AN INVESTIGATION OF AMNIOTIC FLUID MICROVILLAR FRAGMENTS IN AUTOSOMAL TRISOMY PREGNANCIES

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Technician:- A person skilled in a practical art.

(Chambers's Twentieth Century Dictionary)

In Germany they first came for the Communists and I didn't speak up because I wasn't a Communist. Then they came for the Jews and I didn't speak up because I wasn't a Jew. Then they came for the Trade Unionists and I didn't speak up because I wasn't a Trade Unionist. Then they came for the Catholics and I didn't speak up because I was a Protestant. Then they came for me ——— and by that time no one was left to speak up.

——— Pastor Martin Niemöller

TABLE OF CONTENTS Page

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List of Tables Page

List of Figures

Appendices

List of Abbreviations

AF	Amniotic Fluid
AFP	Alphafoetoprotein
ALP	Alkaline Phosphatase
APM	Amino Peptidase M
AST	Aspartate transaminase
CF	Cystic Fibrosis
cDNA	complimentary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
FS	Final Supernatant
GGT	Gamma glutamyl transferase
hCG	human chorionic gonadotrophin
βhCG	free β subunit of human chorionic gonadotrophin
hPL	human placental lactogen
LAP	Leucine amninopeptidase
MV	Microvillar fraction
n	Number of samples
NORM	Normal
PAPP-A	Pregnancy associated placental protein A
PBS	Phosphate Buffered Saline
Q.C.	Quality Control
S.D.	Standard deviation
SP1	pregnancy specific β-1 specific glycoprotein
T21	Trisomy 21
T18	Trisomy 18
T13	Trisomy 13
UE	Unconjugated oestriol
U/g	Units of enzyme activity per gram of protein

SUMMARY

Reduced levels of microvillar enzymes in amniotic fluid have been reported in association with several foetal abnormalities, including urinary tract and gastro-intestinal anomalies, chromosomal disorders and cystic fibrosis. Trisomies 21, 18 and 13 have been reported, by several authors, to show reduced levels of gamma glutamyl transferase (GGT), aminopeptidase M (APM), alkaline phosphatase (ALP) and the disaccharidases; maltase, sucrase, trehalase and lactase. The reasons for this are not understood. Possible explanations include; (a) reduced amounts of enzyme on the microvillar membranes, (b) reduced numbers of microvillar fragments, with the normal complement of enzyme, reaching the amniotic fluid, or (c) normal microvillar fragments present in normal amount but subjected to an abnormal rate of enzyme inactivation in the amniotic fluid.

The aim of this project was to develop the method of Potier *et al.* (1986) for the purification of microvillar fragments from samples of amniotic fluid and to investigate purified microvillar fragments from autosomal trisomy pregnancies in comparison with normal controls, which were matched for sample volume, length of time in storage, gender of the foetus and gestational age.

Confirmation that purified material was rich in microvillar fragments was provided by examination of material using transmission electron microscopy. Microvillar vesicles, as described by other authors, were clearly seen and high resolution electron micrographs taken.

Initial purification utilising aliquots from a large pool of normal amniotic fluid produced results for protein content similar to those found by Potier *et al.* (1986). However, despite improvements in enzyme yield achieved by resuspension of microvillar material in phosphate buffered saline and increased speed and time of centrifugation, levels of enzyme activity in purified samples remained lower than those found by Potier *et al.* (1986).

Each batch of purifications included an aliquot, taken from the above mentioned pool, of normal amniotic fluid as a quality control sample. Twelve trisomy 21, five trisomy 18, four trisomy 13, 34 normal amniotic fluid samples and 36 quality control samples were purified. Lysosomal contamination was excluded by acid phosphatase assay of microvillar fractions which revealed negligible levels of this lysosomal enzyme. The initial fluid, final supernatant and the purified microvillar fractions were tested for protein concentration, gamma glutamyl transferase activity and maltase activity.

Mean protein concentration in trisomy 18 samples was shown to be statistically significantly reduced from normal in initial and final supernatant samples ($p=0.0002$ and $p=0.022$ respectively). This was not reflected in the purified microvillar fraction of trisomy 18 samples which showed no statistically significant difference from normal ($p=0.84$). Mean protein concentration in trisomy 21 and 13 samples showed no statistically significant difference from normal for any of the fractions tested (trisomy 21 samples, initial fluid $p=0.65$, final supernatant $p=0.73$, microvillar fraction $p=0.3$; trisomy 13 samples, initial fluid $p=0.47$, final supernatant $p=0.45$, microvillar fraction $p=0.41$). Thus protein

concentration in purified microvillar fractions for all three forms of autosomal trisomy showed no statistically significant difference from normal.

GGT activity was statistically significantly reduced from normal for all three fractions of trisomy 21 samples (initial sample $p=<0.0001$, final supernatant $p=0.0003$, microvillar fraction $p=0.028$). Trisomy 18 samples showed statistically significant reduction in GGT activity in the initial sample ($p=0.012$) and in the final supernatant ($p=0.012$) but not in microvillar fractions ($p=0.11$) and trisomy 13 samples showed no statistically significant difference from normal GGT activity for any of the fractions tested (initial fluid $p=0.23$, final supernatant $p=0.13$, microvillar fraction $p=0.58$).

Trisomy 21 samples had statistically significantly reduced maltase activity in all three fractions (initial sample $p=<0.0001$, final supernatant $p=0.0002$, microvillar fractions $p=0.0014$). Trisomy 18 samples showed no statistically significant difference from normal in maltase activity for any of the three fractions tested (initial sample $p=0.47$, final supernatant $p=0.7$, microvillar fraction $p=0.25$). Trisomy 13 samples showed a statistically significant reduction in maltase activity in initial sample ($p=<0.0001$) and microvillar fraction ($p=0.0003$) but despite having only 36.1% of normal activity in final supernatant this proved not to be a statistically significant reduction ($p=0.13$).

Post mortem reports for the foetuses with autosomal trisomy were examined for any evidence of developmental anomalies which might have influenced the biochemical findings of this study. Of the nine reports

available for the Down's syndrome foetuses no evidence of any urinary tract anomalies, gastro-intestinal blockage or atresias were found. The trisomy 18 and 13 foetuses however had a high incidence of both gastro-intestinal and urinary tract anomalies.

The findings of this investigation show that in the presence of normal protein concentrations purified microvillar fractions of trisomy 21 samples have statistically significant reductions in microvillar enzyme activity. This suggests that either there is a significant enzyme deficiency in the microvillar material, which reaches the amniotic fluid in normal amounts, or that an increased rate of enzyme inactivation occurs in the amniotic fluid of Down's syndrome pregnancies. In trisomies 18 and 13 the findings were less conclusive and reflect the more complex post mortem findings and limited sample numbers available.

1. INTRODUCTION AND AIMS

1.1 MICROVILLAR MEMBRANE

The specialised striated cell surface of the small intestine lumenal border was first noted by Henle in 1837. However, the limitations of light microscopy meant that the detail of the structure, as a border of microvilli 0.1μm in diameter and 1-2μm in length, was not revealed until 1950 when Granger and Baker used electron microscopy to resolve its true morphology.

Microvillar membrane was first isolated from intestinal epithelial cells by Miller and Crane in 1961 for biochemical investigation of disaccharide hydrolysis. Subsequently brush border vesicles, derived from rat small intestine, were used by Hopfer *et al.* (1973) to study glucose transportation. Lacroix *et al.* (1984) purified brush border membranes according to Schmitz *et al.* (1973) from foetal intestine. Samples from eight weeks gestation to term were used and sucrase, alkaline phosphatase (ALP) and aminopeptidase activity measured. Biopsy material from the same pregnancies was also prepared for scanning electron microscopy and morphological structure and enzymatic function of foetal intestine correlated. More recently, the structure and function of the microvillar membranes and their enzymes have been the subject of detailed investigation by several workers (Semenza 1986, Cowell 1986 Hunziker *et al.* 1986, Danielsen *et al.* 1986, Hu *et al.* 1987, Danielsen 1987 & 1988, Mantei *et al.* 1988, Semenza 1989, Ruf *et al.* 1990 and Danielsen 1990). From their work DNA and amino acid sequences for several

enzymes have been derived and details of the quarternary folding, membrane incorporation and mode of attachment are now known.

1.2 MICROVILLAR ENZYMES AND FOETAL ABNORMALITY

In the field of prenatal diagnosis microvillar enzymes became an area of interest when abnormal levels were noted in amniotic fluids from pregnancies affected by a variety of foetal abnormalities.

1.2.1 Urinary Tract Anomalies

An increased trehalase level in amniotic fluid taken from a pregnancy affected by polycystic kidney disease was reported by Morin *et al.* (1981). The same authors also described elevated trehalase activity in two pregnancies affected by congenital nephrosis (Morin *et al.* 1984). They attributed increased trehalase activity to the breakdown of the microvillar tissue in the proximal tubules of the kidneys. This theory is supported by the normal level, in the same samples, of the intestinal disaccharidase palatinase. Szabo *et al.* (1990) also described elevated amniotic fluid trehalase activity in polycystic kidney disease and in radial-renal disease but in a case of urethral obstruction reduced trehalase and lactase activity were found.

1.2.2 Gastro-intestinal Anomalies

It has been shown by several other authors that gastro-intestinal obstructions resulting in reduced passage of meconium are associated with reduced foetal intestinal microvillar enzymes in amniotic fluid

(Potier *et al.* 1977, Morin *et al.* 1980, Van Diggelen *et al.* 1983, Kleijer *et al.* 1985 and Morin *et al.* 1987).

Potier *et al.* (1977) reported deficiency of disaccharidases in a sample of amniotic fluid taken at 16 weeks gestation from a patient who had a previous pregnancy in which the foetus had a meningocoele. A child with an imperforate anus and duodenal atresia was delivered at 31 weeks gestation following spontaneous onset of labour. Investigation of the stored amniotic fluid supernatant revealed absence of sucrase, trehalase, and lactase activity and significantly reduced levels of maltase and palatinase. These findings were confirmed by Morin *et al.* (1980) who reported reduced disaccharidase activity in four cases of gastro-intestinal anomalies (one case of duodenal atresia, one case of multiple intestinal atresia and two cases of imperforate anus) which were compared to normal ranges derived from 221 normal control fluids. Van Diggelen *et al.* (1983) also reported reduced disaccharidase activity in a case of anal atresia. Morin *et al.* (1987) showed reduced levels of intestinal alkaline phosphatase and GGT as well as disaccharidases in cases of imperforate anus, multiple intestinal atresia, duodenal atresia, jejuno-ileal atresia and in pregnancies affected by gastroschisis, diaphramatic hernia and omphalocoele where intestinal obstruction had occurred. Other foetuses affected by gastroschisis and omphalocoele but without intestinal obstruction showed normal amniotic fluid microvillar enzyme activities. An investigation of maltase activity in amniotic fluid, taken between 16 and 24 weeks gestation by Claass *et al.* (1985) showed that maltase activity is intestinal in origin and attributable to the sucrase-isomaltase complex of the microvillar membrane. They found no evidence of renal or lysosomal contributions. This confirmed Potier's

earlier finding (1978) that amniotic fluid disaccharidase activity is intestinal in origin.

1.2.3 Cystic Fibrosis

Van Diggelen *et al.* (1983) found reduced activity of disaccharidases; lactase, maltase, sucrase and trehalase in amniotic fluid samples from four pregnancies affected by cystic fibrosis (CF). Kleijer *et al.* (1985) found reduced levels of the same four disaccharidases in amniotic fluid from 11 pregnancies affected by CF. Schwartz and Brandt (1985) reported reduced maltase and sucrase activity in three out of four CF samples. Claass *et al.* (1986) used maltase and alkaline phosphatase to test 57 amniotic fluids from pregnancies at a one in four risk of CF and found reduced levels in 88% of affected samples and none of the samples from normal pregnancies gave false positive results. Carbarns *et al.* (1983) used microvillar peptidases GGT and aminopeptidase M (APM) to investigate a group of 132 amniotic fluids of which 16 were from pregnancies at risk of CF. Of this group six were subsequently shown to be from foetuses affected by CF. These six had levels of GGT and APM significantly lower than those in the normal control group. Baker and Dann (1983) confirmed reduced GGT and APM in five pregnancies affected by CF. Brock (1983) achieved a detection rate for CF of 90% by using relative proportions of intestinal and bone/liver/kidney alkaline phosphatase isoenzymes in prenatal diagnosis of CF. Muller *et al.* (1984), in an investigation of alkaline phosphatase isoenzymes, found that increased discrimination between CF and normal samples could be achieved if samples were taken from 17 weeks gestation onwards, those taken earlier having a higher false negative rate. This was confirmed

by Aitken *et al.* (1985) who found that mis-classification of samples was more likely at gestations less than 17 weeks. Further confirmation of the importance of gestational variation was shown by Brock *et al.* (1985) in the course of a comparison of various microvillar enzyme assays and showed APM and intestinal alkaline phosphatase had false positive rates of 4% and 8% respectively but for each of these a detection rate of 96% could be achieved provided sampling occurred after 17 weeks gestation.

Brock *et al.* (1983) proposed that the reduced levels of peptidases, alkaline phosphatase and disaccharidase activity in amniotic fluid were all indicative of an abnormality of the microvilli at the foetal stage of the disease. Carbarns *et al.* (1983) suggested that abnormal development of microvilli or microvillar atrophy would explain reduction in enzyme activity seen in amniotic fluid from CF pregnancies. Van Diggelen *et al.* (1983) hypothesised that reduced gut mobility may be responsible for the reduction in amniotic fluid microvillar enzyme levels in CF. Movement of intestinal contents can be so reduced in a foetus affected by CF that it may result in total obstruction of the small intestine known as meconium ileus (Muller *et al.* 1984 and Muller *et al.* 1985). Reduced levels of amniotic fluid microvillar enzymes in association with various intestinal anomalies (Potier *et al.* 1977, Morin *et al.* 1980, Van Diggelen *et al.* 1983 and Morin *et al.* 1987) and higher than normal disaccharidase activity found in the meconium of neonates with CF, resulting from accumulation of disaccharidases in slow moving and viscid meconium, (Antonowicz *et al.* 1975) appeared to support this theory. However, Aitken *et al.* (1988) used the purification method described by Potier *et al.* (1986) to isolate microvillar fragments from a series of normal amniotic fluids and three samples taken from pregnancies in

which the foetus had cystic fibrosis. The purified CF samples showed reduced levels of maltase, intestinal alkaline phosphatase and GGT by comparison to the control fluids. However protein levels in the purified fractions from the three CF samples were within the same range as those seen in the normal fluids suggesting that there was no deficiency in the amount of microvillar material present in the original sample.

