prenatal monitoring for metachromatic leukodystrophy has required 3-4 weeks (Leroy et al.,1973; Van der Hagen et al.,1973); in the present study diagnosis was performed in 16 and 18 days in two pregnancies studied. A further reduction in this waiting period may be achieved by decreasing the final volume of the enzymatic reaction to 1 - 5 µl and measuring the extinction in a microspectrophotometer (Galjaard et al., 1974[b]). Testing for Maple Syrup Urine disease using a radioactive substrate was completed in 11 days in this study; others have used periods of up to 8 weeks. (Elsas et al., 1974; Hoo et al., 1974).

Using the methods described, the interval between amniocentesis and diagnosis for a number of metabolic diseases is comparable to the period required for prenatal chromosome analysis.

The basic requirements for reliable prenatal diagnosis of metabolic diseases were reviewed by Burton et al.(1974) and Hsu & Hirschhorn (1974). The present experience indicates that the following points should be considered:

1. The expression of the metabolic defect should be characterized in fibroblasts from a previous affected child or relative from the family under study and the level of residual enzymatic activity should be studied, since genetic heterogeneity exists within apparently identical metabolic diseases. From table III one may note, that these data were not available in all cases studied. Many parents will not seek genetic counseling until after the death of an affected child; fibroblast cultures from affected children can be stored in a cell bank to provide control material for a future prenatal diagnosis.

2. A number of variables may influence enzymatic activities in cultured cells and sufficient experience with these factors is essential. The type of tissue culture media (Ryan et al., 1972; Butterworth et al., 1974), its pH (Eagle, 1973; Lie et al., 1973), differences in cell types in amniotic fluid cell cultures (Gerbie et al., 1972) and the effect of growth to confluency (Okada et al., 1971; Russell et al., 1971; Galjaard et al., 1974[e]) may all affect enzymatic activities. The activity of some lysosomal hydrolases may show considerable variations in primary cultures of amniotic fluid cells; in later subcultures the activities increased during prolonged cultivation (Niermeijer et al., 1975). Amniotic fluid cell cultures used as controls in prenatal diagnosis should therefore be carefully matched for their growth stage and passage. Since fibroblast cultures and amniotic fluid cultures have important enzymatic differences, fibroblasts should not be used as the sole controls (Kaback et al., 1971).

Prenatal diagnosis of neural tube defects

The prenatal diagnosis of open neural tube defects by elevated levels of α-fetoprotein in the amniotic fluid (Brock & Sutcliffe, 1972) offers new perspectives in genetic counseling. The risk of open neural tube defects will become a common indication for prenatal diagnosis, since neural tube defects and Down's syndrome have a comparable frequency. Closed lesions or smaller open defects possibly remain undetectable by AFP levels, and the parents should be so informed, however, the clinical effects of these lesions may be milder in some cases (Laurence et al., 1973; Harris et al., 1974). The reliability of the method has been proven in different centers (Allan et al., 1973; Milunsky & Alpert, 1974; Brock et al., 1975). Aspecific elevations in second trimester amniotic fluids may be caused by admixture with fetal blood, which has a high AFP concentration (Field et al., 1974; Ward & Stewart, 1974).In the case observed in this study the amniotic fluid AFP level returned to nor-

mal levels in 10 days. In our laboratory bloody amniotic fluids were tested for fetal Hb in cases with a recurrence risk for neural tube defects.

In our series of 350 pregnancies, selective abortion was performed as a result of the prenatal diagnosis in 36 cases. In 90% of the cases the pregnancies were continuated knowing that the risk of a particular genetic disease was eliminated. Ferguson-Smith (1974) stated: "unfortunately, many parents at risk do not know that the option of prenatal diagnosis is available to them, and a very few are actively discouraged from seeking it by their medical advisers, who may feel that the risks of foetal abnormality are too small to justify the risk of the procedure". Results in the present series again show that these latter fears are unjustified. Improvement of communications between genetic counselors and family doctors might contribute to a better application of present knowledge.

