Analysis of isoenzymes and isoforms of human alkaline phosphatase, hexosaminidase and transferrin by micro column chromatography

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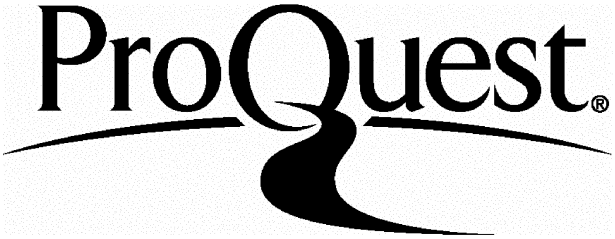

"What is the use of repeating all that stuff?" the Mock Turtle interrupted, "if you don't explain as you go on? It's by far the most confusing thing I ever heard!"

Alice in Wonderland

ABSTRACT

The main objective of this study was to develop a micro column chromatographic system (MCC) for the separation, detection and quantitation of isoenzymes and isoforms of proteins in human plasma. Three proteins (hexosaminidase, alkaline phosphatase and transferrin) were chosen, as they presented different clinical and analytical problems.

Developmental work showed that a 5 mm x 4.6 mm column, packed with a strong polymer-based anion exchanger, used with a step-gradient and a slow flow rate (< 0.55 ml/min) was capable of resolving the different forms of the three proteins. A rapid post-column detection system was designed for the analysis of hexosaminidase and alkaline phosphatase. Transferrin was detected at 460 nm which is specific for iron.

The chromatography was found to be reproducible; the coefficient of variation (CV) for the retention times of the various forms of the three proteins generally being approximately 2%. The precision for the within-batch and between-batch quantitation of individual isoenzymes and isoforms varied greatly from one protein to another (from a CV of 1.2% for hexosaminidase to 48.56% for alkaline phosphatase). This appeared to result principally from problems with the software of the commercial integration system.

When MCC was compared with appropriate reference methods such as agarose electrophoresis and isoelectric focusing (IEF), the following conclusions were made. The percentage contribution of the various forms of hexosaminidase and transferrin following MCC was comparable with that obtained using electrophoresis and IEF respectively. MCC therefore, provided a rapid and easy method for the clinical analysis of these proteins. For alkaline phosphatase, however, MCC and electrophoresis gave poor agreement, particularly in neonatal samples, which may have resulted from different method specificities.

The optimised methods (MCC, IEF and electrophoresis) for the analysis of hexosaminidase and transferrin were applied to a study of patients with Carbohydrate Deficient Glycoprotein Syndrome.

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ABBREVIATIONS

aa	amino acids
Ala	Alanine
ALP	Alkaline Phosphatase
ALT	Alanine ketoglutarate transferase
ARD	Alcohol related disorder
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
bp	Base pair
AST	Aspartate ketoglutarate transferase
BSA	Bovine serum albumin
CDGS	Carbohydrate deficient glycoprotein syndrome
CDT	Carbohydrate deficient transferrin
CSF	Cerebrospinal fluid
Ct	C terminus
Da	Dalton
DEAE	Diethylaminoethyl
DMSO	Dimethylsulphoxide
ER	Endoplasmic reticulum
FABMS	Fast atom bombardement mass spectrometry
FMN	Flavin mononucleotide
FR	Flow rate
GDP	Guanosine diphosphate
Glu	Glutamic acid
GlucNac	N-acetyl-glucosamine
Gly	Glycine
GOT	Glutamate oxaloacetate transaminase
GPI	Glycosylated phosphatidyl inositol
GPT	Glutamate pyruvate transaminase
Hex.A	Hexosaminidase A
Hex.B	Hexosaminidase B
Hex.I1	Hexosaminidase I1
Hex.I2	Hexosaminidase I2
His	Histidine
HMW	High molecular weight
HPLC	High performance liquid chromatography
HSA	Human serum albumin
Kb	kilo base
L-Phe	L-Phenylalanine
L/B/K	Liver/bone/kidney
λem	Emission wavelength
λexc	Excitation wavelength

mALP	Membrane alkaline phosphatase
MCC	Micro column chromatography
Mg^{2+}	Ion magnesium
mRNA	messenger ribonucleic acid
MUP	Methylumbelliferyl phosphate
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide (reduced form)
nt	nucleotide(s)
Nt	N terminus
PI	phosphatidyl inositol
PI-PLC	Phosphatidylinositol phospholipase C
PlALP	Placental alkaline phosphatase
pNPP	para-nitro phenyl phosphate
RER	Rough endoplasmic reticulum
RT	Retention time
sALP	Soluble alkaline phosphatase
SDS	Sodium dodecyl sulfate
Ser	Serine
T0	Asialotransferrin
T2	Disialotransferrin
T4	Tetrasialotransferrin
TALP	Total alkaline phosphatase
TNS	Tissue non-specific
WBC	White blood cells
WGL	Wheat germ lectins
Zn^{2+}	Ion zinc

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Chapter I: Introduction

1 – A brief history of enzymes

In 1833 an active agent which broke down sugar was partially isolated and given the name: 'diastase' (now amylase). A little later, a substance which digested dietary proteins was extracted from gastric juice and called 'pepsin'. The name 'ferment' was given to these and similar compounds soon to be discovered; this name was gradually replaced by 'enzyme'. The latter name, first proposed by Kuhne in 1878 comes from the Greek word ενζυμε, meaning 'in yeast'. Summer crystallised urease from jack-bean extracts in 1926, and in the next few years many enzymes were purified and crystallised. The next stages in the understanding of the structure of enzymes were the first amino acid sequencing which was first carried out on ribonuclease in 1960 followed closely by the first three-dimensional picture by X-ray crystallography (done on lysosyme).

Improvement of separation methods based on differences in net charge of proteins initiated a great interest in enzyme heterogeneity, a phenomenon which was already recognised in the first edition of the authoritative monograph on enzymes by Dixon and Webb in 1958. In 1959 Market and Moller coined the word 'isoenzymes' for different molecular forms of an enzyme with the same substrate specificity.

Modifications of an enzyme's structure without changes in the catalytic properties may arise at different levels; if it is at gene level, the primary structure is changed and such variations are termed isoenzymes; if it is at the post-translational level, it leads to isoforms.

2 – Isoenzymes

2–1 Definitions

According to the IUPAC–IUB (1977), isoenzymes are defined as "multiple molecular forms of an enzyme occurring within a single species, as a result of the presence of more than one structural gene". "Multiple forms of enzymes" were defined as all proteins possessing a particular enzyme activity and occurring naturally in a single species.

The structural differences between members of a family of isoenzymes can be explained by the following variations in the encoding genes :

(i) The existence of multiple gene loci which can be divided in two causes:

- Mutations at unrelated gene loci: resulting in differences in biochemical properties but similar function, e.g. malate dehydrogenase.

- Gene duplication and subsequent independent mutation: resulting in differences in some properties but similarities in function (polypeptide of different but related amino acid sequence), e.g. creatine kinase MM and BB, Lactate dehydrogenase 1 and 5, or hexosaminidase subunits α and β ; the gene encoding the α subunit is located on chromosome 15 (40 Kb – 14 exons) and the gene encoding the β subunit on chromosome 5.

(ii) The occurrence of pairs of alleles at the locus as a result of a point mutation: the polypeptides differ by a single amino acid and the isoenzymes are similar in most properties, e.g. glucose-6-phosphate dehydrogenase variants.

2–2 An example: alkaline phospatase isoenzymes

Isoenzymes of alkaline phosphatase (ALP) arise from at least two of the possibilities mentioned above as some of its isoenzymes are coded by different loci, and some are allelic variations.

