Sanfilippo A disease in the fetus

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Summary. A pregnancy at risk for the Sanfilippo syndrome has been studied in which clear evidence was obtained from the study of amniotic fluid and fetal organs that the fetus was affected. Increased levels of heparan sulphate were found in amniotic fluid and fetal liver, while electronmicroscopy of cultured fetal fibroblasts and fetal liver showed abnormal cytoplasmic inclusions. $^{35}\text{SO}_4$ uptake studies of cultured fetal cells showed abnormal intracellular accumulation of mucopoly saccharide, while both cultured amniotic cells and fetal skin fibroblasts demonstrated deficiency of heparin sulphamidase, the enzyme responsible for the 'A' subtype of the disease. It is suggested that by use of a combination of these methods Sanfilippo A disease can now be diagnosed reliably *in utero*.

In the mucopolysaccharidoses cultured skin fibroblasts show excessive accumulation and prolonged turnover time of sulphated mucopolysaccharides which is due to the inactivity of enzymes involved in their degradation (Fratantoni, Hall, and Neufeld, 1968). The Sanfilippo syndrome (mucopolysaccharidosis III) has been demonstrated to be biochemically heterogeneous (Kresse *et al*, 1971). The inactivity of either a heparin (heparan sulphate) sulphamidase (Sanfilippo A disease—Kresse and Neufeld, 1972; Kresse 1973), or an N-acetyl- α -D-glucosaminidase (Sanfilippo B disease—O'Brien, 1972; von Figura and Kresse, 1972), both enzymes necessary for normal heparan sulphate degradation, leads to the same clinical picture.

Increasing understanding of the metabolic basis of the mucopolysaccharidoses has led to the possibility of their prenatal detection, and successful prenatal diagnosis of the Hurler (mucopolysaccharidosis I) and Hunter (mucopolysaccharidosis II) diseases has been reported (Fratantoni *et al*, 1969; Crawford *et al*, 1973).

We should like to report details of a family in which a fetus affected by Sanfilippo A disease was clearly identified by a number of different methods,

suggesting that reliable prenatal diagnosis of this disorder by amniocentesis is feasible.

Family Details

The pregnancy studied was the second of a healthy unrelated couple from South Wales, the mother being of Maltese origin. Their first child (Fig. 1), a male born in November 1966 was first investigated because of speech delay, behaviour disturbance, and mental retardation at

FIG. 1 Propositus aged 6 years.

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TABLE I
URINE MUCOPOLYSACCHARIDE EXCRETION OF
AFFECTED SIB

(Total 24-hour volume 270 ml.)

Heparan sulphate	{Hexuronic acid {Glucosamine	0.67 mmol/l. (0.002)* 0.62 mmol/l. (0.001)
Chondroitin sulphates including dermatan sulphate	(Galactosamine)	0.13 mmol/l. (0.046)
	Total hexosamine	0.75 mmol/l. (0.150)

* Value for age-matched control.

age 5 years. Coarse facial features and hirsutism suggested the possibility of a mucopolysaccharidosis, but there was no hepatosplenomegaly or corneal clouding. Skeletal x-rays showed slight anterior beaking of the vertebrae but were not diagnostic of mucopolysaccharidosis. No inclusion bodies were seen on the blood film and the karyotype was normal. Initial screening tests for mucopolysaccharides were conflicting, but the diagnosis of Sanfilippo syndrome was established by the finding of large amounts of heparan sulphate in the urine (Table I).

At the time of diagnosis of the first child the mother was again 24 weeks pregnant. Since a confident prenatal diagnosis based on study of amniotic fluid could not be offered, and since there was insufficient time for culture of amniotic cells, a termination of the pregnancy on the basis of a 1 in 4 risk was offered and was carried out at 25 weeks' gestation.

Materials and Methods

Samples of amniotic fluid and fetal tissues were obtained at the time of termination of the pregnancy by hysterotomy. Tissues for biochemical studies were immediately frozen at -20°C and kept at this temperature until analysis. Samples for electronmicroscopy were cut into 1-mm³ fragments and fixed immediately in 6% glutaraldehyde in phosphate buffer for 2 hours at pH 7.4, and post-fixed in 2% osmium tetroxide in veronal acetate buffer (pH 7.4). Tissues to be studied histologically were fixed in formalin and stained

with haematoxylin and eosin, Alcian blue, and periodic acid-Schiff reagent.