CONCLUSIONS

1. Transabdominal amniocentesis in the 14th - 16th week is harmless to the mother and carries a risk for abortion which is as yet unknown but may be estimated as ± 1%.
2. There is a 5% chance that a second amniocentesis after 10 - 14 days will be necessary (4% because of a first "dry tap" and 1% for culture failure). The chance that no diagnosis will be obtained after a repeat amniocentesis is about 1%.
3. When sufficient experience in cell cultivation and chromosome identification techniques are available, prenatal chromosome analysis may be considered a routine technique. The same applies to AFP analysis for open neural tube defects.
4. Prenatal diagnosis of metabolic disorders should be carried out only in centers which have sufficient experience with biochemical analysis of cultured

human cells. Experience with the biochemistry of the metabolic diseases studied is a prerequisite as is the availability of the proper control material in a cell bank. Microchemical techniques have the advantage of reducing the waiting periods for the parents and should be available in the diagnostic center.

Collaboration between different centers is required because of the relative infrequency of most metabolic diseases and the facilities and know-how required.

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REFERENCES

Allan, L.D., Ferguson-Smith, M.A., Donald, I., Sweet, E.M. and Gibson, A.A.M. (1973) Amniotic-fluid alpha-fetoprotein in the antenatal diagnosis of spina bifida. Lancet, II, 522-525.

Allen, H.H., Sergovich, F., Stuart, E.M., Pozsonyi, J. and Murray, B. (1974) Infants undergoing antenatal genetic diagnosis: a preliminary report. American Journal of Obstetrics and Gynecology, 118, 310-313.

Beutler, E., Kuhl, W., Trinidad, F., Teplitz, R. and Nadler, H. (1971) β-Glucosidase activity in fibroblasts from homozygotes and heterozygotes for Gaucher's disease.

Booth, C.W., Gerbie, A.B. and Nadler, H.L. (1973) Intrauterine diagnosis of GM_1 gangliosidosis, type 2. Pediatrics, 52, 521-524.

Borgaonkar, D.S. and Shah, S.A. (1974) The XYY syndrome. Male or syndrome? In: A. G. Steinberg and A.G. Bearn (editors): Progress in medical genetics, Vol. X. Grune & Stratton, New York, pp. 135-222.

Boué, J. and Boué, A. (1973) L'intérêt en diagnostic prénatal des techniques nouvelles d'identification chromosomique dans des translocations et une aneusomie de recombinaison. La Nouvelle Presse Médicale, 2, 3097-3102.

Brock, D.J.H. and Sutcliffe, R.G. (1972) Alpha-fetoprotein in the antenatal diagnosis of anencephaly and spina bifida. Lancet, II, 197-199.

Brock, D.J.H., Scrimgeour, J.B. and Nelson, M.M. (1975) Amniotic fluid alpha-fetoprotein measurements in the early prenatal diagnosis of central nervous system disorders. Clinical Genetics, 7, 163-169.

Burton, B.K., Gerbie, A.B. and Nadler, H.L. (1974) Present status of intrauterine diagnosis of genetic defects. American Journal of Obstetrics and Gynecology, 118, 718-746.

Butterworth, J., Sutherland, G.R., Broadhead, D.M. and Bain, A.D. (1974) Effect of serum concentration, type of culture medium and pH on the lysosomal enzyme activity of cultured human amniotic fluid cells. Clinica Chimica Acta, 53, 239-246.

Butterworth, J., Sutherland, G.R., Broadhead, D.M. and Bain, D. (1973)Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. 1. α-Glucosidase and β-Glucosidase. Life Sciences, 13, 713-722.

Caspersson, T., Lomakka, G. and Zech, L. (1971) The 24 fluorescence patterns of human metaphase chromosomes-distinguishing characters and variability. Hereditas (Lund), 67, 89-102.