1.2.4 Chromosomal Disorders

Jalanko and Aula (1982) analysed GGT activity in 259 amniotic fluid samples, of which 31 were from chromosomally abnormal pregnancies. The remaining 228, from normal pregnancies, provided normal control ranges for GGT activity at 15, 16 and 17 weeks gestation. Autosomal trisomy pregnancies showed significantly reduced levels of GGT activity, most notably in trisomy 18 samples, where seven out of nine samples had levels below the 25th percentile of the normal range. Several other authors have confirmed reduced amniotic fluid GGT activity in association with autosomal trisomies. Each group of authors reported very high percentages of autosomal trisomy samples with GGT activity below the normal median. For all investigations and each form of trisomy between 84% and 100% of samples were found to be in this category. Their findings are summarised in table 1.1.

As in CF, a variety of microvillar enzymes differ from normal in association with chromosomal disorders. Jalanko and Heikinheimo (1983) assayed 173 mid-trimester amniotic fluid samples for alkaline phosphatase activity. Mean alkaline phosphatase from 19 pregnancies affected by trisomy 21 and eight pregnancies affected by trisomy 18 was half of

Author and Year	Trisomy 21		Trisomy 18		Trisomy 13	
	number	% below median	number	% below median	number	% below median
Jalanko and Aula (1982)	19	84	9	100	1	100
Brock *et al.* (1984)	54	85			0	
Buamah *et al.* (1984)	6	100	0		0	
Muller *et al.* (1986)	54	94	12	92	11	91
Morin *et al.* (1987)	13	100	10	100	0	
Macek *et al.* (1987)	35	89	19	100	3	100
Jones and Evans (1988)	16	94	0		0	
Zeitune *et al.* (1989)	37	95	6	83	2	100
Giddy *et al.* (1989)	26	92				
Szabo *et al.* (1990)	1	100	1	100	1	

Table 1.1 Published reports of GGT activity in amniotic fluid from trisomies 21, 18 and 13

that seen in normal controls. Brock *et al.* (1984) used APM, GGT and isoenzymes of alkaline phosphatase, in an investigation of chromosomal anueploidy, with 54 trisomy 21, 14 trisomy 18 and 3 Turner syndrome samples. Significantly reduced levels of these enzymes were seen in all three forms of aneuploidy investigated. Kleijer *et al.* (1985) tested disaccharidases; maltase, sucrase, trehalase and lactase and reported reduced activity in amniotic fluid from five trisomy 13 pregnancies, three out of five trisomy 18 pregnancies and 19 out of 22 trisomy 21 pregnancies. Muller *et al.* (1986) tested amniotic fluids from 54 trisomy 21, 11 trisomy 18, three trisomy 13 and three Turner syndrome pregnancies and found significantly reduced levels of GGT, amino peptidase M and intestinal alkaline phosphatase in all four groups of samples. They proposed that foetal growth retardation may account for this. Morin *et al.* (1987) confirmed reduced microvillar enzymes in amniotic fluids from pregnancies affected by aneuploidy, using disaccharidases; maltase, sucrase, palatinase, trehalase and lactase, GGT and alkaline phosphatase activities. They found reduced microvillar enzyme activity in seven out of 21 trisomy 21 pregnancies, eight out of ten trisomy 18 pregnancies and one out of four Turner syndrome pregnancies. However all seven of the trisomy 21 and five of the trisomy 18 samples were affected by intestinal obstruction and Morin and colleagues concluded that this was the reason for the observed reduction in enzyme activity and not the presence of aneuploidy *per se*. No autopsy was available in the case of the Turner syndrome pregnancy and no explanation was offered for reduced activity in three trisomy 18 samples without intestinal obstruction. Giddy *et al.* (1989) in a study of amniotic fluid from 239 women, of whom 26 had trisomy 21 pregnancies, used leucine aminopeptidase (LAP), GGT, asparate

transaminase (AST) and isoenzymes of alkaline phosphatase (ALP) to look for those pregnancies affected by trisomy 21 and identified LAP as the most reliable predictor of an affected pregnancy. They suggest that enzyme assay of amniotic fluid taken for non-chromosomal indications, could be used to screen samples and in this way to highlight those samples which should than proceed to foetal karyotyping. Szabo *et al.* (1990) used 132 normal amniotic fluids to provide normal ranges for trehalase, lactase, alkaline phosphatase and GGT activity. They reported reduced alkaline phosphatase in amniotic fluid from trisomy 21 and reduced trehalase activity in trisomies 21, 18 and 13. A sample from a Klinefelter syndrome pregnancy had reduced levels of ALP and GGT. They noted that none of the autosomal trisomy foetuses had any evidence of intestinal obstruction.

Thus amniotic fluid samples from pregnancies affected by chromosomal disorders have been shown by several authors to have reduced levels of various microvillar enzymes. To date, however, no study has been undertaken to explain why this should be so and no mechanism which explains reduction in microvillar enzyme activity in amniotic fluid from these pregnancies has been investigated. It is not known if microvillar fragments are present in the same concentration in these samples or if reduced numbers of such fragments reach the amniotic fluid of autosomal trisomy pregnancies or if the loss of enzyme activity is the result of disruption of enzyme production and incorporation into the microvillar membrane or to increased degradation of enzyme once it has reached the amniotic fluid.

1.3 AIMS OF THIS PROJECT

1. To investigate reduced levels of microvillar enzymes in amniotic fluid from autosomal trisomy pregnancies by direct examination of microvillar material purified from amniotic fluid samples.

2. To assess the purification of microvillar fragments from amniotic fluid using the method described by Potier *et al.* (1986).

3. To exclude lysosomal contamination of purified fractions using acid phosphatase activity as a lysosomal marker.

4. To confirm the purity of the microvillar fractions using transmission electron microscopy.

5. To compare protein concentration, GGT and maltase activity in amniotic fluids, prior to purification and in purified fractions, from autosomal trisomies 21, 18 and 13 matched normal controls.

2. MATERIALS AND METHODS

2.1 AMNIOTIC FLUID SAMPLES

Amniotic fluid samples from the West of Scotland, obtained by transabdominal amniocentesis for a variety of indications, are sent to the Duncan Guthrie Institute of Medical Genetics for foetal chromosome analysis and biochemical analysis (approximately 1,500 samples per year).

After centrifugation for the removal of the amniotic fluid cells, used to establish foetal cell cultures for use in determining foetal karyotype, the supernatant fraction (average volume 10 - 20mls) is divided into two parts: a 1.0ml portion for immediate biochemical analysis (Alpha-foetoprotein and Acetyl cholinesterase testing) and the remainder which is stored in universal containers at -20°C.

2.1.1 Quality Control Pool

Periodically, after the outcomes of pregnancies have been confirmed, batches of normal amniotic fluid supernatant (as described above) are pooled and stored frozen in bulk. One five litre container of pooled normal fluids collected over a one year period, was aliquoted into volumes comparable to that of individual samples e.g. 10 15 and 20mls which were then used to develop the microvillar purification protocol and subsequently as a quality control sample in each batch of purifications.

2.1.2 Matched Normal Amniotic Fluids

Each sample of fluid from a chromosomally abnormal pregnancy was purified in parallel with a sample from a normal pregnancy matched, as far as possible, for length of time in storage, sample volume, gestational age and gender of the foetus.

2.2 PURIFICATION OF MICROVILLAR FRAGMENTS FROM AMNIOTIC FLUID

Using the method described by Potier *et al.* (1986) microvilli were purified from both pooled samples of amniotic fluid and individual amniotic fluids.

Samples of between 10ml and 30ml were first dialysed overnight against 5 litres of distilled water at 4°C. The following day each sample was diluted with an equal volume of Tris mannitol buffer (100mM mannitol and 4mM Tris. at pH7) and the appropriate amount of calcium chloride required to bring the concentration to 10mM was added to each. This was then stirred for 10 mins at 4°C after which it was spun in a 4°C centrifuge at 3000*g* for 15 mins. Supernatant was removed and spun, in initial purifications, at 27,578*g* for 45 mins at 4°C. In subsequent purifications the centrifugation protocol was modified in an attempt to increase the yield of enzyme activity in the microvillar fractions, consequently two further batches of purifications had final centrifugation of 32,566*g* and 39,410*g* and respective times of 1 and 1.5 hours. The resulting microvillar pellets were resuspended in Phosphate Buffered Saline pH7.2 and stored at -20°C.

At each stage of the purification process a fraction of the sample was retained and stored for future use. Thus, for each sample, pre-dialysis, post dialysis, calcium chloride pellet, calcium chloride supernatant, final supernatant and microvillar fractions were all retained and stored at -20°C until required for analysis. The purification process can be schematically represented as seen in Fig. 1.1

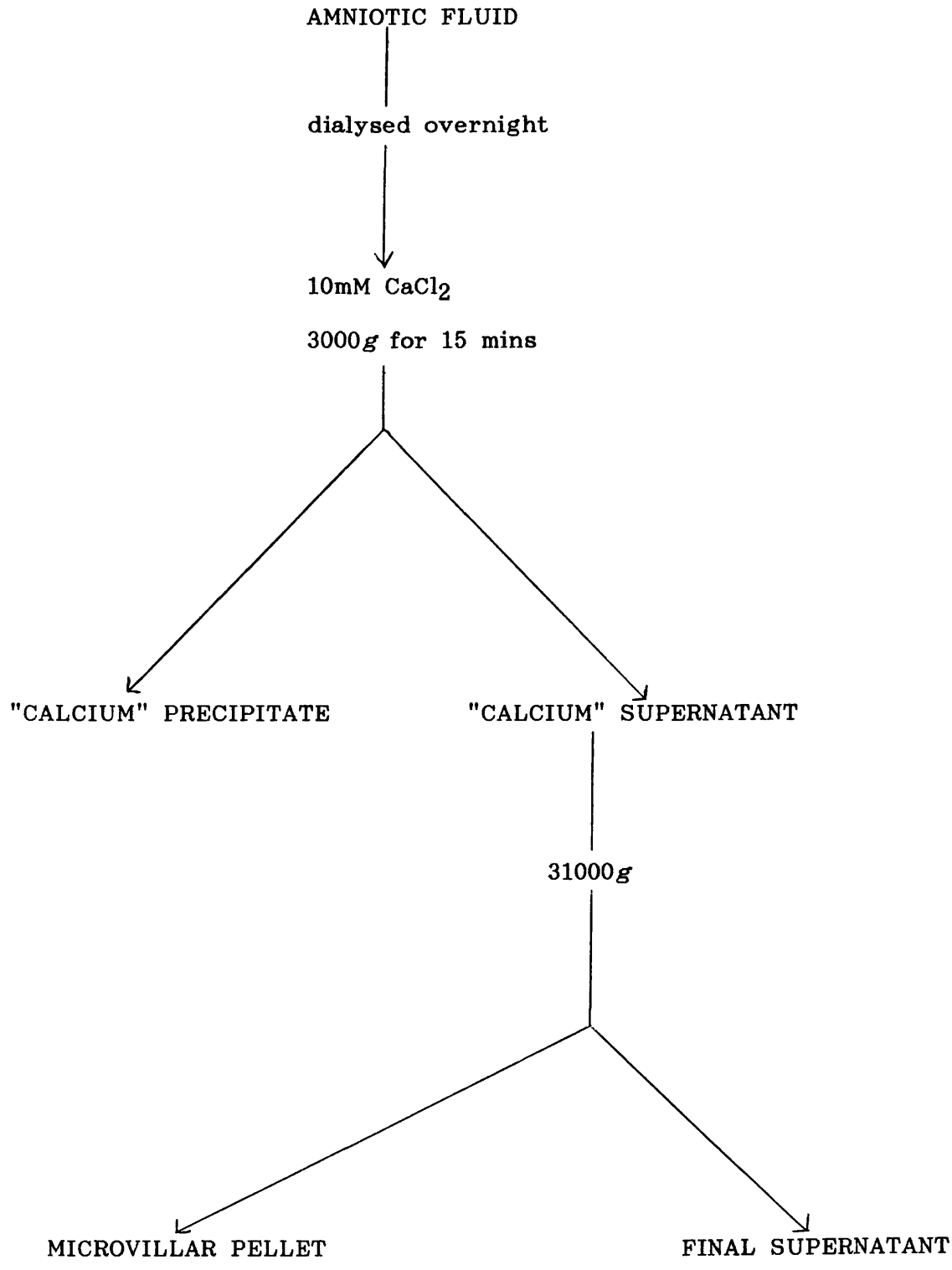

Fig. 1.1 Purification of microvillar fragments from amniotic fluid

2.3 PROTEIN ESTIMATION (Modified Lowry estimation)

Reagents

Solution A 2% Na_2CO_3 in 0.1M NaOH

Solution B1 1% $CuSO_4$ in distilled water

Solution B2 2% Na K Tartrate in distilled water

Solution C 98% of Solution A plus 1% of Solution B1 plus 1% of Solution B2. This solution was freshly made for use each day.

Standard Curve

A standard solution of 500 mg/ml of Bovine Serum Albumin was prepared. Using distilled water this standard was diluted in a series of dilutions from 500 to 50 mg.

Folins reagent

Folins reagent was diluted 1:1 with distilled water.

Assay procedure

Standard curve and tests were done in duplicate. Distilled water was used as a zero point in the standard curve.

800 µl of solution C was added to all tubes.

80 µl of sample/standard was added to appropriate tubes.

Tubes were vortexed and left to stand for 10 minutes.

While holding each tube on the vortex 80 µl of diluted Folins Reagent was added.

Tubes were placed in the dark for at least 45 minutes to allow colour to develop.

These were then read against a reagent blank at O D 750 nm. The standard curve was plotted on a graph and used to calculate the test results.

In each batch of tests a quality control sample of pooled amniotic fluid was run as a measure of assay performance.

2.4 GAMMA GLUTAMYL TRANSFERASE ASSAY

Gamma Glutamyl transferase (GGT) activity was estimated using a modified Sigma diagnostics kit.

Assay Reaction

This utilises the conversion of the artificial substrate gamma glutamyl p-nitroanilide (which is colourless) in the presence of glycylglycine into gamma glutamylglycylglycine and p-nitroaniline which absorbs light strongly at 405 nm. The rate of increase in absorbance is directly proportional to GGT activity in the sample.