Amniotic fluid cells and fetal skin fibroblasts were cultured initially in TC 199 medium with 10% fetal calf serum. The amniotic cell cultures were slow to grow and survived only two subcultures. Fetal skin fibroblasts grew rapidly and were readily established in subcultures. Fibroblasts to be studied by electronmicroscopy were trypsinized and spun at 500 g for 10 min before direct fixation in 2% osmium tetroxide in veronal acetate buffer at pH 7.4 for 3 hours. Amniotic fluid mucopolysaccharides were analysed independently by three laboratories, whose methods in this case have been reported previously (Duncan *et al*, 1973; Ferguson-Smith *et al*, 1973; Whiteman, 1973). The mucopolysaccharide content of fetal tissues was measured after they had been extracted by ethanol, acetone and diethylether, and drying in air. The solid tissues were then digested by deoxyribonuclease and ribonuclease followed by overnight digestion with papain at 65°C (Scott, 1960). Mucopolysaccharides rendered soluble by this treatment and urinary mucopolysaccharides were fractionated on Dowex 1 $\times$ 2 (Cl^{-}) resin and assayed for hexuronic acid (Bitter and Muir, 1962), hexosamines (amino-acid Analyzer), and glucosamine residues with a free or N-sulphated amino group (Lagunoff and Warren, 1962).

Inorganic sulphate uptake by skin fibroblasts was measured by the method of Fratantoni *et al* (1968) using cultured fetal skin fibroblasts from the fetus at risk and from a 17-week normal fetus, each after two subcultures. Protein determinations were done by the method of Lowry *et al* (1951).

Determination of Heparin Sulphamidase and Glycoside Hydrolase Activities. Subcultures of skin fibroblasts and amniotic fluid cells were grown in Eagle's Minimum Essential Medium, supplemented with 10% fetal calf serum (Flow Laboratories), non-essential amino acids, and antibiotics as previously described (Kresse *et al*, 1971).

Skin fibroblasts grown to confluency in a 75-cm² plastic flask (Greiner) were harvested by trypsinization, suspended in 0.5 ml. 0.075 N NaCl, and subjected to 10

TABLE II
MUCOPOLYSACCHARIDE CONTENT OF AMNIOTIC FLUID

	Fetus at Risk (mmol/l.)	Control (mmol/l.)
<i>A. (Cardiff)</i>		
Total uronic acid	0.118	0.052
Total hexosamine	0.110	0.062
Heparan sulphate (expressed as glucosamine)		
Lagunoff and Warren Method	0.021	0.004
Amino-acid Autoanalyzer method	0.061	0.021
<i>B. (Glasgow)</i>		
Hexuronic acid (as glucuronolactone)	0.136	0.059 $\pm$ 0.021
<i>C. (London)</i>		
Total mucopolysaccharide (%)		
Chondroitin sulphates	43%	87%
Hyaluronic acid	5%	13%
Heparan sulphate	52%	None detected

FIG. 2. Cellulose acetate electrophoresis of amniotic fluid. (HS = heparan sulphate; HA = hyaluronic acid; CS = chondroitin sulphate.)

FIG. 2a. Electrophoresis in 0.1 M barium acetate, pH 8.0; 100 V; 2 hr. (Method of Duncan *et al*, 1973).

FIG. 2b. Two-dimensional electrophoresis (method of Whiteman, 1973).

cycles of freezing and thawing. Amniotic fluid cells from a 25-cm² flask were suspended in 0.2 ml. 0.075 N NaCl.

Heparin-N-³⁵SO₄ (charge 32, specific activity 15 μ Ci/mg) was purchased from Amersham-Buchler, o-nitrophenyl- α -D-N-acetylglucosaminide from Koch-Light, and p-nitrophenylglycosides from Sigma; phenyl- α -D-N-acetylglucosaminide was prepared according to the method of Weissmann (1966). From

fetal organs a 20% homogenate was made in 0.15 N NaCl by means of an Ultraturrax. For enzyme measurements a clear supernatant was obtained after centrifugation for 20 mins at 75,000 g.