Cox, D.M., Niewczas-Late, V., Riffell, M.I. and Hamerton, J.L. (1974) Chromosomal mosaicism in diagnostic amniotic fluid cell cultures. Pediatric Research, 8, 679-683.

Doran, T.A., Rudd, N.L., Gardner, H.A., Lowden, J.A., Benzie, R.J. and Liedgren, S.I. (1974) The antenatal diagnosis of genetic disease. American Journal of Obstetrics and Gynecology, 118, 314-321.

Eagle, H. (1973) The effect of environmental pH on the growth of normal and malignant cells. Journal of Cellular Physiology, 82, 1-8.

Ellis, R.B., Ikonne, J.U., Patrick, A.D., Stephens, R. and Willcox, P. (1973) Prenatal diagnosis of Tay-Sachs disease. Lancet, II, 1144-1145.

Elsas, L.J., Priest, J.H., Wheeler, F.B., Danner, D.J. and Pask, B.A. (1974) Maple Syrup Urine Disease: coenzyme function and prenatal monitoring. Metabolism, 23, 569-579.

Ferguson-Smith, M.E., Ferguson-Smith, M.A., Nevin, N.C. and Stone, M. (1971) Chromosome analysis before birth and its value in genetic counseling. British Medical Journal, 4, 69-74.

Ferguson-Smith, M.A. (1974) Prenatal diagnosis. The Practitioner, 213, 655-666.

Field, E., Mitchell, G., Garrett, W. and Kerr, C. (1974) Prenatal diagnosis and selective abortion for anencephaly and spina bifida. Medical Journal of Australia, I, 608-610.

Fratantoni, J.C., Neufeld, E.F., Uhlendorf, B.W. and Jacobson, C.B. (1969) Intrauterine diagnosis of the Hurler and Hunter syndromes. New England Journal of Medicine, 280, 686-688.

Galjaard, H., Mekes, M., Josselin de Jong, J.E. de and Niermeijer, M.F. (1973) A method for rapid prenatal diagnosis of glycogenosis II (Pompe's disease). Clinica Chimica Acta, 49, 361-375.

Galjaard, H., Niermeijer, M.F., Hahnemann, N., Mohr, J. and Sørensen, S.A. (1974[a]) An example of rapid prenatal diagnosis of Fabry's disease using microtechniques. Clinical Genetics, 5, 368-377.

Galjaard, H., Hoogstraaten, J.J. van, Josseling de Jong, J.E. de and Mulder, M.P. (1974[b]) Methodology of the quantitative cytochemical analysis of single or small numbers of cultured cells. Histochemical Journal, 6, 409-429.

Galjaard, H., Hoogeveen, A., Keijzer, W., Wit-Verbeek, E. de and Vlek-Noot, C. (1974[c]) The use of quantitative cytochemical analysis in rapid prenatal detection and somatic cell genetic studies of metabolic diseases. Histochemical Journal, 6, 491-509.

Galjaard, H., Hoogeveen, A., Wit-Verbeek, H.A. de, Reuser, A.J.J., Keijzer, W., Westerveld, A. and Bootsma, D. (1974[d]) Tay-Sachs and Sandhoff's disease: intergenic complementation after somatic cell hybridization. Experimental Cell Research, 87, 444-448.

Galjaard, H., Reuser, A.J.J., Heukels-Dully, M.J., Hoogeveen, A., Keijzer, W., Wit-Verbeek, H.A. de and Niermeijer, M.F. (1974[e]). Genetic heterogeneity and varation of lysosomal enzyme activities in cultured human cells. In: J.M. Tager, G.J. M. Hooghwinkel and W.Th. Daems (editors): Enzyme therapy in lysosomal storage diseases. North Holland Publishing Company, Amsterdam, pp. 35-51.

Gerbie, A.B., Nadler, H.L. and Gerbie, M.V. (1971) Amniocentesis in genetic counseling. Safety and reliability in early pregnancy. American Journal of Obstetrics and Gynecology, 109, 765-770.