Assay Reagent

GGT reagent is reconstituted with distilled water providing a solution of 4.4 mmol/l gamma glutamyl p-nitroanilide and 75 mmol/l glycylglycine in 2-amino-2-methyl propandiol buffer (0.2M) at pH8.2 . This is warmed to between 37°C and 50°C and when completely dissolved the reagent is cooled to assay temperature (30°C) before use.

Assay Procedure

1 ml of GGT reagent was dispensed into a semi-micro glass cuvette and mixed with 0.02 ml of sample. This was placed in a Pye Unicam SP8-100 UV/VIS spectrophotometer with temperature control cuvette chamber and integral chart recorder. One minute pre-incubation at assay temperature (30°C) was allowed to enable the initial "lag phase" to pass before the chart recorder was used to trace the linear increase in absorbance, at 405 nm, over a period of 1-3 minutes of reaction time.

Subsequently the change in absorbance over time was used to calculate the GGT activity in the sample using the following formula.

$$\text{GGT ACTIVITY U/L} = \frac{\text{A PER MIN x TV x 1000}}{\text{9.9 x SV x LP}}$$

A PER MIN = change in absorbance per min at 405 nm

TV = Total reaction volume (1.02 ml)

SV = Sample volume (0.02 ml)

9.9 = Millimolar absorptivity of p-nitraniline at 405 nm

LP = Light path (1 cm)

1000 = Converts units per ml to units per litre

A quality control sample of pooled amniotic fluid was included in each batch of tests as a measure of assay performance.

2.5 MALTASE ASSAY

This method was described by Claass *et al.* (1985) in "Characteristics of Maltase in amniotic fluid" and uses the artificial substrate 4-Methylumbelliferyl apha-D-glucopyranoside which in the presence of maltase is cleaved to release fluorescent methylumbelliferone. The concentration of methylumbelliferone so released is compared with a standard solution of methylumbelliferone and the figures used in the calculation shown.

Reagents

Sodium Maleate Buffer

0.1M Solution of Sodium Maleate (BDH) pH6.0

Substrate Solution

2.2mmol of 4 Methyl umbelliferyl alpha-d-glucopyranoside (Koch Light Ltd.) in 10 ml Sodium Maleate Buffer.

Stopping Buffer

0.5M Bicarbonate / Carbonate Buffer pH10.7

A small volume of 0.5M sodium bicarbonate solution was titrated to pH10.7 with 0.5M sodium carbonate solution. (Stock may be stored frozen at -20°C for future use.)

Methylumbelliferone Standard

2µmolar methylumbelliferone (Koch Light Ltd.) in 0.4M sodium phosphate

buffer with 0.5M glycine was made and dispensed into 1 ml aliquots, stored at -20°C. A fresh aliquot of standard was used for each assay.

Assay Method

Each assay included a water blank and a quality control sample (an aliquot of pooled amniotic fluid). Each estimation was done in triplicate. A solution of 2 micromolar methylumbelliferone (Sigma) in stopping buffer was used as a standard against which the fluorescence in test samples was compared.

20 µl of substrate solution was added to every tube
10 µl of water was added to blank tubes
10 µl of control was added to the control tubes
10 µl of sample was added to the appropriate sample tubes

Tubes were capped and vortexed using a multi-tube vortexer (2601 Scientific Manufacturing Industries) and incubated at 37°C for 1 hour in a waterbath (Grant instrumental, Cambridge, Ltd.).

The reaction was stopped by the addition of 0.5 ml of 0.5M Bicarbonate/Carbonate stopping buffer pH10.7 to each tube.

Readings of the fluorescence were made in a Perkin Elmer MPF-44B Fluorescence Spectrophotometer at 365 nm excitation wavelength 448 nm emission wavelength.

Using an empty cuvette the machine was set to zero, readings of a water blank and standard methylumbelliferone solution were made. Sets

of triplicate readings were taken with reference to the methylumbelliferone standard and any tendency for the machine to drift was corrected before each set of triplicates was read. The value of the water blank was subtracted from the mean of the triplicate readings and Maltase activity was calculated as follows.

$$\frac{\text{Fluorescence - blank}}{60(\text{minutes})} \times \frac{1000}{1} \times \frac{30}{10} \times \frac{2}{S} = \text{Maltase activity U/l}$$

Fluorescence = the chart reading of the sample

blank = the chart reading of the blank

1000 = unit conversion factor

30 = the final assay volume(µl)

10 = the sample volume(µl)

2 = the standard methylumbelliferone value(µM)

S = the chart reading of the standard

A quality control sample of pooled amniotic fluid was included in each batch of tests as a measure of assay performance.

2.6 ACID PHOSPHATASE

Acid phosphatase was measured using a Boehringer Mannheim GmbH diagnostica kit designed for use with serum. It was necessary therefore to adjust the volumes used to allow for the differences in concentration between serum and amniotic fluid.

Assay principle

p-nitrophenylphosphate plus water react in the presence of acid phosphatase to give phosphate and p-nitrophenol.

Reagents

Buffer - citrate buffer 50mmol/litre pH4.8

Substrate - sodium p-nitrophenylphosphate 5.5mmol/litre in tablet form

Preparation and stability of solutions

Buffer was prepared by diluting reagent provided with 100 mls of re-distilled water. This is stable for 1-2 years at plus 8°C.

One tablet of substrate was dissolved in 10 ml of buffer. This is stable for 1 week at 2-8°C.

100 mls of 0.02mol/litre sodium hydroxide solution was also prepared.

Assay Procedure

A sample blank is prepared for comparison with each sample reaction tube.

100 µl of substrate was dispensed into both "blank"and "test" tubes.

20 µl of sample was added to "tests" only.

Tubes were incubated at 37°C in a water bath for 30 minutes exactly.

1 ml of sodium hydroxide was then added to each tube. Finally 20 µl of sample was added to "blank" tubes.

Absorbance in "blanks" and "tests" was read at 405 nm in a PYE UNICAM SP8-100 UV/VIS spectrophotometer. The value obtained for "blank" tubes was subtracted from that of the "test" in each case. The absorbance figures thus obtained were multiplied by 101 to convert to U/l thus:-

$$\text{Absorbance} \times 101 = \text{U/l } (37^{\circ}\text{C})$$

A quality control sample of pooled amniotic fluid was included in each batch of tests as a measure of assay performance.

2.7 STATISTICAL ANALYSIS

The Minitab software program was chosen for statistical analysis of data because of the relatively small numbers involved in any data set, no group having greater than 40 individual results.

2.7.1 Minitab "DESCRIBE"

Using the minitab "DESCRIBE" command minitab will provide a mean, standard deviation, trimmed mean, standard error, median, lowest and highest value in any given data set. The statistical perameters used in this study were derived in this way.

2.7.2 Minitab "TWOSAMPLE" (students's *t* test)

Minitab program "TWOSAMPLE" was used for student *t* testing. This program uses the difference between means of unpaired data and does not assume equal variance for the populations compared. Unless otherwise specified a confidence interval of 95% is used. The formula used for derivation of the t value is as follows:-

$$t = \frac{(x_1 - x_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

Where x = the group mean

s = the group standard deviation

n = the number of samples in the group

On completion of "TWOSAMPLE" analysis Minitab provides for each data set a list of the number of samples, mean, standard deviation, and standard error mean for each set of data. It also shows the Degrees of Freedom and T and p values.

2.8 ELECTRON MICROSCOPY

To examine the morphology of the material present in the pellet of the purified fractions transmission electron microscopy was used.

Pellets of microvilli were scraped into cacodylate buffer containing 2% gluteraldehyde. The material thus fixed was then held in Kranowsky's buffer. Clumps of pellet material were then dehydrated through a graded series of acetones and embedded in EMIX (epoxy resin) embedding agent.

"Thick" (approximately 1 micron) sections were cut from these blocks and examined by light microscope to verify alignment of the block before proceeding to cut "thin" (500 Angstrom) sections. These "thin" sections were then stained with uranyl acetate and lead citrate, mounted on copper grids, and examined using a Philips 200CX Electron Microscope.

Grids were scanned at x14,000 magnification and closer examination of features from x20,000 up to x140,000 magnification.

Photographs of typical fields and structures at both low and high magnification were taken.

3. RESULTS

3.1 INITIAL PURIFICATION

Using the method described by Potier *et al.* (1986), fragments of microvilli were extracted from three pools of amniotic fluid supernatants which had been stored at -20°C. The protein content of each of the fractions in the initial purification gave similar results to those of Potier *et al.* (1986) see table 3.1. The level of gamma glutamyl transferase (GGT) activity in the microvillar pellet was, however, considerably lower than that found by Potier *et al.* (1986). In this study the mean GGT activity in initial samples was 58.3 U/g (S.D.=29.6, n=3) and in the microvillar fraction 89.7 U/g (S.D.=60.5, n=3) with a mean protein content of 2.25 mg (S.D.=0.68, n=3) which represents 2.7% recovery of original total GGT activity, compared to 10.9% recovery found by Potier *et al.* (1986). Modifications to the protocol were introduced in an attempt to improve the enzyme yield in the purified microvillar fractions.

3.2 PURIFICATION MODIFICATIONS

Modifications such as resuspending the microvillar pellet in phosphate buffered saline (PBS), sonicating pellet material and increasing centrifugation speed and time for pelleting microvilli were made in an effort to improve enzyme recovery.

3.2.1 Resuspension of pellets

Initial purifications of nine pooled samples resulted in microvillar pellets

	Initial purification	Potier *et al.* (1986)
Initial pool	100.0%	100.0%
Calcium precipitate	0.4%	0.9%
Calcium supernatant	84.0%	88.0%
Final supernatant	85.0%	75.0%
Microvillar fraction	2.0%	0.6%

Table 3.1 : Mean percentage total protein in initial purification compared to Potier *et al.* (1986)

which had a mean GGT enhancement of 1.6 times the original GGT activity. The use of PBS instead of final supernatant for resuspension of pellet material in the following purification of three pooled samples, resulted in microvillar pellets with a mean enzyme enhancement of 3.2 times the original activity i.e. 100% increase. This modification was adopted for all further purifications.

3.2.2 Sonication

Sonication of the resuspended microvillar pellet before enzyme assay did not provide a consistent increase in enzyme activity and was not used in future enzyme assays (Table 3.2).

3.2.3 Centrifugation

Increasing centrifugation force from 27,578*g* through 32,566*g* to 39,410*g* and time from 45 minutes through one hour to one and a half hours provided increased mean protein yield from 1.3% to 1.7% to 2.1% and improved enzyme recovery in microvillar pellets from x3.0 to x6.8 to x8.7 in quality control samples (Table 3.3). These increases in centrifugal force and time were therefore adopted as modifications to the purification protocol.

3.3 EXCLUSION OF LYSOSOMAL CONTAMINATION

In the course of purification lysosomal material was removed by precipitation with calcium chloride. To ensure that this process had

GGT ACTIVITY (U/L)

sample	without sonication	with sonication	percentage difference
POOL 8	213	240	+12
POOL 9	247	316	+28
POOL 10	206	206	0
POOL 11	282	344	+22
873983	240	186	-23
874302	234	164	-30

Table 3.2 : Effect of sonication on GGT activity in microvillar fractions

	BATCH 1 27,578*g* 45 mins.	BATCH 2 32,566*g* 1 hour	BATCH 3 39,410*g* 1.5 hours
Protein recovered	1.3%	1.7%	2.1%
GGT enhancement	x3.0	x6.8	x8.7
No of Samples	n=7	n=10	n=7

Table 3.3 : The effects of increasing centrifugal force and time on protein content and GGT activity in microvillar pellets of quality control samples

been successfully completed microvillar pellets were tested for the lysosomal enzyme acid phosphatase. Twenty four samples from six purifications gave mean acid phosphatase activity of 2.8 U/gm of protein (S.D.=2.1, n=24) in microvillar fractions, indicating negligible lysosomal contamination.

3.4 ELECTRON MICROSCOPY

Purified material from the quality control sample was prepared for transmission electron microscopy as described in materials and methods (section 2.8). Examination of this material confirmed the presence of microvillar vesicles as described by Potier *et al.* (1986) and Schmitz *et al.* (1973). Both smooth and rough vesicles of variable circumference were seen with clearly defined double membrane structure and, on rough vesicles, the outer electron-dense structures responsible for the "cogwheel" appearance described by Potier *et al.* (1986). Photomicrographs of typical fields can be seen in figures 3.1, 3.2 and 3.3 .

3.5 QUALITY CONTROL SAMPLE (EXCLUSION OF NORMAL GROUP BIAS)

The quality control sample, derived from a pool of at least 500 normal amniotic fluid supernatants, was included in each batch of purifications. Using student's *t*-test the quality control sample data were compared with the normal amniotic fluid group. Mean protein concentration for the quality control group was 5,240 mg/l (S.D.=761, n=36) and for the normal sample group was 5,285 mg/l (S.D.=1,644, n=34), mean GGT activity for the quality control group was 67.7 U/g (S.D.= 9.4, n=31) and for the

normal sample group was 67.5 U/g (S.D.=34.1, n=35) and mean maltase activity for the quality control group was 44.2 U/g (S.D.= 14.4, n=17) and for the normal sample group was 58.3 U/g (S.D.=32.4, n=24). P values for each of these parameters were 0.66, 0.98 and 0.068 respectively confirming that there was no statistically significant difference between the two groups and indicating the absence of selection bias in the normal sample group.