The incubation mixture for the determination of heparin sulphamidase activity was as follows (values for organ extracts are given in parentheses): 50 (50) μ l. cell homogenate, 0.1 M acetate buffer, pH 5.0, 0.02% NaN₃, 7(15) μ g heparin-N-³⁵SO₄, final volume 65 (80) μ l.

TABLE III
MUCOPOLYSACCHARIDE CONTENT OF FETAL ORGANS
(μmol uronic acid/g dry tissue)

Site	Fetus at Risk		Control Fetus	
	Heparan Sulphate Fraction	Chondroitin Sulphate Fraction	Heparan Sulphate Fraction	Chondroitin Sulphate Fraction
Liver	15.9	5.7	0.50	7.9
Brain	3.25	4.8	1.48	5.5
Spleen	4.2	2.4	—	—

Skin fibroblast extracts were incubated for 6 hours, amniotic fluid cell extracts for 16 hours, and organ extracts for 16–24 hours at 37° C. At the end of the incubation period the sulphate released was determined by paper electrophoresis in a barbital-acetate-N-cetyl pyridinium chloride buffer according to Kresse (1973).

For the determination of N-acetyl- α -D-glucosaminidase activity 100 μl . cell homogenate were incubated for

6–15 hours at 37° C in the presence of 0.01 M o-nitrophenyl- α -D-N-acetylglucosaminide or 0.01 M phenyl- α -D-N-acetylglucosaminide. At the end of the incubation period N-acetylglucosamine liberated was determined by the Morgan–Elson reaction (Reissig, Strominger, and Leloir, 1959). Other glycoside hydrolases were determined as described by Werries, Pott, and Buddecke (1972).

FIG. 3. Electron micrographs of fetal liver showing hepatocytes and erythroblasts ($\times 3,600$).

FIG. 3a. Normal fetus. Contents of hepatocytes evenly distributed.

FIG. 3b. Affected fetus. Contents of hepatocytes interrupted by masses of electron-lucent, spherical inclusions.

TABLE IV
HEPARIN SULPHAMIDASE AND N-ACETYL- α -D-GLUCOSAMINIDASE
ACTIVITIES IN FETAL SKIN FIBROBLASTS AND AMNIOTIC FLUID CELLS

	Heparin Sulphamidase (pmol/mg protein/min)	N-acetyl- α -D- Glucosaminidase (nmol/mg protein/min)	
<i>Skin fibroblasts</i>			
Fetus at risk	0.21	0.083*	0.053†
Control	7.82	0.090*	0.070†
<i>Amniotic fluid cells</i>			
Fetus at risk	0.20	nd	nd
Control (32-week gestation)	7.59	0.366*	0.291†

* α -Nitrophenyl- α -D-N-acetylglucosaminide as substrate.

† Phenyl- α -D-N-acetylglucosaminide as substrate.

nd = not determined.

Results

Mucopolysaccharide Analysis of Amniotic Fluid and Fetal Organs. The amniotic fluid mucopolysaccharides, analysed independently in three laboratories, are shown in Table II; all agree in showing a marked increase in mucopolysaccharide content and in particular in heparan sulphate, compared to normal samples.

The electrophoretic pattern is shown in Fig. 2. Fig. 2a shows absence of the well-marked hyaluronic acid component normally present at this stage of gestation and the presence of increased amounts of material with mobility slightly less than that of the heparan sulphate standard. Due to the diversity of molecular species of heparan sulphate, the mobility of material in subjects with Sanfilippo syndrome has sometimes been found to be less than that

of the standard heparan sulphate preparation. In Fig. 2b two-dimensional electrophoresis demonstrates a component in the fetus at risk with mobility comparable to that of heparan sulphate.

The mucopolysaccharide content of liver, brain, and spleen is given in Table III. The 30-fold increase of heparan sulphate in the liver compared with the two-fold increase in the brain is of interest in view of the severe cerebral damage and lack of clinical hepatic involvement in the Sanfilippo syndrome.