Gerbie, A.B., Melancon, S.B., Ryan, C. and Nadler, H.L. (1972) Cultivated epithelial-like cells and fibroblasts from amniotic fluid: their relationship to enzymatic and cytologic analysis. American Journal of Obstetrics and Gynecology, 114, 314-320.

Golbus, M.S., Conte, F.A., Schneider, E.L. and Epstein, C.J. (1974) Intrauterine diagnosis of genetic defects: results, problems and follow up of one hundred cases in a prenatal genetic detection center. American Journal of Obstetrics and Gynecology, 118, 897-905.

Hall, C.W. and Neufeld, E.F. (1973) α-L-Iduronidase activity in cultured skin fibroblasts and amniotic fluid cells. Archives of Biochemistry and Biophysics, 158, 817-821.

Harris, R., Jennison, R.F., Barson, A.J., Laurence, K.M., Ruoshlahti, E. and Seppälä, M. (1974) Comparison of amniotic fluid and maternal serum alpha-fetoprotein levels in the early antenatal diagnosis of spina bifida and anencephaly. Lancet, I, 429-433.

Hirschhorn, K., Lucas, M. and Wallace, I. (1973) Precise identification of various chromosomal abnormalities. Annals of Human Genetics (London), 36, 375-379.

Hoo, J.J., Latta, E. and Schaumlöffel, E. (1974) Antenatal diagnosis of Maple Syrup Urine disease. Zeitschrift für Kinderheilkunde, 118, 225-229.

Hook, E.B. (1973) Behavioral implications of the human XYY genotype. Science, 179, 139-150.

Hsu, L.Y.F., Dubin, E.C., Kerenyi, T. and Hirschhorn, K. (1973) Results and pitfalls in prenatal cytogenetic diagnosis. Journal of Medical Genetics, 10, 112-119.

Hsu, L.Y.F. and Hirschhorn, K. (1974) Prenatal diagnosis of genetic disease. Life Sciences, 14, 2311-2336.

Jacobs. P.A. (1974) Correlation between euploid structural chromosome rearrangements and mental subnormality in humans. Nature, 249, 164-165.

Jacobs, P.A., Melville, M., Ratcliffe, S., Keay, A.J. and Syme, J. (1974) A cytogenetic survey of 11,680 newborn infants. Annals of Human Genetics (London), 37, 359-376.

Jacobson, C.B. and Barter, R.H. (1967). Intrauterine diagnosis and management of genetic defects. American Journal of Obstetrics and Gynecology, 99, 796-807.

Javert, C.T. (1957) Spontaneous and habitual abortion. Mc Graw-Hill, New York, p. 204.

Kaback, M.M., Leonard, C.O. and Parmley, T.H. (1971) Intrauterine diagnosis: comparative enzymology of cells cultivated from maternal skin, fetal skin, and amniotic fluid cells. Pediatric Research, 5, 366-371.

Kaback, M.M., Sloan, H.R., Sonneborn, M., Herndon, R.M. and Percy, A.K. (1973) GM_1 gangliosidosis type I: in utero detection and fetal manifestations. Journal of Pediatrics, 82, 1037-1041.

Kardon, N.B., Chernay, P.R., Hsu, L.Y., Martin, J.L. and Hirschhorn, K. (1972) Pitfalls in prenatal diagnosis resulting from chromosomal mosaicism. Journal of Pediatrics, 80, 297-299.

Laurence, K.M., Turnbull, A.C., Harris, R., Jennison, R.F., Ruoslahti, E. and Seppälä, M. (1973) Antenatal diagnosis of spina bifida. Lancet, II, 860.

Lee, C.L.Y., Gregson, N.M. and Walker, S. (1970) Eliminating red blood-cells from amniotic fluid samples. Lancet, II, 316-317.