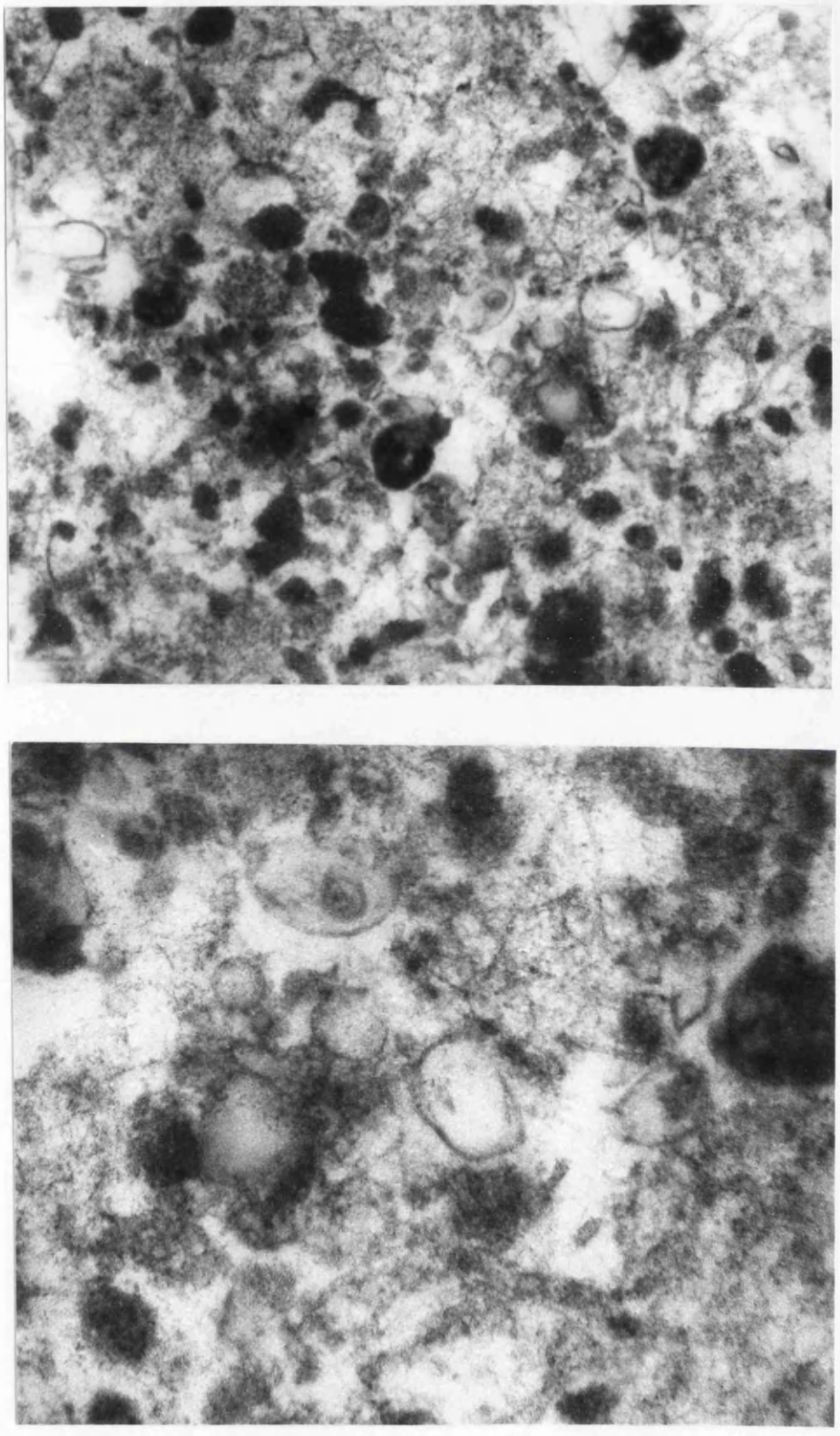

Fig 3.1 Transmission electron micrographs showing several 'smooth' microvillar vesicles. Magnification, above x20,000 : below x40,000

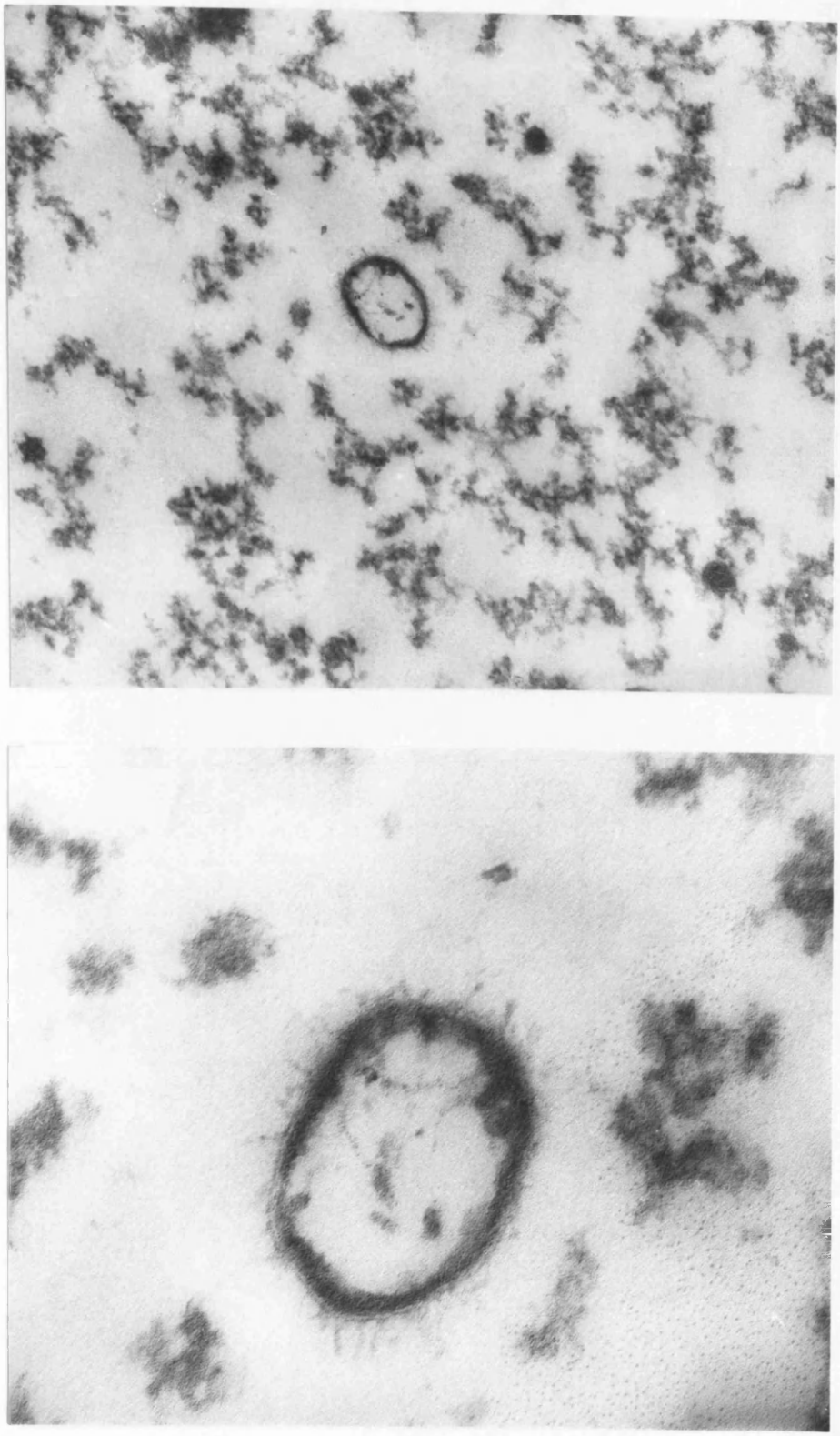

Fig. 3.2 Transmission electron micrographs showing a single 'rough' microvillar vesicle with visible double membrane structure and electron dense projections. Magnification, above x20,000 : below x67,000

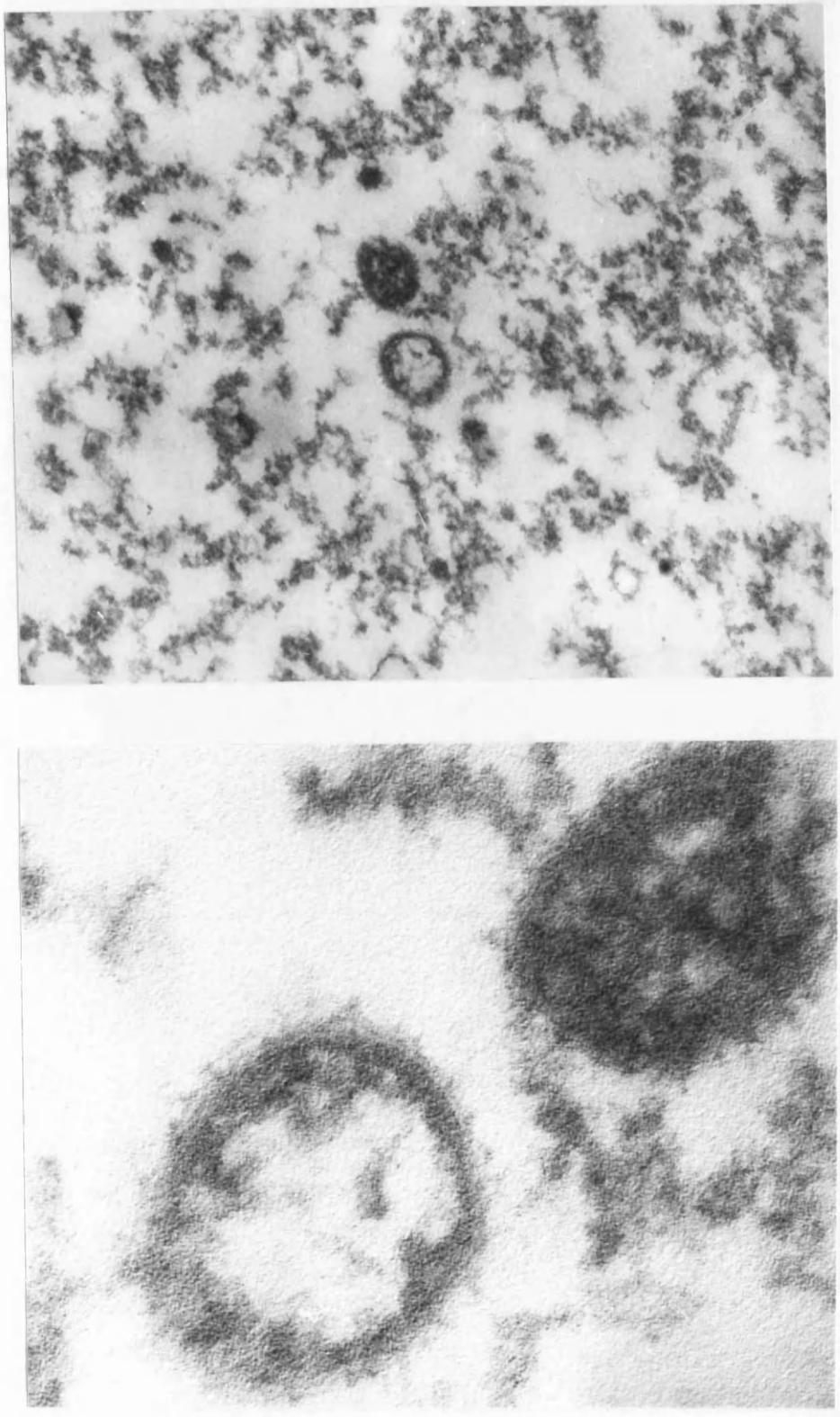

Fig. 3.3 Transmission electron micrographs of 'rough' microvillar vesicles, high magnification clearly shows double membrane structure and electron dense projections. Magnification, above x20,000 : below x100,000

3.6 PROTEIN CONCENTRATION

Means and standard deviations of protein concentration in the initial samples, final supernatants and microvillar fractions for each group of amniotic fluid samples (quality control, normal, trisomy 21, trisomy 18 and trisomy 13) are shown in table 3.4

Scattergrams comparing the distribution and range of protein concentrations for each of the above groups were drawn for initial fluid (Fig. 3.4), final supernatant (Fig. 3.5) and microvillar fractions (Fig. 3.6).

3.6.1 Protein concentration in normal and trisomy 21 samples

Initial protein concentration in normal fluids had a mean of 5,285 mg/l (S.D.=1,644, n=34). Trisomy 21 fluids fall within this normal range having a mean value of 5,068 mg/l (S.D.=1,351, n=12). A p value of 0.65 confirms that there is no statistically significant difference between these results.

A similar pattern was found in the final supernatant with, for normal fluids, a mean of 2,319 mg/l (S.D.=813, n=34) and for trisomy 21 fluids a mean value of 2,418 mg/l (S.D.=856, n=12). There is no statistically significant difference between these distributions (p=0.73).

Microvillar fractions had a mean protein content of 709 mg/l (S.D.=469, n=34), for normal samples, and 578 mg/l (S.D.=333, n=12) for trisomy 21 fluids. Again there is no statistically significant difference between normal and trisomy 21 fluids (p=0.3).

INITIAL FLUID

Samples	MEAN	S.D.	n
Q.C.	5420	761	36
NORMAL	5285	1644	34
TRISOMY 21	5068	1351	12
TRISOMY 18	3706	490	5
TRISOMY 13	6440	2782	4

FINAL SUPERNATANT

Samples	MEAN	S.D.	n
Q.C.	2458	298	36
NORMAL	2319	813	34
TRISOMY 21	2418	856	12
TRISOMY 18	1687	414	5
TRISOMY 13	3050	1680	4

MICROVILLAR FRACTION

Samples	MEAN	S.D.	n
Q.C.	720	299	36
NORMAL	709	469	34
TRISOMY 21	578	333	12
TRISOMY 18	678	279	5
TRISOMY 13	500	406	4

Table 3.4 Mean protein concentration (mg/l) for each group. (S.D.=standard deviation, n=number of samples, Q.C.=quality control)

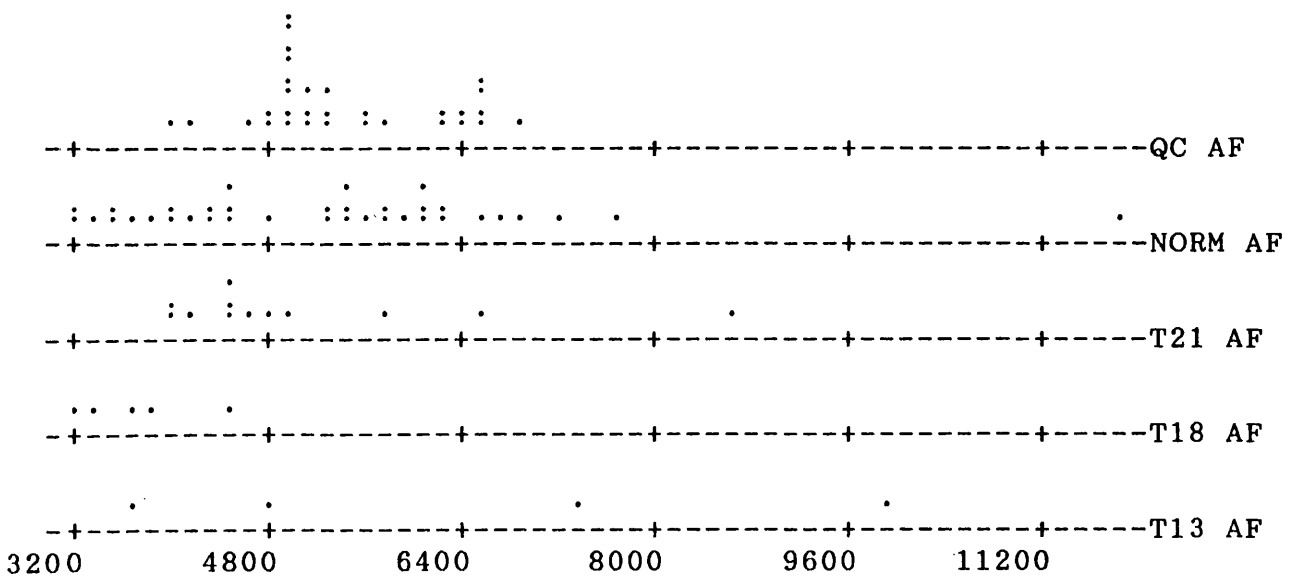

Fig. 3.4 Protein concentrations (mg/l) for each group of initial samples (AF=initial amniotic fluid, NORM=normal samples, T21=trisomy 21 samples, T18=trisomy 18 samples, T13=trisomy 13 samples, Q.C.=quality control samples)