Histological and Electronmicroscopic Studies of Fetal Organs and Cultured Cells. The female fetus (PM.CH.20/73) weighed 970 g and measured 25 cm in crown/rump and 36 cm in crown/heel length. It showed no gross external or internal abnormalities, with solid organs all being

FIG. 4. Electron micrographs of cultured fetal skin fibroblast ($\times 8,600$).

FIG. 4a. Normal fetus. Cytoplasm densely granular, with a normal complement of organelles.

FIG. 4b. Affected fetus. Cytoplasm electron-lucent, with extensive Golgi complex and numerous lysosome-like, densely osmiophilic bodies.

of normal weight (lungs 14 g, heart 5 g, liver 44 g, thymus 4 g, suprarenals 1.8 g, kidneys 5 g, and brain 112 g).

Histological examination (using haematoxylin and eosin, cresyl fast violet, toluidine blue, periodic acid-Schiff, and alcian blue at both pH 1 and pH 2.5) of all internal organs as well as cerebral cortex, basal nuclear complex, cerebellum, pons medulla, spinal cord and spinal root ganglia, nerves, lymph glands, muscle, bone, and cartilage, did not reveal structural abnormalities nor any abnormal mucopolysaccharide or other constituent. The electronmicroscopic appearance of the liver of the at risk fetus and a normal control fetus are shown in Fig. 3. Cytoplasmic inclusion bodies distending the hepatocytes are demonstrated. Abnormal inclusions are also seen in the cultured fibroblasts (Fig. 4). A full report of

electronmicroscopic findings is to follow (G. R. Newman, in preparation).

Cellular Incorporation of ^{35}S Sulphate. Figure 5 shows the uptake of ^{35}S inorganic sulphate by cultured skin fibroblasts of the at risk fetus compared with those of a normal fetus of 17 weeks' gestation. Continued accumulation of isotope is seen at 4 days, in contrast to the levelling off in the control cells, suggesting failure of release of mucopolysaccharide from the lysosomal pool.

Determination of Heparin Sulphamidase and Glycoside Hydrolases. Figure 6 shows that fibroblasts cultured from the skin of the fetus at risk for the Sanfilippo syndrome do not release inorganic sulphate from heparin- $\text{N-}^{35}\text{SO}_4$ in significant amounts. Fibroblasts from a control fetus exhibit

TABLE V
ACTIVITIES OF HEPARIN SULPHAMIDASE AND DIFFERENT GLYCOSIDE HYDROLASES IN FETAL ORGANS

	Heparin Sulphamidase*	N-acetyl- α -D-Glucosaminidase†	N-acetyl- β -D-Glucosaminidase†	β -D-Galactosidase†	β -D-Glucuronidase†
<i>Heart</i>					
Fetus at risk	0.030	0.080	118	0.99	0.095
Control	0.889	0.073	43	1.77	0.316
<i>Lung</i>					
Fetus at risk	0.043	0.103	245	0.49	0.153
Control	0.347	0.065	60	1.05	0.183
<i>Brain</i>					
Fetus at risk	0.033	0.050	29	0.41	0.052
Control	0.235	0.057	28	0.43	0.305
<i>Adrenal gland</i>					
Fetus at risk	0.015	0.119	220	4.45	0.091
Control	1.390	0.081	136	9.03	0.188
<i>Small intestine</i>					
Fetus at risk	0.112	0.249	368	1.67	0.235
<i>Placenta</i>					
Fetus at risk	0.067	0.348	18	0.10	0.311

* Expressed as pmol/mg protein/min.

† Expressed as nmol/mg protein/min.

FIG. 5. ³⁵S uptake of cultured fibroblasts from affected and normal fetus.

normal sulphamidase activity (Table IV). For eight skin fibroblast lines of other than Sanfilippo A genotype the mean heparin sulphamidase activity was 9.41 ± 2.55 pmol/mg protein/min, whereas five Sanfilippo A cell lines had an activity of 0.30 ± 0.19 pmol/mg protein/min (Kresse, 1973).