ALP isoenzymes are present in nearly all plants and animals. In humans they are ubiquitous plasma membrane-bound glycoproteins (see chapter IV) encoded by at least three gene loci: liver/bone/kidney, intestinal, placental (see Table I-1) with possibly a fourth locus which can be considered, coding for placental-like.

Multiple forms of isoenzymes resulting from the existence of multiple gene loci have become disseminated throughout the whole species during the course of evolution. In some cases however, allelic variations at a particular locus may have conferred recognisable inherited modifications of its product, thus indicating their related origin and distinguishing them from analogous products from other loci. It is believed that all the human ALP genes evolved from a common ancestral gene since the introns of all the ALP isoenzymes genes occur in the same position.

The Liver/ Bone /Kidney (L/B/K) isoenzymes

The human L/B/K gene locus has been extensively investigated in osteosarcoma cells and cultured fibroblasts (see Table I-1) and the nucleotide sequence has been fully determined [Weiss et al, 1988].

The main biochemical characteristic of the L/B/K isoenzymes is their tissue non-specificity. Since the gene is expressed as a single copy all the small variations (e.g. different carbohydrate chains) have been attributed to post-translational modifications (see Section 3–3).

Table I-1: *Features of the alkaline phosphatase genes*

	Liver/Bone/Kidney	Placental	Intestinal
Number of exons	12 over more than 50 Kb	11	11 67-192 bp
Number of introns		10 -very short	10 62-242 bp
Location	map to the distal short arm of chrom. 1 band p-36.1 to p-3	map to band q-34-q-37 of chromosome 2	
Number of gene	locus exists as a single copy	PLALP related gene family contains 3 genes, all located at the same place	one copy for the adult form possibly an other gene for the fetal
Intron compos-ition	introns begin with GT and end with AG	very A+T rich regions	
mRNA	about 2.5 Kb, levels from these sources correspond with level of activity	the control of the level of activity seems to be the regulation of mRNA abundance	length varies between 2522 & 3231 nt several different species existing
% identity	52/57% identity with Intestinal, Placental (25 Kb)	90% similarity for the structural region but expressed in a highly tissue-specific fashion	
Structure of polypep-tide	524 aa MW 57 KDa [from cDNA cloned from mouse carcinoma cells]	dimer of identical subunits 1subunit=glycosylated peptide of 513 aa	homodimeric structure, MW=143,00 fetal & 72,000 adult

Placental isoenzyme

The placental ALP resides on the surface of syncitiotrophoblasts [Knoll et al, 1988] and its expression in placenta is controlled by the fetal genome. In contrast to the other forms of ALP it is very polymorphic, with three common variants arising from 18 alleles at a single gene locus [Moss, 1977]. It has been shown that the leader sequence and the membrane-anchoring domain are encoded by different exons. Two isoforms of placental type, or placental-like, isoenzymes are used in oncology because of their tumour-associated characteristics. They have been named from the first patients diagnosed, Regan and Nagao isoenzymes [Higashino et al, 1989].

Intestinal isoenzyme

Biochemically-distinct isoenzymes can be isolated from fetal and adult intestinal tissues [Henthorn et al, 1988]. The first is a sialylated hetero-dimer whereas the latter is a non-sialylated homodimer. A fifth gene, coding for the fetal intestinal isoenzyme cannot be excluded but the comparison between the two proteins leads to equivocal results as there are only minor differences which could result from dissimilar post-translational modifications [Moss, 1992]. The amino acid composition of fetal and adult forms of ALP are the same.

The gene promoters for intestinal and placental ALP are similar but different from the L/B/K isoenzyme. Taking into consideration the completely different location of the latter gene this is not surprising.

3 – Isoforms

3–1 Definition

Isoforms occur by post–translational modifications of a single gene product like proteolysis, phosphorylation, glycosylation or attachment of an anchor, e.g. attachment of an inositol-phosphatidyl anchor to alkaline phosphatase [Hahnel and Schultz, 1989]. Glycosylation is of particular importance in this work as it is the main reason for the differences between liver, bone and kidney isoforms of ALP and between the isoforms of transferrin which were also investigated in this study.

3–2 An example: transferrin isoforms

The gene for transferrin is believed to be the product of a gene duplication estimated to have occurred in prochordates 500 million years ago [Yang et al, 1984]. It has been mapped to the long arm of chromosome 3 in the region 3q21–25. A genetic polymorphism of the transferrin molecule was first demonstrated in 1957. Since then 22 functional variants have been detected; the most common was designated TfC (found in 95% of the European population) and referred to as 'normal' in this study; the others were named by their migration on electrophoresis, the most anodal has been designated TfB and the most cathodal TfD [de Jong et al, 1990]. The amino acid substitutions in some of the variants have been determined (see Table I-2), but only one type of transferrin protein is synthesised in a particular individual. The isoforms occur only by modification of the glycosylation of the molecule.

3–3 A post-translational modification: glycosylation

A glycosylated protein, or glycoprotein, is composed of the protein core and a varying number of carbohydrate chains made of 2 to 18 monosaccharides organised in a tree-like structure. Two kinds of protein-carbohydrate linkage can be found:

- O-glycans: The carbone 1 (C1) of N-acetylglucosamine is linked to the hydroxyl of serine, threonine, hydroxyproline or hydroxylysine
- N-glycans: C1 of N-acetylglucosamine (GlcNac) is linked to the amide group of asparagine.

Generally three types of structures would be found for an N-linked glycoprotein (see Fig.I-1):

a high-mannose (composed of 4-7 mannose residues linked to GlcNac-GlcNac-N),

a hybrid-chain type (GlcNac occupies a second position on an α-mannosyl residue, and in some cases there is a further substitution by a galactose residue),

and a complex-type (fewer mannose residues with asymmetry in the structure and containing N-acetyllactosamine units) [Hughes, 1983].

When N-acetyl neuraminic acid is present at the end of a chain, a charge heterogeneity appears within the same chain.

Table I-2: *Amino acid changes in some of the genetic variants of transferrin* [Mac Gillivray et al, 1983]

Variant	Modification
Tf D1	277 (Asp->Gly)
Tf Dchi	300 (His->Glu)
Tf D2	652 (Gly->Glu)

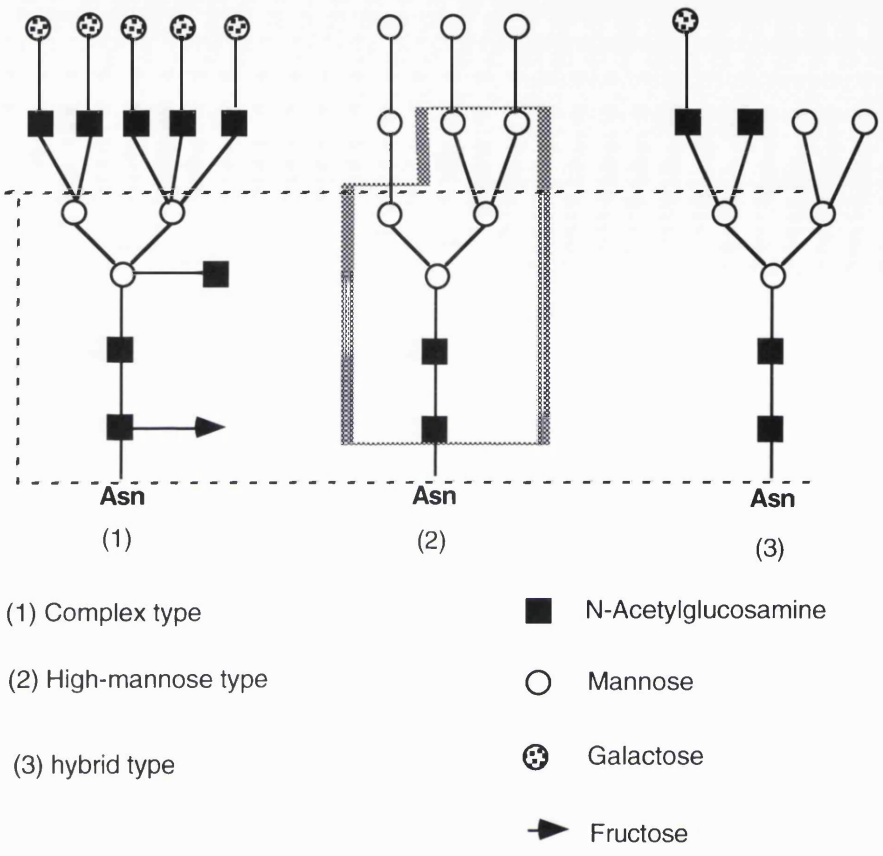


Fig.I-1: *Schematic representation of the different types of carbohydrate chain.* The dashed line encloses the common structure to all N-linked sugar and the shaded line the structure common to high-mannose type.