Leroy, J.G., Van Elsen, A.F., Martin, J.J., Dumon, J.E., Hulet, A.E., Okada, S. and Navarro, C. (1973) Infantile metachromatic leukodystrophy. Confirmation of a prenatal diagnosis. New England Journal of Medicine, 288, 1365-1369.

Lie, S.O., Schofield, B.H., Taylor Jr., H.A. and Doty, S.B. (1973) Structure and function of the lysosomes of human fibroblasts in culture: dependence on medium pH. Pediatric Research, 7, 13-19.

Littlefield, J.W. (1971) Problems in the use of cultured amniotic fluid cells for biochemical diagnoses. Birth Defects: Original Article Series, VII, no. 5, 15-17.

Lowden, J.A., Cutz, E., Conen, P.E., Rudd, N. and Doran, T.A. (1973) Prenatal diagnosis of GM_1-gangliosidosis. New England Journal of Medicine, 288, 225-228.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193, 265-275.

Milunsky, A. and Littlefield, J.W. (1972) The prenatal diagnosis of inborn errors of metabolism. Annual Review of Medicine, 23, 57-76.

Milunsky, A., Atkins, L. and Littlefield, J.W. (1972) Amniocentesis for prenatal genetic studies. Obstetrics & Gynecology, 40, 104-108.

Milunsky, A. (1973) The prenatal diagnosis of hereditary disorders. Charles C. Thomas Publisher, Springfield, Ill.

Milunsky, A. and Alpert, E. (1974) The value of alpha-fetoprotein in the prenatal diagnosis of neural tube defects. Journal of Pediatrics, 84, 889-893.

Milunsky, A. and Atkins, L. (1974) Prenatal diagnosis of genetic disorders. An analysis of experience with 600 cases. Journal of the American Medical Association, 230, 232-235.

Nadler, H.L. (1968) Antenatal detection of hereditary disorders. Pediatrics, 42, 912-918.

Nadler, H.L. (1969) Prenatal detection of genetic defects. Journal of Pediatrics, 74, 132-143.

Nadler, H.L. and Messina, A.M. (1969) In-utero detection of type II glycogenosis (Pompe's disease). Lancet, II, 1277-1278.

Nadler, H.L. and Gerbie, A.B. (1970) Role of amniocentesis in the intrauterine detection of genetic disorders. New England Journal of Medicine, 282, 596-599.

Nadler, H.L. (1972) Prenatal detection of genetic disorders. In: H. Harris & K. Hirschhorn (editors): Advances in Human Genetics, 3, 1-37; Plenum Press, New York.

Navon, R. and Padeh, B. (1971) Prenatal diagnosis of Tay-Sachs genotypes. British Medical Journal, 4, 17-20.

Niermeijer, M.F., Fortuin, J.J.H., Koster, J.F., Jahodova, M. and Galjaard, H. (1974) Prenatal diagnosis of some lysosomal storage diseases. In: W.Th. Daems, G.J.M. Hooghwinkel & J.M. Tager (editors): Enzyme Therapy in Lysosomal Storage Diseases, pp. 25-33. North-Holland Publishing Company, Amsterdam.

Niermeijer, M.F., Koster, J.F., Jahodova, M., Fernandes, J., Heukels-Dully, M.J. and Galjaard, H. (1975). Prenatal diagnosis of type II glycogenosis (Pompe's disease) using microchemical analyses. Pediatric Research, in press.

O'Brien, J.S., Okada, S., Chen, A. and Fillerup, D.L. (1970) Tay-Sachs disease: detection of heterozygotes and homozygotes by serum hexosaminidase assay. New England Journal of Medicine, 283, 15-20.

Okada, S., Veath, M.L., Leroy, J. and O'Brien, J.S. (1971) Ganglioside GM_2 storage disease: hexosaminidase deficiencies in cultured fibroblasts. American Journal of Human Genetics, 23, 55-61.

Paris Conference (1971) Standardization in human cytogenetics. Birth Defects: Original Article Series, 8, No. 7, 1972. The National Foundation-March of Dimes, New York.