Since essentially normal values for N-acetyl- α -D-glucosaminidase activity have been measured in skin fibroblasts of the fetus at risk, the results obtained establish the diagnosis of Sanfilippo A disease. This was confirmed by correction studies kindly carried out by Dr Elizabeth Neufeld on cultured fetal skin fibroblasts.

The deficiency of heparin sulphamidase activity was also found in cultured amniotic fluid cells. Shortage of material prevented the determination of N-acetyl- α -D-glucosaminidase activity in those cells.

The deficiency of a heparin sulphamidase can also be demonstrated in organ extracts of the affected fetus (Table V). The somewhat higher residual activity compared with skin fibroblasts and amniotic fluid cells might be due to the existence of sulphamidase isoenzymes, the presence of which has not yet been investigated. It should be noted that heparin-N-³⁵SO₄ contains some labelled O-sulphate groups which could be removed by the action of a heparin sulphate ester sulphatase.

The measurements of N-acetyl- β -D-glucosaminidase, β -D-galactosidase and β -D-glucuronidase show that the secondary activity changes of lysosomal hydrolases in the mucopolysaccharidoses which have been reported in the past, are already present to some extent during fetal life. In the brain, however, in which only a moderate increase of

FIG. 6. Electrophoretic pattern of heparin-N-³⁵SO₄ after incubation with skin fibroblast homogenates from a fetus at risk for Sanfilippo syndrome (●—●) and from a normal control (○—○).

heparan sulphate content has been observed, normal N-acetyl- β -D-glucosaminidase and β -D-galactosidase activities were found. Kint *et al* (1973) have suggested that the activity of lysosomal enzymes is altered by excessive storage of acid mucopolysaccharides.

Discussion

Various techniques for prenatal diagnosis of mucopolysaccharidoses, especially the determination of mucopolysaccharide content in the amniotic fluid and the pattern of ³⁵SO₄ incorporation into amniotic cells, have been attempted.

The analysis of total mucopolysaccharide content of amniotic fluid and their fractionation, which avoids the uncertainty of growth of amniotic cells and the delay involved, bears the risk of false negative results. Matalon *et al* (1970) reported raised

total mucopolysaccharide content of amniotic fluid at 14 weeks' gestation in a pregnancy affected by Hurler disease, and also found a high proportion of heparan sulphate, not detectable in normal samples. Subsequently, however, they found normal levels of mucopolysaccharide in two abnormal pregnancies; one studied at 10 weeks' gestation, resulted in an infant with Sanfilippo syndrome; the other, at risk for Hunter disease, was later shown to have abnormal ³⁵SO₄ cellular uptake, and a termination was performed (Matalon, Dorfman, and Nadler, 1972). Brock *et al* (1971) also found normal values at 18 weeks in a pregnancy resulting in a child with Hurler disease. Nevertheless an abnormal result may be of great value, particularly in more advanced pregnancies where, as in the present case, there may not be time to study cultured cells before the decision has to be made whether or not to terminate the pregnancy. Details of variation in the

acid mucopolysaccharide content of amniotic fluid throughout pregnancy have been given by Duncan *et al* (1973).

Using the method of measuring the $^{35}\text{SO}_4$ uptake of cultured amniotic cells no misdiagnosis has been reported so far. Successful prenatal diagnoses of Hurler and Hunter diseases have been reported by Fratantoni *et al* (1969) and Crawford *et al* (1973).

Because of the late onset of clinical symptoms in the Sanfilippo syndrome the possibility of its prenatal diagnosis was not predictable. The results reported in this paper, which were obtained by various techniques in different laboratories, leave no doubt that the fetus exhibited an abnormal heparan sulphate metabolism at the 25th week of pregnancy.

In addition to the above mentioned methods, the determinations of heparin sulphamidase and N-acetyl- α -D-glucosaminidase activities were used for the first time to establish prenatal diagnosis. The present case was shown to be type A by the profound deficiency of heparin sulphamidase in all tissues investigated, whereas the N-acetyl- α -D-glucosaminidase activity was normal. Correction tests confirmed the enzymatic diagnosis.

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