4 – Aims of the thesis

The main objective of this thesis has been to investigate the suitability of micro column anion-exchange chromatography (MCC) for the separation, the detection, and the quantitation of isoenzymes and isoforms of proteins. This work has included:

- A study of the general behaviour of chromatography in small columns with regard to packing materials, gradient and post-column reaction (Chapter II).
- The application of MCC for the separation of isoenzymes of hexosaminidase and alkaline phosphatase and isoforms of transferrin. These proteins were chosen respectively for their varied clinical importance in monitoring inherited lysosomal storage disorders, bone and liver disorders (especially in paediatric), and alcoholism. The analytical performance of MCC was evaluated by a comparison with electrophoresis and isoelectric focusing, two established methods for the separation of the selected proteins (Chapter III, IV, V).
- A study of abnormal isoenzymes and isoforms of hexosaminidase and transferrin in the Carbohydrate Deficient Glycoprotein Syndrome (CDGS).

Each chapter contains the individual theoretical and clinical background, together with the results and their interpretation.

Chapter II: Analytical developments

A – Methods available for the separation and detection of isoenzymes and isoforms

1 – Introduction

Enzyme assays based on the determination of catalytic activity can distinguish between active and inactive forms of enzyme, but they cannot distinguish between isoenzymes. Therefore the activity measured will be the sum of the contributions of all the active forms of the enzyme being assayed. Differences in physical-chemical properties, such as heat stability or net charge, and in sensitivity towards inhibitors are used to obtain measures of the relative activities of the individual isoenzymes. Some of these methods are reviewed.

2 – Separation of isoenzymes and isoforms

2–1 Chemical methods

2–1–1 Heat denaturation

Enzyme activity depends on the tertiary structure of a protein which is primarily dependent on hydrogen bonds and hydrophobic interactions. These interactions can be disrupted by heat treatment. The tertiary structure of a protein varies between different isoforms and therefore they react to temperature in different ways. This property can frequently be used to distinguish between isoforms and isoenzymes.

The resistance to heat treatment for the ALP isoenzymes increases in the following order: Bone < Liver < Intestinal < Placental (the only one to be resistant at 65°C). Hexosaminidases A and S are thermolabile (3 hours at 50°C) whereas hexosaminidase B is resistant to the same treatment.

2–1–2 Inhibitors

ALP isoenzymes can be identified by using specific inhibitors, some methods using this property have been developed (see Table II-1). A number of amino acids are inhibitors and studies have shown that this inhibition is stereo specific (only the L–isomers are active inhibitors) and non-competitive; often one amino acid can inhibit two isoforms, e.g. L-phenylalanine inhibits intestinal and placental ALP (see Table II-1).

Table II-1: *Specific inhibitors of ALP isoenzymes*

Inhibitor	Isoforms inhibited
L–Phenylalanine	intestinal
	placental
L–Homoarginine	bone
Imidazole	liver
Levamisole	
L–Tryptophane	placental
L–Leucine	placental but not Regan

Table II-2: *Degree of sialylation of ALP, hexosaminidase and transferrin isoforms*

Protein	Non sialylated isoform	Sialylated isoforms
Alkaline phosphatase	Intestinal	Bone, liver, Placental partial treatment can differentiate between bone and liver [Moss and Edwards, 1984]
Hexosaminidase	Hexosaminidase A	Hexosaminidase B and I
Transferrin	Asialotransferrin	the 5 other forms (from monosialo to hexasialotransferrin)

2–1–3 Digestion with neuraminidase

ALP, hexosaminidase, and transferrin are all glycosylated molecules. Neuraminidase, an enzyme which can hydrolyse neuraminic acid, has been commonly used to differentiate between isoenzymes or isoforms of a protein. By digesting the glycan branch either partially or completely the charge surface of the protein is modified, allowing a better resolution when methods such as chromatography, electrophoresis or IEF are used. A summary of the normal degree of sialylation in these proteins is presented in Table II-2.

2–2 Electrophoresis

The earliest, and most common method for separating and quantifying isoenzymes and isoforms of a protein was by electrophoresis.

Electrophoretic techniques have been used extensively to separate isoenzymes of ALP but the plethora of methods indicates that there is not an optimal method for resolving every single isoenzyme known without prior sample preparation. In contrast, the different molecular forms of hexosaminidase and transferrin seem to be resolved accurately by standard electrophoresis.

A summary of electrophoretic methods commonly used for the separation of the isoenzymes and isoforms investigated in this study is given in Tables II-3A to 3E.

2–3 Isoelectric focusing (IEF)

Isoelectric focusing involves sending a direct current through a system of ampholytes so that the pH increases gradually from anode to cathode.

Proteins are zwitterions, i.e. they have a neutral charge at their isoelectric point (pI). If, instead of a constant pH, the gel is made with a pH gradient (established between two electrodes and stabilised with carrier ampholytes) the proteins will migrate until they align themselves with their pI. This allows a very high resolution of components differing by as little as 0.001 of a pH unit.

The ampholytes have to obey three criteria :

- to be soluble (at pI)
- to be conductive
- to have a buffer capacity

Table II-3A : *Electrophoretic methods for separating ALP isoenzymes–Standard methods.*

Gel	Buffers	Voltage/time	Staining solution	Staining	Reference
1 – Agarose 1% w/v	Barbital pH= 8.4 ionic strength= 0.1	20 V/cm (50 min) 100-120 mA	α-Naphthyl Phosphate, 20 mg 4-aminodiphenyl aminediazomium, 50 mg $MgSO_4$, 60 mg in 100 ml Tris Maleate, pH= 9.8	24 hours 5°C	Johansson 1972
2 – Starch gel	gel : Tris-HCl 0.05 mol/L pH= 8.8 bridge: Tris-HCl 0.3 mol/L pH= 8.6	8-10 V/cm (5 hours) or 4 V/cm (16 hours)	α-naphthyl phosphate, 10 mg Brentamine Fast Red TR, 5 mg $MgCl_2$, 5 mM in 100 ml 0.1 M Tris-HCl or 0.1 mol/L bicarbonate pH= 9.5		Skillen et al 1972
3 – Difco Agar Noble- 0.9g/100 ml	Barbital pH= 8.4 ionic strength= 0.1 containing 10 mg bovine albumin	5.5-6 V/cm (4 hours)	α-Naphthyl phosphate, 20 mg 4-aminodiphenylaminediazomium sulphate, 50 mg MgSO4, 60 mg in 100 ml Tris-Maleate buffer, pH= 9.8	24 hours 5°C	Sundblad et al 1973
4 – Agarose 1% w/v	Barbital 0.075 mol/l pH= 8.6 2 mmol/l Calcium Lactate Triton X-100 0.5%	20 V/cm (1 hour) 200 mA (Albumin migration 5–6 cm).	Naphthol AS-TR phosphoric acid or Naphthol AS-BI, 0.02-0.05% Fast-Red TR or 4-aminodiphenyl aminediazomium sulphate, 0.05% in Tris Maleate pH= 9.8	30 min 37°C 30 min 40°C	Hagerstrand 1976
5 – Cellogel membrane (Chemetron,Milano)	Veronal pH= 8.6 ionic strength= 0.06	6 mA per membrane (5 x 6 cm) (60-90 min)	Naphthol AS-MX phosphate, 20 mg $MgCl_2$, 100 mg in 100 ml of 2-amino-2-methyl-1,3-propanediol-HCl buffer, 0.1 mol/l pH= 9.5 or fast-violet B salt, 5 mg (same buffer as above)	1 hour 37°C 5-10 min RT	Saga & Kano 1979
6 – Agarose 1% w/v	See 1 above	28 V/cm (1 hour)	α-Naphthyl phosphate pH= 9.8, according to Sunblad and al	24 hours 5°C	Danielsson & Von Schenck. 1984