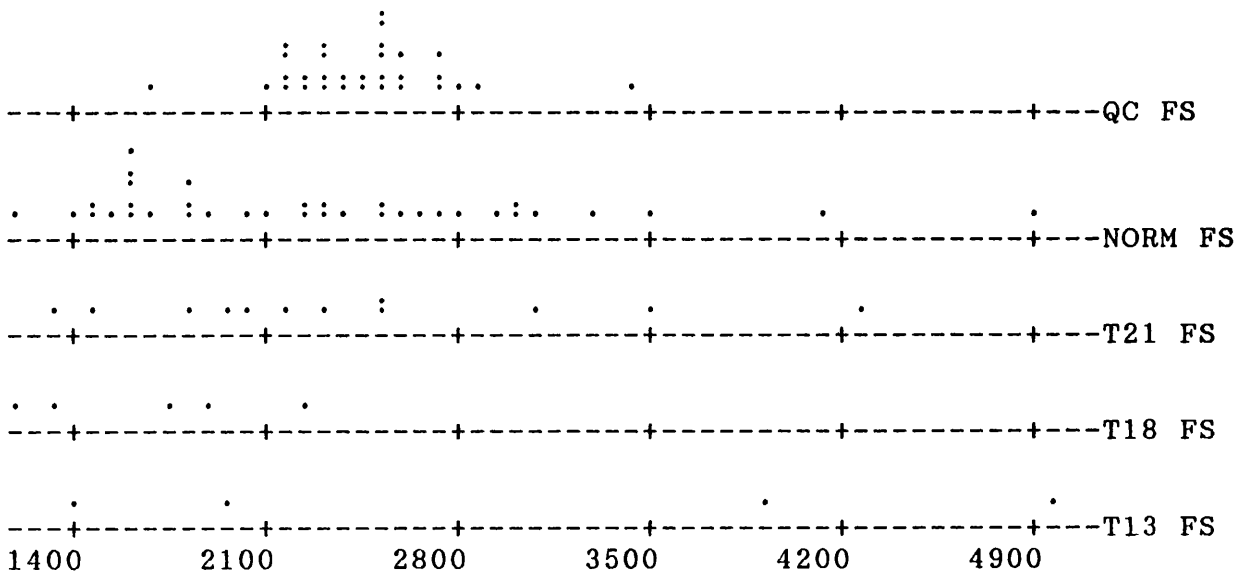

Fig. 3.5 Protein concentration (mg/l) for each group of final supernatant fractions (FS=final supernatant fraction, NORM=normal samples, T21=trisomy 21 samples, T18=trisomy 18 samples, T13=trisomy 13 samples, Q.C.=quality control samples)

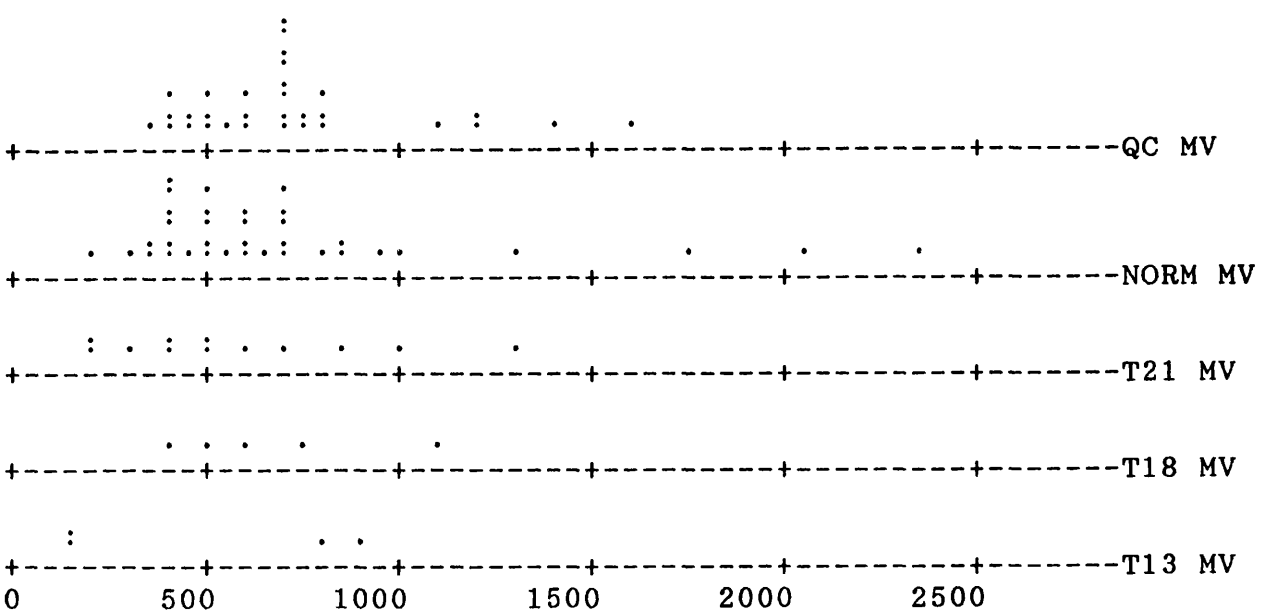

Fig. 3.6 Protein concentration (mg/l) for each group of microvillar fractions (MV=microvillar fraction, NORM=normal samples, T21=trisomy 21 samples, T18=trisomy 18 samples, T13=trisomy 13 samples, Q.C.=quality control samples)

Thus no statistically significant difference in protein concentration between normal amniotic fluids and those of trisomy 21 samples was seen in any of the fractions tested.

3.6.2 Protein concentration in normal and trisomy 18 samples

Five trisomy 18 samples had an initial protein concentration between 3,000 mg/l and 4,000 mg/l, with a mean of 3,706 mg/l (S.D.=490, n=5). This is 73.1% of the normal mean of 5,285 mg/l (S.D.=1,644, n=34) and is a statistically highly significant reduction ($p = 0.0002$).

Final supernatant samples show a similar relationship between normal and trisomy 18 samples with a mean value for trisomy 18 samples of 1,687 mg/l (S.D.=414, n=5). This is 72.7% of the normal mean of 2,319 mg/l (S.D.=813, n=34) and is a statistically significant reduction ($p=0.022$). This difference is not seen in the microvillar fractions of trisomy 18 samples. No reduction in protein concentration is apparent when trisomy 18 microvillar fractions are compared with the normal microvillar fractions. Mean protein concentration for trisomy 18 microvillar fractions was 678 mg/l (S.D.=279, n=5) which is 95.6% of the normal mean which was 709 mg/l (S.D.=469, n=34). There is no statistically significant difference ($p=0.84$) between the two groups of samples.

Therefore although protein concentration prior to purification and in final supernatant shows a statistically significant reduction from normal, there is no such statistically significant reduction in purified microvillar fractions of trisomy 18 samples.

3.6.3 Protein concentration in normal and trisomy 13 samples

The four trisomy 13 samples showed no consistency regarding protein concentration in the initial sample, each sample being widely separated from the others, sometimes by greater than 1,000 mg/l. The mean of 6,440 mg/l (S.D.=2,782, n=4) is 27% higher than the normal mean of 5,285 mg/l (S.D.=1,644, n=34) but not statistically significantly different (p=0.47) from it.

In the final supernatant fraction, the mean of the trisomy 13 samples, 3,050 mg/l, (S.D.=1,680, n=4) is 31.5% higher than that of the normal mean 2,319 mg/l, (S.D.=813, n=34). This is not a statistically significant difference (p=0.45).

In the microvillar fractions, the normal mean is 709 mg/l (S.D.=469, n=34) and the trisomy 13 mean is 500 mg/l (S.D.=406, n=4) which is 29.5% lower than normal but this is not a statistically significant reduction (p=0.41).

Thus, as with trisomy 21 samples, no significant difference in protein concentration between normal amniotic fluids and those of trisomy 13 samples is seen in any of the fractions tested although in trisomy 13 the number of samples is small and the standard deviation is wide.

3.7 GAMMA GLUTAMYL TRANSFERASE (GGT) ACTIVITY

Means and standard deviations of GGT activity in the initial samples, final supernatants and microvillar fractions for each group of amniotic

fluid samples (quality control, normal, trisomy 21, trisomy 18 and trisomy 13) are shown in table 3.5.

Scattergrams comparing the distribution and range of GGT activity for each of the above groups of samples were drawn for initial fluid (Fig. 3.7), final supernatant (Fig. 3.8), and microvillar fraction (Fig. 3.9).

3.7.1 Gamma glutamyl transferase activity in normal and trisomy 21 samples

All twelve trisomy 21 amniotic fluid samples had initial GGT activity which was less than the normal group mean of 67.5 U/g (S.D.=34.1, n=35). The highest GGT activity in a trisomy 21 sample was 44.3 U/g. The trisomy 21 group mean was 30.1 U/g (S.D.=10.0, n=12) which is 44.6% of the normal group mean and is statistically a highly significant reduction ($p=<0.0001$).

Trisomy 21 final supernatant samples show the same pattern of distribution relative to normal samples as that seen in the initial samples. All twelve trisomy 21 samples had less GGT activity than the normal group mean of 52.3 U/g (S.D.=36.2, n=35). The highest activity in a trisomy 21 sample was 46.0 U/g. The trisomy 21 group mean was 24.8 U/g (S.D.=11.0, n=12) which is 47.5% of the activity seen in the normal group. This is statistically a highly significant redution ($p=0.0003$).

Comparison of trisomy 21 microvillar fractions with those from normal pregnancies again show a similar pattern of GGT activity. Only two

INITIAL FLUID

Samples	MEAN	S.D.	n
Q.C.	67.7	9.4	31
NORMAL	67.5	34.1	35
TRISOMY 21	30.1	10.0	12
TRISOMY 18	29.8	21.6	5
TRISOMY 13	41.1	33.0	4

FINAL SUPERNATANT

Samples	MEAN	S.D.	n
Q.C.	51.7	11.6	31
NORMAL	52.3	36.2	35
TRISOMY 21	24.8	11.0	12
TRISOMY 18	19.7	18.6	5
TRISOMY 13	25.8	25.4	4

MICROVILLAR FRACTION

Samples	MEAN	S.D.	n
Q.C.	412.7	210.9	31
NORMAL	330.1	226.1	35
TRISOMY 21	216.4	110.7	12
TRISOMY 18	200.7	133.3	5
TRISOMY 13	417.0	268.0	4

Table 3.5 Mean GGT activity (U/g) for each group of samples (S.D.=standard deviation, n=number of samples, Q.C.=quality control samples)

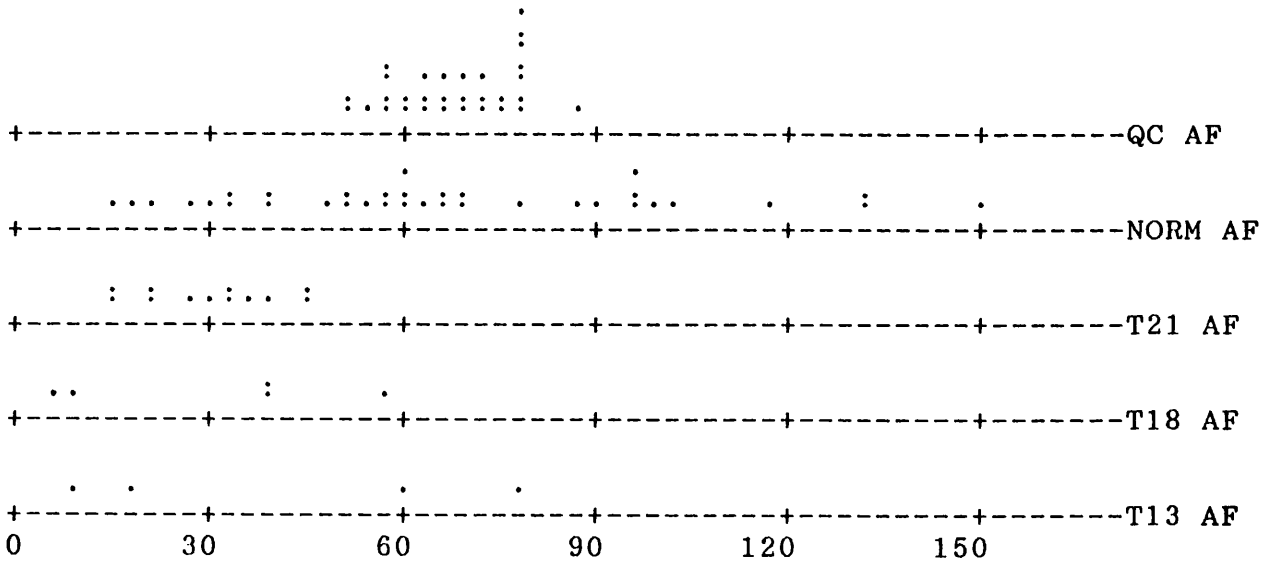

Fig. 3.7 Gamma glutamyl transferase activity (U/g) for each group of initial samples (AF=initial amniotic fluid, NORM=normal samples, T21=trisomy 21 samples, T18=trisomy 18 samples, T13=trisomy 13 samples, Q.C.=quality control samples)