Petterson, F. (1968) Epidemiology of early pregnancy wastage. Scandinavian University Books, Stockholm.

Philip, J., Bang, J., Hahnemann, N., Mikkelsen, M., Niebuhr, E., Rebbe, H. and Weber, J. (1974) Chromosome analysis of fetuses in risk pregnancies. Acta Obstetrica et Gynecologica Scandinavica, 53, 9-14.

Prescott, G.H., Pernoll, M.L., Hecht, F. and Nicholas, A. (1973) A prenatal diagnosis clinic: an initial report. American Journal of Obstetrics and Gynecology, 116, 942-948.

Punnett, H.H., Kistenmacher, M.L., Toro-Sola, M.A. and Kohn, G. (1973) Quinacrine fluorescence and Giemsa banding in trisomy 22. Theoretical and Applied Genetics, 43,

134-138.

Queenan, J.T. (1970) Intrauterine Diagnosis of Down's syndrome. Annals of the New York Academy of Sciences, 171, 617-626.

Robinson, A., Bowes, W., Droegemuller, W., Puck, M., Goodman, S., Shikes, R., and Greenshur, A. (1973) Intrauterine diagnosis: potential complications. American Journal of Obstetrics and Gynecology, 116, 937-941.

Russell, S.B., Russell, J.D. and Littlefield, J.W. (1972) β-Glucuronidase activity in fibroblasts cultured from persons with and without cystic fibrosis. Journal of Medical Genetics, 8, 441-443.

Ryan, C.A., Lee, S.Y. and Nadler, H.L. (1972) Effect of culture conditions on enzyme activities in cultivated human fibroblasts. Experimental Cell Research, 71, 388-392.

Schneider, E.L., Stanbridge, E.J., Epstein, C.J., Golbus, M., Abbo-Halbasch, G. and Rodgers, G. (1974) Mycoplasma contamination of cultured amniotic fluid cells: potential hazards to prenatal chromosomal diagnosis. Science, 184, 477-480.

Shapiro, S., Jones, E.W. and Densen, P.M. (1962) A life table of pregnancy terminations and correlates of fetal loss. Milbank Memorial Fund Quarterly, 60, 7-45.

Skovby, F. and Niebuhr, E. (1974) Presumably balanced translocations involving the same band of chromosome No. 4 found in two mentally retarded, dysmorphic individuals. Annales de Génétique, 17, 243-249.

Steele, M.W. and Breg Jr., W.R. (1966) Chromosome analysis of human amniotic fluid cells. Lancet, I, 383-385.

Therkelsen, A.J., Petersen, G.B., Steenstrup, O.R., Jonasson, J., Lindsten, J. and Zech, L. (1972) Prenatal diagnosis of chromosome abnormalities. Acta Paediatrica Scandinavica, 61, 397-404.

Van der Hagen, C.B., Børessen, A.L., Molne, K., Oftedal, G., Bjøro, K. and Berg, K. (1973) Metachromatic leukodystrophy. I. Prenatal detection of arylsulphatase A deficiency. Clinical Genetics, 4, 256-259.

Van der Ploeg, M. and Ploem, J.S. (1973) Filter combinations and light sources for fluorescense microscopy of quinacrine mustard or quinacrine stained chromosomes. Histochemie, 33, 61-70.

Wahlström, J., Bartsch, F.K. and Lundberg, J. (1974) Prenatal chromosome determination. Clinical Genetics, 6, 184-191.

Ward, A.M. and Stewart, C.R. (1974) False-positive results in antenatal diagnosis of neural tube disorders. Lancet, II, 345.

Wendel, U., Wöhler, W., Goedde, H.W., Langenbeck, U., Passarge, E. and Rüdiger,

H.W. (1973) Rapid diagnosis of Maple Syrup Urine disease (branched chain ketoaciduria) by micro-enzyme assay in leukocytes and fibroblasts. Clinica Chimica Acta, 45, 433-440.