Table II-3A: *Continued.*

Gel	Buffers	Voltage/time	Staining solution	Staining	Reference
Cellulose acetate membranes (Sephraphore III)	Tris-Barbitone pH= 8.8 Ionic strength= 0.05	250 V (60 min)	p-Toluidinium-5-bromo-4-chloro-3-indoxylphosphate, 1.25 mmol/l in 2-amino-2-methyl-1,3-propanediol 1 mol/l, pH= 10.2 + Magnesium sulphate 1 mmol/l in 10 g/l agar gel	60 min 37°C	Karmen et al 1984
Cellulose acetate membranes (Sephraphore III)	Tris/barbital/sodium barbital pH-8.8 Ionic strength= 0.05	260 V (60 min)	5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt 1.25 mmol/l in 2-amino-2-methyl-1,3-propanediol 1 mol/l, pH= 10.2 + Magnesium sulphate 1 mmol/l	1 hour 37°C	Rosalki & Ying-Foo 1984
Cellulose acetate membranes	Tris barbital-sodium-barbital buffer pH= 8.8	180V (35 min)	a-Naphthyl phosphate with fast blue RR dissolved in electrophoresis buffer. Helena Lab. kit	30 min 37°C	Schreiber & Whitta 1986
Agarose gel from Isopal kit	Tris /H_3BO_3, final conc 0.38 mol Tris 0.06 mol/l boric acid pH= 9.45	150 V (25 min)	BCIP 1.89 mmol/l, pH= 10.4	30 min 45°C	Van Hoof et aL 1988

Table II-3B: *Electrophoretic methods for separating ALP – Affinity electrophoresis*

Gel	Buffers	Voltage/time	Staining solution	Staining	Reference
Cellulose acetate membranes (Sephraphore III) soaked in buffer + 50g/l of WGL	Tris/barbital/sodium barbital pH= 8.8 Ionic strength= 0.05	260 V (60 min)	5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt 1.25 mmol/l in 2-amino-2-methyl-1,3-propanediol 1 mol/l, pH= 10.2 + Magnesium sulphate 1 mmol/l	1 hour 37°C	Rosalki & Ying Foo 1984
Agarose Seakem, Marine colloid 0.8%	0.075 mol/L barbital calcium lactate 2 mmol/L WGL 0.025 to 0.2 mg/ml if Triton, 0.1% final conc. pH= 8.6	20 V/cm (1 hour) 130 mA albumin migration 5 cm	a-Naphthyl phosphate pH= 9.8 according to Sunblad	24 hours 5°C	Onica et al 1986
Agarose 1%	Tris-barbital pH= 8.9 Triton 100 mmol/L WGL 4 mg per gel= minimum	180V (35 min)	2.3 mmol/L BCIP + 0.15 mol/L 2-amino2-Methyl-propanol + 0.2 mmol/L magnesium aspartate	1 hour 37°C	Schreiber & Whitta 1986

Table II-3C: *Electrophoretic methods for separating ALP – Immunofixation (IFE)*

Cellogel membrane (Chemetron, Milano)	Phosphate 0.03 mol/L pH= 7.4	30 min Ag/Ab reaction (anti human serum) and 3 hours in buffer		3-6 hours	Saga-Kano 1979
Agarose 1%	Barbital pH= 8.4 Ionic strength= 0.1	1 hour reaction with Ab against IgG, IgGA, IgM	a-Naphtyl phosphate pH 9.8 according to Sunblad et al, to visualise ALP activity	24 hours 5°C	Danielsson & Von Schenck 1984

Table II-3D: *Electrophoresis methods for separating hexosaminidases isoenzymes*

Gel	Buffers	Voltage/time	Staining solution	Staining	Reference
Cellulose acetate (Cellogel)	K phosphate 0.04 mol/L pH= 6.5	200 V (2 hours per band) 3 mA	4-MuGlcNac 500 µM in citrate- phosphate 0.04 mol/L, pH= 4.4 Stop : Gly-NaOH 1 mol/L, pH= 10.0 read on UV lamp	10 to 30min RT	Poenaru & Dreyfus, 1973
Cellulose acetate (Cellogel)	K phosphate 0.05 mol/L pH= 6.6	1 hour, 4°C	according to Okada & O'Brien 1969		Hoeksema et al, 1977
Cellulose acetate			same conditions as above scanned for fluorescence		Saratawi et al, 1986

Table II-3E: *Electrophoretic methods for separating transferrin isoforms*

Agarose (Beckman) 'high resolution agarose- Panagel'	barbital buffer pH= 8.6	100 V (30 min) 200 V (40 min)	immunofixation		Zaret et al, 1992
Gelatinized cellulose acetate membrane	barbital buffer pH= 8.6	200 V (50 min)	immunofixation (modification of Ritchie et al, 1976)		Irjala et al, 1979
agarose 1%	barbital buffer pH= 8.6	200 V (60 min)	immunobloting (nitro-cellulose membrane coated with antibody overnight)		Fransen et al, 1991
agarose gel thickness 0.5 mm	barbital buffer pH= 8.6	500 V (40 min) @95 mA	immunoblotting: (see text for details)		Keir et al, 1992

An ideal pH gradient would be a line passing through the origin (0;0) but in practice the configuration is dependent on the number of carrier ampholytes present in the mixture.

Two different media are generally used: agarose or polyacrylamide. The pH gradient can be created in two ways, either by carrier ampholytes or by immobilised gradient. The former can be applied to polyacrylamide [Griffith and Black, 1987] or to agarose [van Hoof et al, 1991] and the latter is used with polyacrylamide only, where the gradient is covalently linked to the acrylamide backbone [Sinha et al, 1986].

The running conditions for the separation of ALP isoenzymes have again varied considerably but are fairly consistent for the separation of transferrin and hexosaminidases (see Table II-4A to 4C).

At one point, isoelectric focusing was expected to develop into the preferred method for isoenzymes but the abundance of resulting bands has made the interpretation difficult.

2–4 High Performance Liquid Chromatography (HPLC)

HPLC is based on using particulate material with high resolution, high loading capacity and high recovery, through which a mobile phase is run at a high pressure and a controlled flow rate. The key to HPLC was the development of suitable stationary phases. The support material has to withstand high pressure allowing eluants to be pumped through at high flow rate to give quick and sharp resolution. The first method for the separation of isoenzyme was developed by Chang et al using a packing material created and patented by F. E. Regnier [Schlabach et al, 1979]. In 1990 Toren and Smith attempted to define the 'ideal' packing material for the separation of isoenzymes on HPLC. Three criteria should be observed :

- The pore size of the material should be > 100 Å to allow sufficient interaction with the protein.
- Coating must include a hydrophilic coupling moiety so as not to denature the protein.
- The coating must contain an acidic or basic group for cation or anion exchange, respectively.