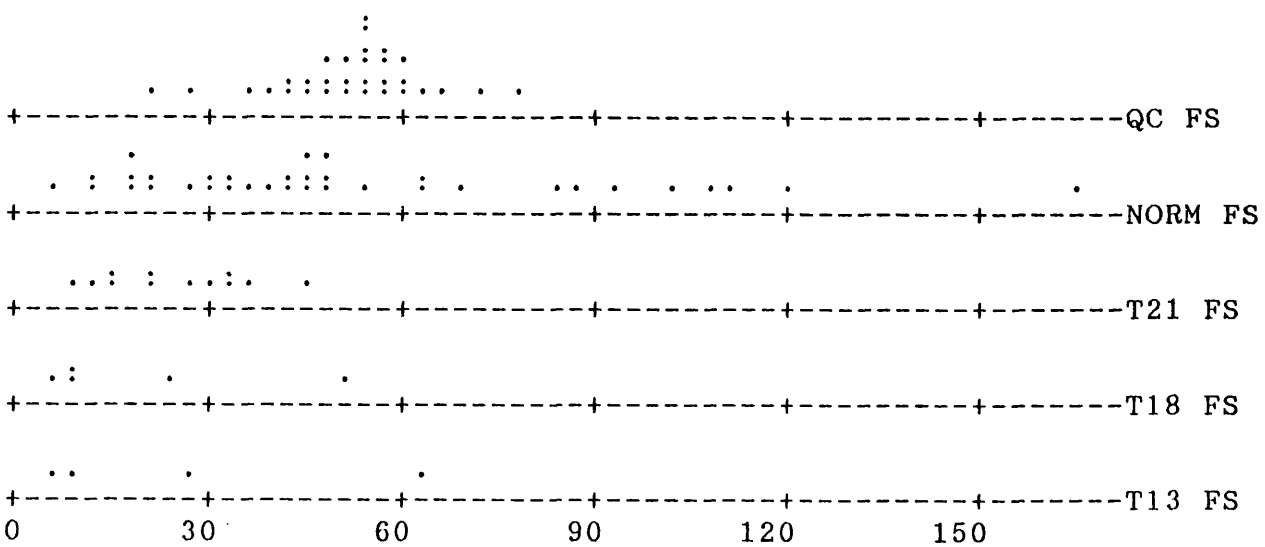

Fig. 3.8 Gamma glutamyl transferase activity (U/g) for each group of final supernatant fractions (FS=final supernatant fraction, NORM=normal samples, T21=trisomy 21 samples, T18=trisomy 18 samples, T13=trisomy 13 samples, Q.C.=quality control samples)

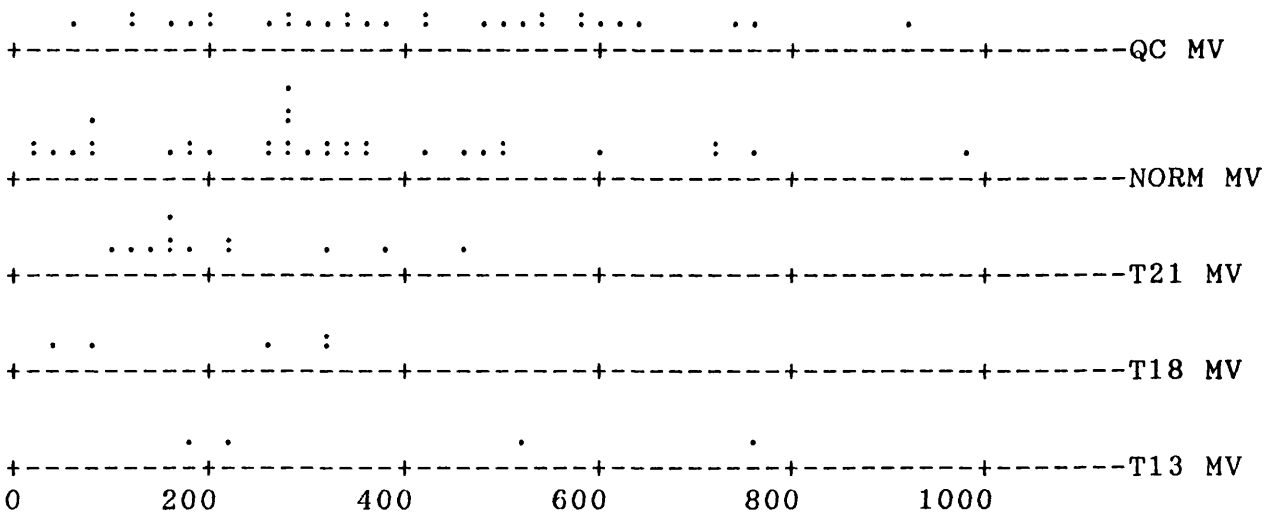

Fig. 3.9 Gamma glutamyl transferase activity (U/g) for each group of microvillar fractions (MV=microvillar fraction, NORM=normal samples, T21=trisomy 21 samples, T18=trisomy 18 samples, T13=trisomy 13 samples, Q.C.=quality control samples)

(16.6%) trisomy 21 microvillar samples have GGT activity higher than the normal group mean of 330.1 U/g (S.D.=226.1, n=35). The trisomy 21 group mean of 216.4 U/g (S.D.=110.7, n=12) is 65.6% of the normal mean. This represents a statistically significant reduction (p=0.028).

Thus for all three fractions tested, initial sample, final supernatant and microvillar fraction, trisomy 21 samples show statistically significant reductions from normal in GGT activity.

3.7.2 Gamma glutamyl transferase activity in normal and trisomy 18 samples

All five trisomy 18 samples have GGT activity which is lower than the normal group mean of 67.5 U/g (S.D.=34.1, n=35). The highest trisomy 18 sample had GGT activity of 57.0 U/g. The trisomy 18 group mean was 29.8 U/g (S.D.=21.6, n=5). This represents 44.1% of normal GGT activity and is a statistically significant reduction (p=0.012).

The same pattern is seen in final supernatant samples where the highest trisomy 18 sample had GGT activity of 50.3 U/g. The normal group mean was 52.3 U/g (S.D.=36.2, n=35) and the trisomy 18 group mean was 19.7 U/g (S.D.=18.6, n=5). This represents 37.7% of normal GGT activity and is a statistically significant reduction (p=0.012).

Although all five samples of trisomy 18 microvillar fractions have GGT activity which is less than the normal sample mean of 330.1 U/g (S.D.=226.1, n=35), the trisomy 18 group mean was 200.7 U/g (S.D.=133.3, n=5) which is 60.8% of normal. This is not statistically significantly

different (p=0.11) from normal.

Thus trisomy 18 samples show a statistically significant reduction in GGT activity in the initial fluid and final supernatant samples but although activity is also reduced in the microvillar fractions this reduction is not sufficient to be statistically significant.

3.7.3 Gamma glutamyl transferase activity in normal and trisomy 13 samples

Only one trisomy 13 sample has GGT activity higher than the normal group mean in the initial amniotic fluid samples. The mean GGT activity in trisomy 13 amniotic fluid samples was 41.1 U/g (S.D.= 33.0, n=4) this is 60% of the normal amniotic fluid group mean of 76.5 U/g (S.D.=34.1, n=35) but is not a statistically significant reduction (p=0.23).

Again in final supernatant samples only one trisomy 13 sample has GGT activity higher than the normal group mean. The mean GGT activity in final supernatant of trisomy 13 samples was 25.8 U/g (S.D.=25.4, n=4) which is 49% of the normal final supernatant mean of 52.3 U/g (S.D.=36.2, n=35). However this is not a statistically significant reduction (p=0.13).

Mean GGT activity in microvillar fractions of trisomy 13 samples was 417.0 U/g (S.D.=268, n=4) which was 26% higher than that of the normal group mean of 330.1 U/g (S.D.=226.1, n=35) but this is not a statistically significant difference (p=0.58).

Thus no statistically significant difference was found in GGT activity

between trisomy 13 and normal samples for any of the fractions tested. This may be a reflection of the small number of trisomy 13 samples available and the wide standard deviations for protein concentration in these samples.

3.8 MALTASE ACTIVITY

Means and standard deviations of maltase activity in the initial sample, final supernatant and microvillar fractions for each group of amniotic fluid samples (quality control, normal, trisomy 21, trisomy 18 and trisomy 13) are shown in table 3.6.

Scattergrams comparing the distribution and range of maltase activity for each of the above mentioned group of samples were drawn for initial sample (Fig. 3.10), final supernatant (Fig. 3.11) and microvillar fractions (Fig. 3.12).

3.8.1 Maltase activity in normal and trisomy 21 samples

Prior to purification all 12 trisomy 21 samples had maltase activity which was less than the normal sample group mean of 58.3 U/g (S.D.=32.2, n=24). The highest value for a trisomy 21 sample was 47.0 U/g. The trisomy 21 group mean was 21.2 U/g (S.D.=14.5, n=12) which is 36.3% of the normal group mean and is a statistically highly significant reduction ($p=<0.0001$).

The same pattern is seen in final supernatant samples where the highest trisomy 21 sample had maltase activity of 14.0 U/g which is lower than

INITIAL FLUID

Samples	MEAN	S.D.	n
Q.C.	44.2	14.4	17
NORMAL	58.3	32.2	24
TRISOMY 21	21.2	14.5	12
TRISOMY 18	45.9	32.0	5
TRISOMY 13	9.4	8.4	4

FINAL SUPERNATANT

Samples	MEAN	S.D.	n
Q.C.	14.9	6.2	17
NORMAL	16.6	11.9	24
TRISOMY 21	5.2	3.9	12
TRISOMY 18	14.8	8.8	5
TRISOMY 13	6.0	10.1	4

MICROVILLAR FRACTION

Samples	MEAN	S.D.	n
Q.C.	599.9	381.5	17
NORMAL	457.0	385.3	24
TRISOMY 21	154.2	119.9	12
TRISOMY 18	300.5	221.4	5
TRISOMY 13	84.7	77.7	4

Table 3.6 Mean maltase activity (U/g) for each group (S.D.=standard deviation, n=number of samples, Q.C.=quality control samples)

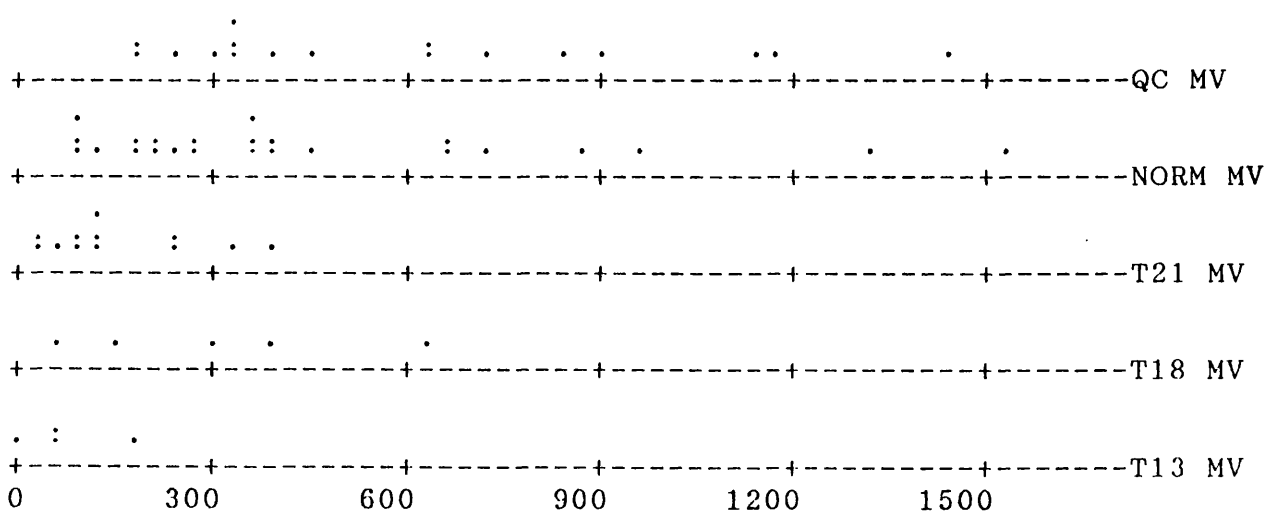

Fig. 3.10 Maltase activity (U/g) for each group of initial samples (AF=initial amniotic fluid, NORM=normal samples, T21=trisomy 21 samples, T18=trisomy 18 samples, T13=trisomy 13 samples, Q.C.=quality control samples)

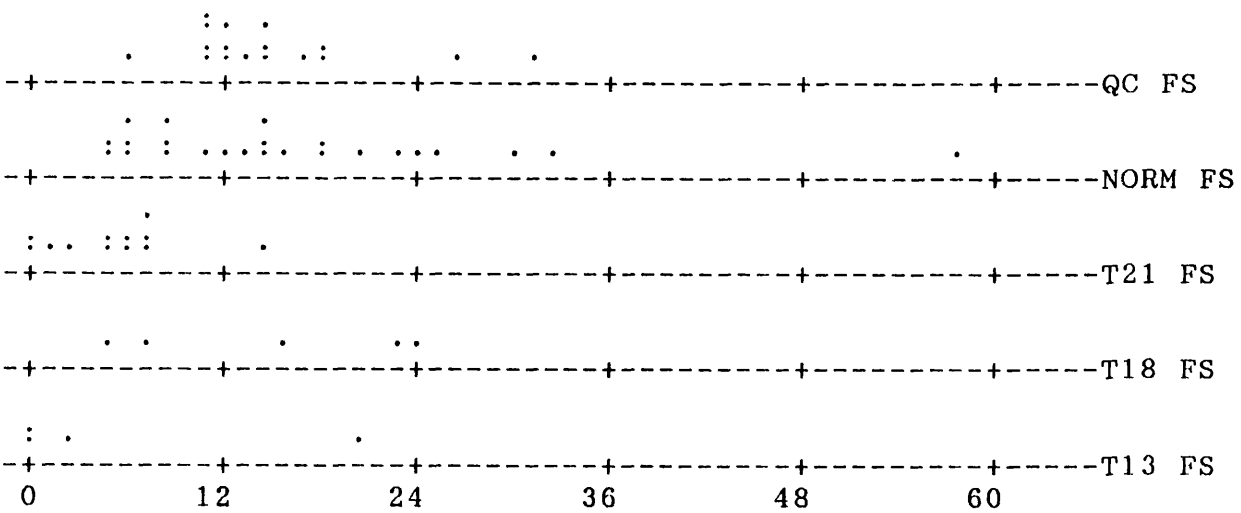

Fig. 3.11 Maltase activity (U/g) for each group of final supernatant fractions (FS=final supernatant fraction, NORM=normal samples, T21=trisomy 21 samples, T18=trisomy 18 samples, T13=trisomy 13 samples, Q.C.=quality control samples)