Considering that isoenzymes catalyse the same reaction in different locations (or tissues), the main application of chromatography of isoenzymes will be diagnostic information on the nature and extent of tissue damage. Most commonly the amount of activity will be

Table II-4A : *Isoelectric focusing methods for separating isoenzymes and isoforms of ALP.*

GEL TYPE	GRADIENT RANGE	ELECTROPHORETIC CONDITIONS	ENZYME STAINING	TIME TEMPERATURE	REFERENCE
Mixed type polyacrylamide support containing immobilised gradient with superimposed carrier ampholyte gradient.	pH= 3.5 to 6.0 apply about 30 µl of sample	2W constant power (12 hours at 10°C) anolyte: 10 mmol/L glutamic acid catholyte: 10 mmol/L lysine	gel incubated for 10 min in 1mol/L DEA + 1 mmol/L ZnSO4 + 1 mmol/L MgSO4 then add 1 g/l of BCIP + 0.5 g/l NBT	2 hours room temperature	Sinha et al 1986
Ready-made ampholine PAG plates-5% acrylamide and 2.2% ampholine (w/v)	pH= 4 - 6.5 pH= 5.5 - 8 apply 20 µl of sample depending on activity	prefocusing 500 V (0.5 to 1.5 hour) 1300 V for 6.5-7.5 hours 1200 V for 4.5 hours	0.75 mmol/L α-Naphthyl phosphate 1mmol/L 4-amino-diphenylamine diazomium sulphate 5mmol/LMgSO4 + 0.5 mmol/L ZnAcetate in Tris Maleate buffer pH= 9.8	4-12 hours 4°C in the dark	Rosendhal et al 1987
agarose for IEF from Litex	C.A pH= 3.5 to pH= 9.5 apply 15 µl of sample	15 W constant power (35 min-5°C) anolyte: 0.5 M AceticAc. catholyte: 0.5 M NaOH	4.4 mmol/L α-Naphthyl phosphate, 1 g/l 4-aminodiphenyl amine diazomium sulphate and 3 mmol/L MgCl2 in 1 mol/L MAP buffer pH= 10.3	5ml of solution for 15 min at 37°C-decant 5ml fresh for 15 other min	Griffiths and Black 1987
Use of Isopal system (Beckamn Analys, Namur, Belgium) – No details given					Van Hoof et al 1991

Table II-4B: *Methods for separating hexosaminidase isoenzymes by IEF*

	pH 5-8	16 hours 800 V			Sandhoff 1968
5% w / v acrylamide	3% v/v Ampholine (LKB) pH= 3.5-9.5	Constant power 25 W (1 hour) anolyte: 1M H3PO4 catholyte: 1M NaOH	use of Naphtol-ASBI 2-acetamido-2-deoxy-β-D-glucopyranoside method of Hayashi 1965		Emiliani et al 1990
? 40 ml column	2% v/v Ampholine (LKB) pH 5-7 or 5-8 sucrose grad. 0-67% w/v	600V (17 to 19.5 hours) from 3.5-4.5 mA to less than 0.5 mA	4-MuGlcNac 6 mM in citrate pH= 4.4 stop solution : 0.085M Glycine carbonate pH= 9.8	20 min 37°C	Plucinsky et al 1986
4.8 w/v polyacrylamide	2% v/v Servalytes (Serva) pH 2-11		Fast Garnet GBC + Naphtol AS_BI-N-Acetyl β-D-glucosaminide in citrate buffer, pH= 4.5	90 min	Drexler et al 1986

Table II-4C: *Isoelectric focusing methods for separating transferrin isoforms*

GEL TYPE	GRADIENT RANGE	ELECTROPHORETIC CONDITIONS	STAINING	REFERENCE
Polyacrylamide gel	2.2 to 11		Direct immunofixation	Stibler et al, 1986
Polyacrylamide	5 to 7 ampholine		CIEF (crossed immunoelectro focusing)	Van Eijk et al, 1982
Polyacrylamide immobiline dry plates (LKB)	pH 4.9 to 6.1 ampholine	Anode : glutamic acid 10 mmol/L Cathode : NaOH 10 mmol/L	CIEF	de Jong et al, 1988

monitored after elution of the different isoforms which implies an ACTIVE enzyme. This will be the factor dictating the choice of stationary and mobile phase. To maintain biological activity aqueous buffers must be used, and the stationary phase must respect the tertiary conformation of the protein which excludes reversed phase chromatography. Size exclusion and affinity are generally used for purification but, some analytical applications of affinity gels have recently been used for ALP and transferrin [Anderson et al, 1990 & Gonchoroff et al, 1991](see Table II-5B). Generally, isoenzymes being coded by different genes have slightly different amino acid compositions and different patterns of glycosylation conferring different pIs. The separation is therefore based on the principle of ion exchange chromatography. As can be seen in Table II-5A & B, different packing materials give different elution profiles.

2–5 Low pressure chromatography

2–5–1 Soft gels

Packing materials such as cellulose, dextran agarose, polyamide and other hydrophilic synthetic polymers (known as soft gels) have traditionally been used for low pressure chromatography of proteins. They were gradually replaced in the past decades by improved products with superior physical and chemical properties such as cellulose.

DEAE (derivatized cellulose) was introduced in 1958 [Peterson and Sober, 1962], and is still commonly used for the resolution of isoenzymes (e.g. hexosaminidase analysis – see Table II-5B).

2–5–2 Silica / polymer based packing

Bonded silica and organic polymers have frequently been used for the chromatography of biomolecules over the years. It would be impossible to list all their features in this chapter [for more details see Unger, 1990], but in general they were found to satisfy most of the requirements mentioned above for chromatography of macromolecules.

The first low pressure system using a silica-based packing material and columns 5 to 20 mm long was described in 1986 for haemoglobin analysis [Ersser et al, 1986]. It was shown that using a constant flow rate and continuous monitoring of the eluate, high-resolution chromatography providing quantitative data could be obtained. This presents advantages such as speed and simplicity of analysis, low sample volumes and low cost, which are of importance in a clinical environment. Although HPLC columns as small as 50 mm are used for ALP analysis [Shoenau et al, 1986], no true low pressure system exists