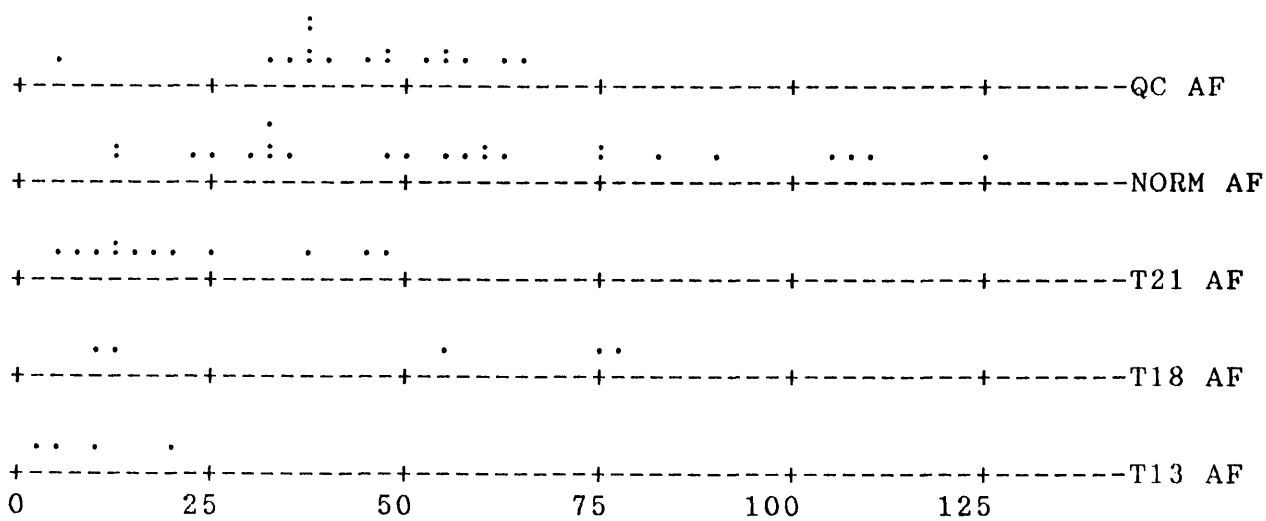

Fig. 3.12 Maltase activity (U/g) for each group of microvillar fractions (MV=microvillar fraction, NORM=normal samples, T21=trisomy 21 samples, T18=trisomy 18 samples, T13=trisomy 13 samples, Q.C.=quality control samples)

the normal group mean of 16.6 U/g (S.D.=11.9, n=24). The trisomy 21 group mean for maltase activity in final supernatant samples was 5.2 U/g (S.D.=3.9, n=12), this represents 31.1% of the normal group mean and is statistically a highly significant reduction (p=0.0002).

Similarly trisomy 21 microvillar fractions all have less maltase activity than the normal group mean of 457.0 U/g (S.D.=385.3, n=24). The highest trisomy 21 value was 400.0 U/g. The trisomy 21 group mean was 154.2 U/g (S.D.=119.9, n=12) which is 33.7% of the normal group mean and is statistically a highly significant reduction (p=0.0014).

Thus trisomy 21 samples show a statistically highly significant reduction from normal maltase activity in all three fractions tested.

3.8.2 Maltase activity in normal and trisomy 18 samples

Trisomy 18 samples had a mean maltase activity, prior to purification, of 45.9 U/g (S.D.=32.0, n=5), this is 78.8% of the normal group mean which was 58.3 U/g (S.D.=32.2, n=24). There is no statistically significant difference between the groups (p=0.47).

The final supernatant mean maltase activity for trisomy 18 samples was 14.8 U/g (S.D.=8.8, n=5) which is 88.8% of the normal group mean 16.6 U/g (S.D=11.9, n=24). This is not a statistically significant difference (p=0.7).

The microvillar fraction mean maltase activity for trisomy 18 samples was 300.5 U/g (S.D.=221.4, n=5) which is 65.8% of the normal group mean of

457.0 U/g (S.D.=385.3, n=24). This is not a statistically significant difference (p=0.25).

Thus, in all three fractions tested, trisomy 18 samples showed no statistically significant difference from normal samples for maltase activity.

3.8.3 Maltase activity in normal and trisomy 13 samples

Even the highest maltase activity seen in a trisomy 13 sample had less than half the activity of the normal group mean. The highest trisomy 13 sample had maltase activity of 21.0 U/g and the normal group mean was 58.3 U/g (S.D.=32.2, n=24). The trisomy 13 group mean was 9.4 U/g (S.D.=8.4, n=4). This represents only 15.4% of normal activity and is statistically a highly significant reduction (p=<0.0001).

Mean maltase activity in final supernatant samples for trisomy 13 was 6.0 U/g (S.D.= 10.1, n=4) which is 36.1% of that seen in final supernatant of normal samples which was 16.6 U/g (S.D.=11.9, n=24), however there is no statistically significant difference between the two groups (p=0.13).

As seen in initial samples even the highest trisomy 13 sample has less than half the normal group mean maltase activity. The highest trisomy 13 sample had 194.4 U/g of maltase activity and the normal group mean was 457.0 U/g (S.D.=385.3, n=24). The mean maltase activity in the microvillar fractions of trisomy 13 samples was 84.7 U/g (S.D.=77.7, n=4), this is 18.5% of the normal group mean and is statistically a highly significant reduction (p=0.0003).

Thus trisomy 13 samples have a statistically highly significant reduction of maltase activity in initial sample and purified microvillar fractions.

3.9 POST MORTEM FINDINGS IN FOETUSES WITH AUTOSOMAL TRISOMY

All available post mortem (PM) records for foetuses affected by autosomal trisomy used in this study were checked for evidence of gastro-intestinal obstruction or developmental anomaly which might contribute to reduced microvillar enzyme levels in amniotic fluid.

Of the twelve trisomy 21 foetuses PM reports were avilable for nine. Two recorded cardiac defects and two others were noted to have "tiny" or "small" Meckel's diverticulum. For the others no developmental anomalies were noted and none of the nine had recorded blockage of intestinal contents or any gastro-intestinal atresias.

Of the five trisomy 18 foetuses PM reports were available for three. One had horseshoe kidney but no other developmental anomalies, one was recorded as having a small Meckel's diverticulum and the third had a jejunal atresia with the distal segment of bowel largely unexpanded. The fourth foetus for which no PM report was available had shown evidence of exomphalos on ultrasound scanning during pregnancy.

Of the four trisomy 13 foetuses one had no available PM report and the remaining three all showed evidence of gastro-intestinal disorder. One had a low ano-rectal atresia without fistula, one had an imperforate anus and also a ventricular septal defect of the heart, the third had

malrotation of the gut and large dysplastic kidneys which lacked corticomedullary definition.

4. DISCUSSION

4.1 AMNIOTIC FLUID MICROVILLAR ENZYME ACTIVITY IN AUTOSOMAL TRISOMIES

Several previous studies have noted reduced microvillar enzyme activity in amniotic fluid in association with autosomal trisomy (Jalanko and Aula 1982, Jalanko and Heikinheimo 1983, Brock *et al.* 1984, Baumah *et al.* 1984, Kleijer *et al.* 1985, Muller *et al.* 1986, Morin *et al.* 1987, Macek *et al.* 1987, Jones and Evans 1988, Zeitune *et al.* 1989, Giddy *et al.* 1989 and Szabo *et al.* 1990).

Attention was first drawn to reduced GGT activity in association with trisomy 18 and 21 by Jalanko and Aula (1982). They reported that nine trisomy 18 samples had mean GGT activity which was 33.3% of the normal control group mean and 19 trisomy 21 samples had mean GGT activity which was 53.8% of the normal control group mean. Baumah *et al.* (1984) reported six trisomy 21 samples with mean GGT activity which was 38% of the normal control mean. Zeitune *et al.* (1989) reported 37 trisomy 21, six trisomy 18 and two trisomy 13 samples all of which had group mean GGT activity which was 44% of the normal group mean.

GGT activity for amniotic fluids from autosomal trisomies, as reported in this thesis, is in agreement with these findings; 12 trisomy 21 samples had mean GGT activity which was 44.6% of the normal group mean, five trisomy 18 samples had 44.1% of the normal group mean and four trisomy 13 samples had 60% of the normal group mean.

In all previous studies of GGT activity 84-100% of trisomy 21 samples, 83-100% of trisomy 18 samples and 91-100% of trisomy 13 samples had values below the median. Results of this study compare favourably with these findings, as shown in table 4.1 . (Median values are given in appendix A).

Only two other authors previously investigated maltase activity in amniotic fluid from autosomal trisomy pregnancies. Kleijer *et al.* (1985) reported reduced maltase activity in 19 of 22 trisomy 21 samples, three of five trisomy 18 samples and five trisomy 13 samples. A group of samples with extremely low maltase activity was also identified. These had less activity than any of the normal control samples. Within this category were nine of the 22 trisomy 21 samples (40.9%), one of the five trisomy 18 samples (20%) and four of the five trisomy 13 samples (80%). Morin et al. (1987) measured maltase activity in 21 trisomy 21 and 13 trisomy 18 amniotic fluid samples. Eight of the trisomy 21 samples (38%) and five of the trisomy 18 samples (38%), had maltase activity which was less than any of the normal control samples, however, all of these samples were from pregnancies in which the foetus had intestinal obstruction. Data for the remaining samples, uncomplicated by intestinal obstruction, had values within the normal control range but of these, nine of the trisomy 21 samples and three of the trisomy 18 samples had maltase activity below the normal median. Thus in total 17 of the 21 trisomy 21 samples (80.9%) and eight of the 13 trisomy 18 samples (61.5%) had maltase activity which was below the normal median.

Comparable distributions for trisomy 21 and trisomy 18 samples were found in the course of this study as shown in table 4.2 . All 12 trisomy

Author and Year	Trisomy 21		Trisomy 18		Trisomy 13	
	number	% below median	number	% below median	number	% below median
Jalanko and Aula (1982)	19	84	9	100	1	100
Brock *et al.* (1984)	54	85			0	
Buamah *et al.* (1984)	6	100	0		0	
Muller *et al.* (1986)	54	94	12	92	11	91
Morin *et al.* (1987)	13	100	10	100	0	
Macek *et al.* (1987)	35	89	19	100	3	100
Jones and Evans (1988)	16	94	0		0	
Zeitune *et al.* (1989)	37	95	6	83	2	100
Giddy *et al.* (1989)	26	92				
Szabo *et al.* (1990)	1	100	1	100	1	
Present study (1992)	12	100	5	100	4	75

Table 4.1 Investigations of GGT activity in amniotic fluid from trisomies 21, 18 and 13

	Trisomy 21		Trisomy 18		Trisomy 13	
	n	<normal range	n	<normal range	n	<normal range
Kleijer et al. (1985)	22	9 (40.9%)	5	1 (20%)	5	4 (80%)
Morin et al. (1987)	21	8 (38.1%)	10	4 (40%)	-	-
Present study (1992)	12	5 (41.6%)	5	1 (20%)	4	3 (75%)

Table 4.2 Investigations of maltase activity in amniotic fluids from trisomies 21, 18 and 13 (n=number of samples)

21 samples (100%) investigated had maltase activity which was less than the normal median and five (41.7%) had activity which was less the lowest control value. Three of the five trisomy 18 samples (60%) had maltase activity which was less than the normal median while one of the five trisomy 18 samples (20%) and three of the 4 trisomy 13 samples (75%) had activity which was less than the lowest control value. (Median values are shown in appendix B).

4.2 PURIFICATION OF MICROVILLAR FRAGMENTS FROM AMNIOTIC FLUID

The reduction in GGT and maltase activity found in amniotic fluid samples from autosomal trisomies in this study therefore confirm previous reports by other authors. To date, however, no study of microvillar tissue itself, as found in amniotic fluid, from autosomal trisomy pregnancies has been made. The purpose of this project was to investigate purified microvillar material from autosomal trisomies in comparison with normal amniotic fluids.

An initial purification although having similar protein content at each stage of purification to those of Potier *et al.* (1986) failed to yield the same level of enzyme activity in the microvillar material. Some improvement was made by using phosphate buffered saline in which to resuspend the microvillar material and increased speed and duration of centrifugation also showed increases in enzyme yield.

Acid phosphatase activity was used as a marker of lysosomal contamination in purified microvillar material. Potier *et al.* (1986) reported 8.2 U/g (S.D.=5.0, n=4) of activity in microvillar fractions. In

this study acid phosphatase activity was 2.8 U/g (S.D.=2.1, n=24) and so lysosomal contamination was considered to be negligible.

Confirmation that purified material was rich in microvillar fragments was provided by transmission electron microscopy which revealed typical microvillar vesicles of both rough and smooth appearance with clearly visible double membrane structure and on rough vesicles projections of electron dense material giving them their characteristic "cogwheel" appearance. Comparison of photomicrographs of typical fields with those of other authors (Schmitz *et al.* 1973, Jalanko *et al.* 1985. and Potier *et al.* 1986) show identical microvillar vesicle morphology.

4.3 BIOCHEMICAL ANALYSIS OF PURIFIED FRACTIONS

Purification of microvillar fragments from amniotic fluids has not previously been reported in the investigation of autosomal trisomies and so this is the first investigation to show statistically significant reductions in the enzyme activity of microvillar material found in the amniotic fluid of these pregnancies.

By examining the specific activities (U/g of protein) for both GGT and maltase in the course of purification of normal amniotic fluid samples an important difference between the characteristics of these enzymes can be seen. By comparing the level of activity in the final supernatant fraction to that in the initial fluid it is seen that GGT activity in the final supernatant of normal samples has a specific activity which is 78% of the original sample. The same figure for maltase activity is 29%. The subsequent increase in specific activity in the microvillar fraction for

GGT is 500% while for maltase it is 790%. This shows that a larger proportion of maltase activity is associated with microvillar tissue than is true for GGT. Removal of microvillar fragments from final supernatant fractions leaves only minimal maltase activity in this fraction but still leaves substantial GGT activity. This confirms that maltase is a membrane bound enzyme while GGT occurs to a greater extent in a soluble form. This is in keeping with the findings of Jalanko *et al.* (1983) and Muller *et al.* (1988) who showed high levels of GGT in foetal bile, a rich source of soluble enzyme. Maltase however, is known to be almost exclusively from the brush border membrane of the small intestine and it is from fragments of this membrane that the maltase activity of amniotic fluid is derived (Claass *et al.* 1985).