Table II-5A: *HPLC methods for separating isoenzymes and isoforms of ALP.*

PACKING	LENGTH & INSIDE DIAMETER OF THE COLUMN	ELUTION BUFFERS A AND B	GRADIENT	POST COLUMN & DETECTION	ELUTION SEQUENCE	REFERENCE
Anion exchange Resin DEAE cellulose	8 cm 8 mm i.d.	Tris 10 mmol/l A + 100 mmol NaCl B + 300 mmol NaCl pH= 7.5	2 steps : 100 & 300 mmol (450 mmol to be sure all eluted)	Collect fractions Enzyme assay pNPP in DEA 405 nm	F1: hepatic, intestinal, bone F2: biliary	[Karmen, 1984]
Mono.Q HR 5/5 strong exchanger Resin+ $CH_2N(CH_3)_3$ Ionic capacity= 0.28-0.36 mmol/ml particle size 10µm	5 cm 4.6 mm i.d	Tris HCl 20 mmol/l A + 0 mmol/l LiCl B + 500 mmol/l LiCl pH = 8.2	Stepwise 4 min-0 mmol LiCL 6 min 90 mmol/l 9 min 105 mmol/l 12 min 130 mmol/l 33 min linear up to 500	pNPP 5 mmol/l in 0.5M DEA pH= 9.8 + 0.5 mmol/l $MgCl_2$ FR= 0.5ml/min inc. =1 min at 25°C detection 405 nm	bone 1 bone 2 liver 1 liver 2 HMW liver	[Shoenau et al, 1986]
Same conditions used	idem	idem	only 2 steps 10 min 175 mmol/l LiCl 5 min linear, up to 500 mmol/L LiCl	MUP 1 mmol/l in DEA/HCL 0.5mol/L pH= 9.8, FR= 0.05 ml/min Incubation: 1.5 min at 37°C Ex= 405nm, Em= 451nm	Bone + Liver in 1 peak HMW Liver	[Shoenau et al, 1988]
same conditions work on urine	idem	idem	3 steps 10 min 175 mmol/l LiCl 5 min up to 500 mmol/l 5 min at 500 mmol/l	same as 1988 see above	10 min: soluble AP fraction 22 min: particulate AP fraction	[Shoenau et al, 1990]
AX 300 Synchropack Weak exchanger silica, quaternary amine	25 cm 4.6 mm i.d	Tris acetate 20 mmol/L A+ 0 mmol/L NaAc B + 0.5 mol/L NaAc pH= 7.9	linear from 13% B to 97% B in 15 min	pNPP, 176 mmol/l in 0.1mol/L DEA +2 mmol/l $MgCl_2$ & 5 ml of non-ionic wetting agent detection 405 nm	bone 1 (+intestinal) bone 2 liver 1 liver 2 HMW L & placental	[Parviainen et al, 1988]
AX 300 Synchropak Weak exchanger silica, quaternary amine	25 cm 4.6 mm id	Tris acetate 20 mmol/L A + 0 mmol/L NaAc B + 0.6 mol/L NaAC pH= 7.8	Linear from 18% to 45% in 7 min then to 95% in 7 min + isocratic for 10 min	pNPP 1.8 mmol/L in DEA 0.25mol/L 1 mmol/L $MgCL_2$ pH= 10.1 + Triton X-405 6 g/l FR 0.8 ml/min	bone + intestinal bone 1 bone 2 liver 1 liver 2 liver 3	[Magnusson et al, 1992]

Table II-5A – *continued*

PACKING	LENGTH & INSIDE DIAMETER OF THE COLUMN	ELUTION BUFFERS A & B	GRADIENT	POST COLUMN & DETECTION	ELUTION SEQUENCE	REFERENCE
PAK DEAE 5PW Weak exchanger resin (Water cie)	7.5 cm 7.5 mm i.d	Tris HCl 20 mmol/L A + 0 mmol/L LiCl B + 0.5 mol/L LiCl pH= 8.2	linear from 0% B to 100% B in 20 min	collect fractions pNPP 10 mmol/l in DEA 0.6 mol/L + $MgCl_2$ pH= 9.8 detection 405 nm	Liver Bone Intestinal Placental	[Ragougi-Sengler et al, 1990]
HPAC WGL on 7 mm silica particles	5 cm 4.6 mm i.d	N,N(2-hydroxyethyl)2-amino ethane sulfonic acid- 50 mM + NADG pH= 7.0	stepwise 0g/l NAG 2g/l NAG 50g/l NAG	pNPP 2 mmol/L in DEA 1.23 mol/L 0.62 mmol/L $MgCl_2$ pH= 10.15 FR= 1 ml/min 30°C at 405 nm	0g/l, 1.6 min no ALP 2g/l, 5.7 min liver 50g/l, 15.1 min bone Intestinal inhibited by L-Phe 10 mmol/L	[Gonchoroff et al, 1991]
HPAC WGL on 7 mm silica particles	5 cm 4.6 mm i.d	N,N(2-hydroxy-ethyl) 2-aminoethane sulfonic acid 50 mmol/L + 4 g/l NADG + 100 g/l NADG pH= 7.0	linear exponential	pNPP 2 mmol/L in DEA 1.23 mol/L 0.62 mmol/L MgCl2 pH= 10.15 FR= 1 ml/min 30°C at 405 nm	P1 non retained P2-liver isomer P3 bone isomer	[Anderson et al, 1990]

Table II-5B: *Methods for separating hexosaminidase isoenzymes by liquid chromatography*

PACKING	LENGTH & INSIDE DIAMETER OF THE COLUMN	ELUTION BUFFERS A & B	GRADIENT	POST COLUMN & DETECTION	ELUTION SEQUENCE	REFERENCE
Anion exchange DEAE cellulose	4.5 cm 0.35 cm i.d	NaH_2PO_4 10 mmol/L pH = 6 A + 0 mmol/L KCl B + 0.5 mol/L KCl	Linear 30 min	fluorometric : 4-MuGlcNac Substrate buffer= 90 mmol/L Na_2HPO_4 50 mmol/L citric acid pH= 4.5 + 0.5 mol/L Gly.NaOH pH 10.4	Hex B, Hex I1, Hex I2, Hex A	[Ellis et al, 1975]
DEAE cellulose (from Biorad)	7 cm 0.5 cm i.d	Phosphate 10 mmol/L pH = 6 A + 0 mol/L NaCl B + 0.5 mol/L NaCl	linear 40 min	idem 8 min incubation 37°C /stop with carbonate-glycine pH= 10 Ex= 365 nm, Em= 450 nm	Hex B, Hex I1, Hex I2, Hex A	[Nakagawa et al, 1978]
DEAE cellulose (DE-52 Wathman)	10 cm 0.5 cm i.d	NaPhosphate 10 mM pH = 6 A + 0 mol/L NaCl B + 0.3 mol/L NaCl	linear 90 min	25 mmol/L Na citrate + 50 mmol/L Kphosphate pH= 4.5 + 1 mmol/L 4-MuGlcNac + 0.1g/l HSA stop GlyNaOH pH= 10.6	Hex B, Hex I1, Hex I2, Hex A, Hex AS	[Datti et al, 1991]
DEAE cellulose DE-52 wet type Wathman	3 cm 0.5 cm id.	Citrate phosphate 10 mM pH = 6 A + 0 mol/L NaCl B + 0.3 mol/L NaCl	linear 32 fractions of 0.3 ml	Citrate Phosphate 0.1mol/L pH= 4.4 4-MuGlcNac 0.264 mmol / fraction + 15% HSA incubation 30 min 37°C stop Gly-NaOH 0.2M pH 10.6	Hex B, Hex I1, Hex I2, Hex A	[Yabuuchi et al, 1974]
DEAE-cellulose DEAE-22 powder Wathman	4.5 cm 0.6 cm id	Kphosphate 10 mmol/L pH = 6 A + 0 mol/L NaCl B + 0.5 mol/L NaCl	linear 60 min	Na-Citrate 0.5mol/L pH 4.0 4-MuGlcNac 8 mg/40 ml buffer stop Gly-Carbonate pH= 10.0	Hex B, Hex I1, Hex I2, Hex I3(in carrier, pregnant & Tay-Sachs), Hex A (some I detected in WBC)	[Saifer et al, 1975]

Table II-5C: *Methods for separating transferrin isoforms by liquid chromatography*