The results from the investigation of trisomy 21 purified microvillar fractions show that there is a statistically significant reduction from normal GGT and maltase activity in the microvillar tissue of pregnancies in which the foetus has Down's syndrome. However no statistically significant difference from normal was seen for protein content in any of the fractions tested. In this study, of the nine Down's syndrome foetuses for which post mortem findings were available, no evidence of blockages or atresias within the gastro-intestinal tract was found and so it seems unlikely that mechanical obstruction of intestinal contents provides an explanation of enzyme deficiency seen in these pregnancies. Normal protein content in these samples provides further evidence to suggest that the routes by which microvillar fragments reach the amniotic fluid in trisomy 21 pregnancies were unimpaired and that either the microvillar fragments from the Down's syndrome foetuses lacked enzyme activity or an abnormally rapid degradation of enzyme occurs

within the amniotic fluid of these pregnancies. In view of the different sources and characteristics of the two enzymes already described the deficiency of both enzymes in foetuses with Down's syndrome may suggest disruption of a common controlling mechanism which results in depletion of both. GGT has a key role in transportation of amino acids (Nemesanszky and Lott 1985) and utilises glutathione as an acceptor in the transport of amino acids accross membranes (Moniz *et al.* 1984). Lejeune *et al.* (1992) reported an imbalance in normal serum levels of amino acids of 79 adult Down's syndrome patients. The deficiency seen in purified microvillar fragments, as purified from amniotic fluid, in Down's syndrome pregnancies may be the first evidence of the same process seen in adult patients.

Although results from trisomy 18 and 13 samples show statistically significant differences between these groups and normal controls the small number of samples available for these two groups of samples (five and four respectively) require caution to be observed in any conclusions which might be drawn from these data sets. Further investigation of greater numbers would be needed before any substantive conclusion could be reached. Also, three out of four trisomy 13 foetuses showed evidence of gastro-intestinal disorders which may have influenced the movement of intestinal microvillar fragments into the amniotic fluid and of the three trisomy 18 foetuses, for which PM reports were available, they too showed developmental anomalies which may have influenced microvillar enzyme levels. Therefore caution is again required in interpretation of reduced levels of enzyme activity seen in these samples.

4.4 SYNTHESIS AND PROCESSING OF MICROVILLAR ENZYMES

The mechanisms which underlie the deficiencies seen in this study of the purified microvillar fractions of autosomal trisomies as yet remain obscure. However, much has previously been reported regarding the synthesis and post translational processing of enzymatically active proteins and their incorporation into microvillar membranes (Reviewed Semenza 1989). Maltase activity is a common property of all but one alpha-glucosidase (Gray 1978) however Claass *et al.* (1985) using monoclonal antibodies and heat inactivation studies showed that in amniotic fluids from 16-24 weeks gestation maltase activity originated solely from the foetal intestine and was attributable to the sucrase-isomaltase (SI) complex of the intestinal brush border membrane. Sucrase-isomaltase comprises 8-10% of intestinal brush border membrane protein content (Kessler *et al.* 1978) and because of its importance in the digestive process has been the subject of detailed investigation. The DNA sequence was determined by Hunziker *et al.* (1986) who cloned the gene; from the cDNA sequence the complete amino acid sequence was derived which enabled confirmation of the quarternary structure and orientation of the molecule on the microvillar membrane.

Danielsen *et al.* (1984) used tunicamycin, an N-glycosylation inhibitor, to induce failure of post translation glycosylation in sucrase-isomaltase and aminopeptidase N which resulted in non-incorportation of the enzymes into the microvillar membrane and to their intracellular persistence. Sucrase-isomaltase also requires modification by pancreatic proteases, after incorporation into the microvillar membrane, to provide the final form of the enzymatically active molecule (Semenza 1989). Failure of

either co-translational glycosylation or post membrane incorporation hydrolytic modification result in reduced levels of active maltase molecules on the microvillar membrane.

Further investigation of these mechanisms in tissue from Down's syndrome foetuses might highlight whether failure of these or attendant events in co- and post-translational modifications of enzyme molecules are responsible for the reduced levels of microvillar enzymes seen in microvillar fragments purified from the amniotic fluid of Down's syndrome pregnancies.

4.5 BIOCHEMICAL SCREENING OF MATERNAL SERUM FOR PRENATAL DETECTION OF CHROMOSOMALLY ABNORMAL PREGNANCIES

Biochemical differences between trisomy 21 and normal pregnancies have become increasingly important for use in screening of expectant mothers in the hope of identifying those women at high risk of carrying a Down's syndrome foetus. Merkatz *et al.* (1984) were first to note an association between low maternal serum alphafoetoprotein (AFP) and foetal chromosome abnormality. This allowed existing screening programmes for neural tube defects in which high levels of maternal serum AFP indicated an at risk pregnancy, to be adapted to provide an estimate of the individual risk for each woman of carrying a child affected by autosomal trisomy (Cuckle *et al.* 1987, Zeitune *et al.* 1991 and Knight *et al.* 1988). Such screening programmes use a patient's population risk, calculated from her age, combined with a risk figure (likelihood ratio) derived from the maternal serum AFP level to provide

an individual overall risk of a pregnancy being affected by autosomal trisomy.

Previously, patients with no family history or known predisposition to autosomal trisomy, such as translocation carriers, could only use the increased risk associated with increasing maternal age to provide information on which to base the decision whether or not to seek diagnostic testing of their pregnancy, by either amniocentesis or chorionic villus sampling, for foetal karyotyping. Within the U.K. by offering testing to older mothers ($\geqslant$35 years) only 30% of affected pregnancies could be detected (Aitken and Crossley 1992). The introduction of a biochemical parameter (AFP) can improve possible detection of affected pregnancies to 37% (Zeitune *et al.* 1991), and reduce the age at which diagnostic testing could be offered to 25 years.

Other pregnancy associated analytes have been investigated in the hope that more definitive screening can be offered. These included human chorionic gonadotrophin (hCG) (Bogart *et al.* 1987 and Petrocik *et al.* 1989) and its subunits alpha and beta hCG (Macri *et al.* 1990a), unconjugated oestriol (UE) (Canick *et al.* 1988 and Canick 1990), progesterone, human placental lactogen (hPL) (Knight *et al.* 1989), pregnancy specific β-1 glycoprotein (SP1) (Bartels and Lindemann 1988 and Graham *et al.* 1992), pregnancy associated placental protein-A (PAPP-A) (review Stabile *et al.* 1988). From these investigations markers which provided the highest detection rate for the lowest false positive rate were selected as additional analytes to AFP, for prenatal screening. On this basis the foeto-placental products human chorionic gonadotrophin (hCG) and unconjugated oestriol (UE) have both been

used as additional analytes in maternal serum screening programmes. Although there is clear evidence that hCG provides an improved estimation of risk of autosomal trisomy, and indeed can provide differential risk being elevated in Down's syndrome and reduced in Edwards' syndrome, opinion regarding the usefulness of UE, as an additional analyte differs (Wald *et al.* 1988, Macri *et al.* 1990b and Crossley *et al.* 1991).

Current use of maternal serum screening for autosomal trisomy pregnancies occurs in the second trimester of pregnancy, typically between 15 and 21 weeks gestation. For women who chose termination of an abnormal pregnancy the lateness of the diagnosis contributes substantially to their distress. The usefulness of markers in the first trimester has been explored and hCG (van Lith 1992) free β-hCG (Spencer 1991) and PAPP-A (Wald *et al.* 1992) have been identified as potential screening analytes in early pregnancy.

4.6 MECHANISMS CONTROLLING BIOCHEMICAL VARIATION

The mechanisms which modulate the level of various pregnancy markers in chromosomally abnormal pregnancies are poorly understood. Complex patterns of variation in different tissues and between different types of abnormality are beginning to emerge (table 4.3) Kronquist *et al.* (1990) have presented evidence that in trisomy 21 reduced levels of AFP synthesis may occur in the foetal liver, while Kratzer *et al.* (1991) found no evidence of altered hCG bioactivity in trisomic pregnancies.

	Second Trimester			First Trimester			
	T21	T18	T13	T21	T18	T13	
MS	d	d	d	d	N	N	AFP
AF	d	N	N	d	-	-	
MS	+	d	N	N	d	d	hCG
AF	+	d	-	-	-	-	
MS	+	d	-	+	-	-	FβhCG
AF	-	-	-	-	-	-	
MS	d	d	N	d	-	-	UE
AF	d	-	-	-	-	-	
MS	+	N	N	d	-	-	SP1
AF	-	-	-	-	-	-	
MS	+	-	-	d	-	-	hPL
AF	-	-	-	-	-	-	
MS	-	-	-	d	-	-	PAPP-A
AF	-	-	-	-	-	-	

Table 4.3 Relative levels of pregnancy associated markers in autosomal trisomy pregnancies in Maternal Serum (MS) and Amniotic Fluid (AF) (d=decreased, N=normal, +=increased)

By purifying available foetal tissue in the form of microvillar fragments from amniotic fluid, this study has shown that useful information can be derived from such tissue. A more extensive investigation of other foetal proteins purified from amniotic fluid in this way may highlight other deficiencies or imbalances which may be used in piecing together a more complete picture of the differences found between normal and trisomy pregnancies. The findings of this study also suggest that more direct investigation of foetal tissues e.g. small intestine, may provide a useful avenue of future research.

It is hoped that by pursuing further research into foetal and pregnancy associated metabolites in foetal and maternal blood, amniotic fluid, placental and foetal tissues, some of the mechanisms which will differentiate autosomal trisomy pregnancies from normal pregnancies will be highlighted and by investigation of these materials in the first trimester, this may lead to improved screening methods and earlier diagnosis of autosomal trisomy. By this means it is hoped to reduce the numbers of false positive tests in screening and thus the number of women (and their families) who are exposed to unnecessary anxiety. The alleviation of this anxiety and the sound basis of re-assurance given by negative findings are the aims which improved screening accuracy seeks to achieve.

Improved prenatal screening will not prevent the birth of individuals with Down's syndrome. Some affected pregnancies will remain undetected, some women will present too late in pregnancy to allow intervention, for some the concept of screening is not acceptable and for others although alerted by screening they will chose to continue with autosomal trisomy

pregnancies. It is known that individuals with Down's syndrome are at increased risk of several disease processes including leukaemias (Rowley 1981) in which it has been shown that chromosome 21 is involved in the leukaemic process (Rosner and Lee 1972) and premature senile dementia (Oliver and Holland 1986 and Vassipoulos 1990) in which recent investigations by Goate et al. (1989) mapped the familial form of Alzheimer's disease to chromosome 21. Investigation of basic biochemical differences between individuals with autosomal trisomy and normal individuals may provide information leading to improved understanding of disease processes not only for Down's syndrome individuals but for others too.

	N	MEAN	MEDIAN	TRMEAN	STDEV	SEMEAN
QC AF	31	67.66	69.30	67.75	9.41	1.69
QC FS	31	51.74	53.40	52.02	11.55	2.07
QC MV	31	412.7	387.0	405.4	210.9	37.9
NORM AF	35	67.54	60.80	66.05	34.07	5.76
NORM FS	35	52.29	44.10	49.34	36.22	6.12
NORM MV	35	330.1	288.0	314.8	226.1	38.2
T21 AF	12	30.13	31.20	30.12	10.03	2.90
T21 FS	12	24.83	24.20	24.22	11.02	3.18
T21 MV	12	216.4	169.5	203.3	110.7	32.0
T18 AF	5	29.82	37.50	29.82	21.57	9.65
T18 FS	5	19.66	9.80	19.66	18.61	8.32
T18 MV	5	200.7	263.3	200.7	133.3	59.6
T13 AF	4	41.1	38.7	41.1	33.0	16.5
T13 FS	4	25.8	17.4	25.8	25.4	12.7
T13 MV	4	417	367	417	268	134

	MIN	MAX	Q1	Q3
QC AF	51.00	86.20	59.00	76.50
QC FS	21.80	78.20	46.00	58.30
QC MV	68.3	910.0	256.4	583.0
NORM AF	13.50	150.60	39.50	95.00
NORM FS	5.40	164.20	27.70	69.00
NORM MV	21.9	980.0	188.6	463.0
T21 AF	16.10	44.30	20.37	38.53
T21 FS	9.70	46.00	14.48	33.93
T21 MV	108.6	455.0	135.0	292.7
T18 AF	6.50	57.00	7.75	48.05
T18 FS	5.00	50.30	7.00	37.25
T18 MV	32.7	313.0	57.6	312.5
T13 AF	8.4	78.6	11.0	73.6
T13 FS	6.8	61.6	7.2	52.8
T13 MV	181	750	191	691

APPENDIX A : Statistical analysis of GGT data for each group of samples (median values used in comparison of this data with other authors)

	N	MEAN	MEDIAN	TRMEAN	STDEV	SEMEAN
QC AF	17	44.21	45.30	45.47	14.39	3.49
QC FS	17	14.85	13.00	14.37	6.15	1.49
QC MV	17	599.9	454.0	572.3	381.5	92.5
NORM AF	24	58.26	56.50	57.34	32.19	6.57
NORM FS	24	16.62	13.95	15.28	11.86	2.42
NORM MV	24	457.0	350.3	424.9	385.3	78.7
T21 AF	12	21.17	16.50	20.30	14.50	4.18
T21 FS	12	5.17	5.50	4.80	3.92	1.13
T21 MV	12	154.2	113.5	142.1	119.9	34.6
T18 AF	5	45.9	55.6	45.9	32.0	14.3
T18 FS	5	14.76	15.10	14.76	8.81	3.94
T18 MV	5	300.5	288.0	300.5	221.4	99.0
T13 AF	4	9.37	7.05	9.37	8.43	4.22
T13 FS	4	6.00	1.50	6.00	10.10	5.05
T13 MV	4	84.7	66.7	84.7	77.7	38.9

	MIN	MAX	Q1	Q3
QC AF	4.60	64.90	36.95	56.00
QC FS	5.80	31.10	10.95	17.05
QC MV	181.0	1432.5	312.1	863.3
NORM AF	11.80	125.00	32.33	81.20
NORM FS	4.60	58.00	8.68	22.40
NORM MV	88.3	1530.6	197.2	667.8
T21 AF	4.10	47.00	11.07	33.45
T21 FS	0.00	14.00	1.85	7.30
T21 MV	30.0	400.0	53.0	243.7
T18 AF	10.4	76.3	11.9	75.1
T18 FS	4.50	24.20	5.95	23.40
T18 MV	58.0	617.6	98.0	509.3
T13 AF	2.40	21.00	2.80	18.27
T13 FS	0.00	21.00	0.00	16.50
T13 MV	11.0	194.4	24.3	163.0

APPENDIX B : Statistical analysis of Maltase data for each group of samples (median values used in comparison of this data with other authors)

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