TYPE OF CHROMATOGRAPHY	PACKING	LENGTH & INSIDE DIAMETER OF THE COLUMN	ELUTION BUFFERS A & B	GRADIENT	ELUTION SEQUENCE	REFERENCES
HPLAC Detection: UV, 280nm	SelectiSpher-10 activated Tresyl column (Perstorp Biolytica) particle size: 10 mm coupled to polyclonal antibody to Transferrin	10 cm 0.5 cm i.d & 5 cm 0.5 cm i.d	citric acid 0.1 mol/L + isopropanol 100 g/l pH= 1.3 FR = 3 ml/min	none	Only one peak for total transferrin.	[Ohlson et al, 1988]
Anion exchange 'Chromatofocusing' detection : double antibody immunoassay	Sepharose based buffering anion exchanger of PBE type. *prepared by Pharmacia, Swedish patent*	125 ml of suspension	dilution of serum 1/25 Piperazine-HCOOH 20 mmol/L, pH= 5.65	isocratic at pH= 5.65 chosen so only component with pI= 5.7 and above are eluted (main transferrin, 5.4)	Only one peak for disialo and asialotransferrin.	[Stibler et al, 1986]
Anion Chromatofocusing Detection: UV 280nm or RIA	Mono P column (Pharmacia)	§	10 fold diluted Polybuffer 74-pH= 4.5 1 ml /min	pH gradient between 5.1 and 6.1 (according to diagram)	From hexasialo pH 5.4 to asialo pH 5.9	[Storey et al, 1985]
Anion exchange detection: RIA	DEAE-Sephacel anion exchanger (Pharmacia)	200 ml *(0.5 cm x4.6 mm by experience)*	2-N-Morpholino-ethanesul-fonic acid (MES) 20 mmol/L pH= 5.65	isocratic and calculate ratio total T4/ T2.		[Kwoh-Gain,1990]
Anion exchange Detection: 460 nm	MonoQ HR 5/5 (Pharmacia)	5 cm 0.5 cm i.d	Bis-Tris 20 mmol/L pH= 6.2 A + 0 mmol/L NaCl B + 350 mmol/L NaCl FR= 1 ml/min	two steps: 10% B 34% B	Complete resolution of a-di-, tri-, tetra-, and pentasialylated forms	[Jeppsson et al, 1993]

for isoenzyme analysis. The key to the development of this kind of technology seems to be the quality of the packing material used.

3 – Detection of isoenzymes and isoforms

Sensitivity and specificity of detection are two major factors determining the quality of an analytical procedure and this will clearly be influenced by the type of detection system used. Tables II-3, II-4, and II-5 list the detection systems commonly used for the isoenzymes and isoforms of alkaline phosphatase, hexosaminidase (based on their activity) and transferrin. Two of these detection systems (fluorescence and immunostain) which were used in this study will now be reviewed.

3–1 Fluorescence detection

Fluorescence detection can not easily be adapted for use following electrophoresis or IEF because of the rapid diffusion of the product in gels or nitro-cellulose membranes. However, the gain in sensitivity, e.g. compared with the p–nitrophenyl–substrates (colorimetry), makes fluorescent substrates the preferred method for the detection of enzyme activity in continuous flow analysis.

Fluorescence detection does have two main problems: quenching and intrinsic fluorescence, both of which are likely to interfere with the detection of the products of interest.

- Quenching

Some molecules are able to interfere with the fluorescence, they are defined as quenchers. Many molecules are known in chemistry to act as quenchers, but the only one of relevance in this study would have been oxygen as proteins and membranes are highly permeable to molecular oxygen. No problems were however encountered due to the quenching by oxygen during this work.

- Intrinsic fluorescence

Human serum is composed of a large number of components, but fortunately only a few of them make a significant contribution to overall fluorescence. Most of these compounds, e.g. tryptophane, NAD(P)H, pyridoxic acid lactone, pyridoxal phosphate Schiff base, and protein bound bilirubin are present at low concentrations, and therefore have a very low

fluorescence efficiency and are mostly detectable at wavelengths within the U.V spectrum. In the visible range (λexc= 320 nm to λem= 500 nm) serum diluted 1:20 does not show significant absorption maxima except in the case of high bilirubin concentrations; moreover, in human serum, fluorescence is highly pH-dependent and is low at pH >9.

Table II-6 shows some examples of compounds in human serum with their respective excitation/emission maxima. This table is of particular importance as it shows that none of these wavelength coincide with those used in this work (exc= 360 nm / em= 450 nm) suggesting that interferences should not be a problem.

- Fluorescence in enzyme activity detection

In 1955 Mead, Smith and William found that when umbelliferone (7–hydroxycoumarin) or 4–methylumbelliferone were fed to rabbits, the animals excreted this material in urine as β–D glucuronide conjugates. These glucuronides were much less fluorescent than the parent phenols and the authors proposed the use of 4–methylumbelliferyl glucuronide as a fluorogenic substrate for very sensitive assays [Leaback, 1974]. The main advantage of this system is that a common detection system can be used for a wide range of applications as the product released is always 4–methylumbelliferone (λexc= 360 nm, λem = 450 nm). 4–methylumbelliferyl compounds have since been used extensively as enzyme substrates.

3–2 Immunostain

3–2–1 Immunofixation

This technique was first published in 1969 by Alper and Johnson, their primary purpose being the detection of genetic polymorphism of ceruloplasmin, and was introduced into clinical laboratories from 1976 [Ritchie & Smith, I-III].

Immunofixation consists of electrophoresis followed by a fixation phase in which antiserum is used to precipitate a protein. As long as the antibody is in slight excess or near equivalency, the complex antigen/antibody remains insoluble. The reaction can then be detected by protein staining, visual inspection, or antibodies labelled with fluorescein, enzyme or isotopes. For example, by applying horseradish peroxidase conjugated antibody directly onto the agarose gel, Cawley could detect monoclonal immunoglobulin of 2.6 ng/μl of sample [Cawley et al, 1976]. This peroxidase-conjugated system can also be used with western blotting, as will be seen in this study.

Table II-6: *Some fluorescent compounds in human serum and their excitation/emission maxima.*

COMPOUND	REPORTED VALUES Excitation/emission maxima (in nm)
Tyrosine	275/303
free Tryptophane	278/348
bound Tryptophane	278/322–345
indoxyl sulphate	290/380
3–Hydroxyanthranilic acid	320/415
5–Hydroxyanthranilic acid	340/430
4–Pyridoxic acid	315–317/425–440
Pyridoxal phosphate Schiff base (imine form)	325/430
free NADPH	340/462–470
Pyridoxic acid lactone	340–350/440–478
Riboflavine/FMN	360/430
Pyridoxal phosphate Schiff base (enamine form)	410/505
Bilirubin	448–460/525,455/520, 460/515

The numerous advantages compared with immunoelectrophoresis, such as a much higher resolution (limited diffusion), speed, great sensitivity and economy (it does not require equipment beyond standard agarose electrophoresis), result in this technique being widely used.

3–2–2 Western blotting

Molecules separated by electrophoresis (or isoelectric focusing) can be transferred onto a nitro-cellulose membrane, to which they bind in an identical pattern; this process can be done either by transverse electrophoresis (electroblotting), or by simple diffusion from gel to nitro-cellulose. An antibody is then applied to the membrane rather than the gel, a process known as immunostaining. The main advantage of this method is the almost complete lack of diffusion as proteins bind strongly to nitro-cellulose.

Immunofixation and western blotting are both methods used to examine the structure of the protein but cannot be used to estimate activity.

4 – Definitions regarding the evaluation of a method

The analytical performance of a method can be described in terms of, (i) its accuracy, (ii) its precision, (iii) its practical aspect like turnover time, and (iv) its cost.

Accuracy and precision have been respectively defined as "agreement between the mean estimate of a quantity and its true or accepted value" and "agreement between replicate measurement" - IUPAC definitions [Guilbault and Hjelm, 1989] but are usually measured as inaccuracy – "numerical difference between the mean of a set of replicate measurement and the true value" and imprecision – "variations of the results in a set of replicate measurements".

Inaccuracy and imprecision of an analytical procedure can be determined by comparison with a reference method, which is a state-of-the-art method, where systematic errors have been eliminated as far as possible, and random errors minimised as far as practicable. An example of a commonly-used reference method is mass spectrometry, which can now be used to quantify both low molecular-mass, and high molecular-mass compounds such as proteins.

Inaccuracy can also be determined by using a certified control material, defined as "a material to be used for the assessment of the performance of an analytical procedure or part thereof" - IUPAC definition. At present there are only a limited number of control

materials for proteins and no such control material was available for the three proteins analysed in this study.

In the absence of acknowledged reference methods and reference materials [Moss, 1994], the performance of a new method had to be compared with that of established routine methods. Such a comparison will not provide information about inaccuracy and imprecision of the new method *per se* but will give an idea of the transferability of values between the methods. There are no absolute criteria for deciding which degree of inaccuracy or imprecision is acceptable and often a balance has to be struck between several factors, such as the quality of analytical performance related to the clinical needs, and costs. High analytical performance is directly related to cost, as methods with high accuracy and precision usually require staff-time and expensive equipment. Chromatographic methods have been devised to separate molecules of interest from their matrix and should, in theory, give accurate results provided detection is adequate. In practice this may not always be the case as (i) two or several compounds may be co-eluted in the same fraction, (ii) the packing material may change its properties with time due to interactions with eluants and compounds in the sample matrix, and (iii) the chemical composition of the eluant may deteriorate with time.

B – Development of a low pressure chromatographic method suitable for the analysis of isoenzymes and isoforms

1 – Aim

The aim of the work described in this section was to study the general behaviour of chromatography in small columns, as a basis for attempting to develop micro column chromatographic method for separating isoenzymes and isoforms of enzymes and proteins. This has included:

(i) adaptating two commercial systems for chromatography of the selected proteins,

(ii) establishing basic principles for post-column detection of enzyme activities,

(iii) testing a range of packing materials, and

(iv) examining the effect of the size and shape of the column and of the flow rate on the chromatographic resolution.

2 – Chromatographic equipment

Two commercial systems, the Glycomat (GMAT) and the G15 (Drew Scientific), originally developed for the analysis of haemoglobin by micro column chromatography [Ersser et al, 1986], were modified for this work and used mainly as a pump coupled to a gradient maker.

2–1 The Glycomat (GMAT)

The GMAT system (Fig.II-1) has two syringes, of 20 ml each, driven by software programmed to give a suitable gradient. The variables are flow rate, and the ratio of buffers A and B. The main limitation was the fixed capacity of the syringes which resulted in a maximum length of analysis time dependent on the used flow rate.

Every cycle the syringes refill with buffer, which stops the flow through the whole system and creates artefactual peaks (labelled X on figures). This problem is discussed in following sections.

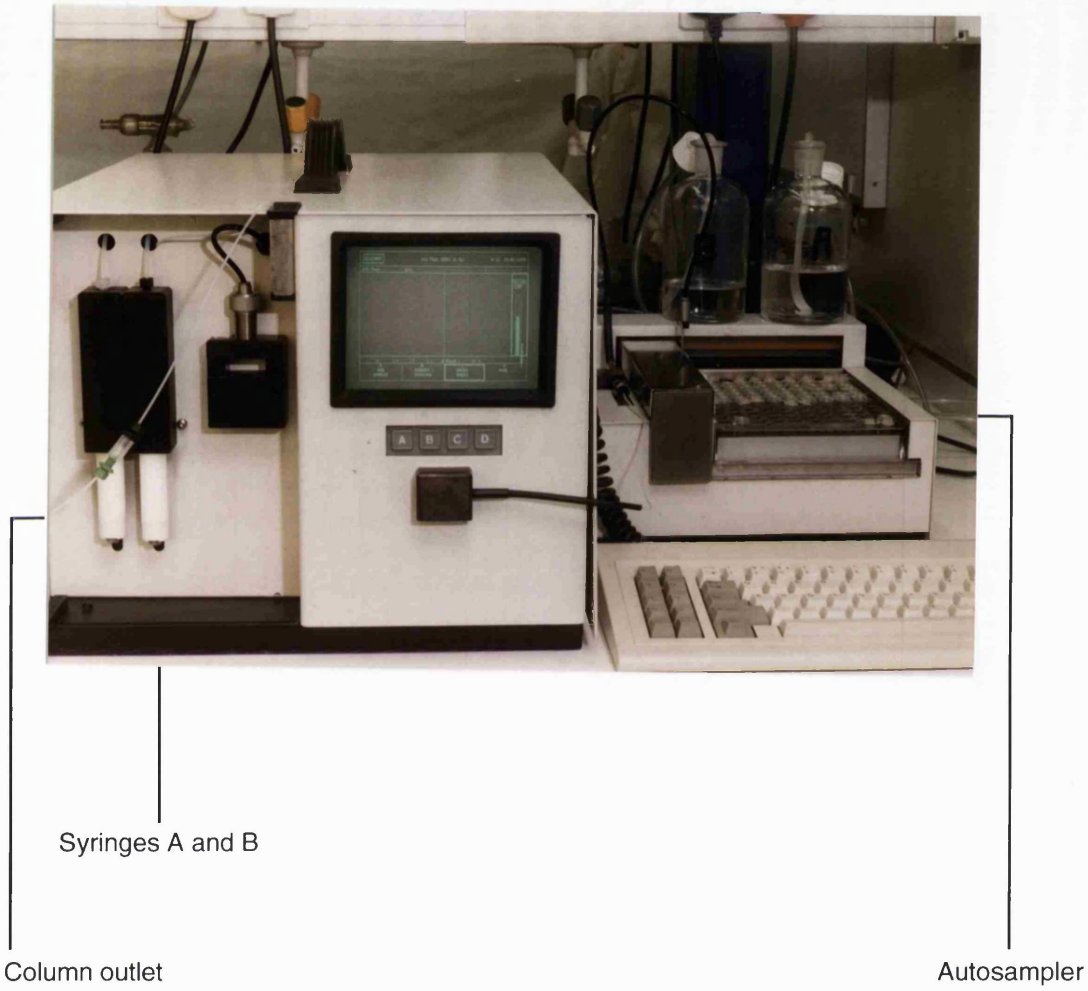


Fig.II-1 : *Glycomat system*

2–2 G15

G15 (Fig.II-2) is composed of a peristaltic pump coupled with the same software as used by the GMAT. The flow is continuous and should, in theory, allow a better analysis of any peak in the void volume. However the flow rate is not easily varied as the pump works at a fixed speed and the most appropriate size of the pump tubing had to be used. One of the limitations common to both systems was the number of channels available. Only two buffers could be delivered and as a result it was impossible to use a wash solution different from the eluting buffers or to use extra reagents.

2–3 A post-column for isoenzymes analysis

The enzyme analysis required the development of a suitable post-column detection system. An in-house system consisting of a coiled teflon tubing and T-piece was designed as shown in Fig.II-3. One coil was equivalent to 1 min incubation when run at 1 ml/min and as many coils as required were put in series. Because of the different types of reactions required for ALP and hexosaminidase, a multi-channel peristaltic pump had to be used to add the complementary reagents. More details are given for the respective enzymes in the relevant chapters.

2–4 Detectors and data handling

Data Collection Unit 3040 and Software DS 4000 used to analyse data (Drew Scientific, Barrow in Furness, Cumbria), Fluorometer RF 535 (Shimadzu, Dyson Instrument, Tyne and Wear, UK), U.V detector: Spectromonitor 3100 with variable wavelength (LDC Analytical, Staffordshire, UK).

3 – Selection of packing materials

The following packing materials were used: Synchropack-AX 300 and DuPont Biotechnology-Zorbax SAX (FSA chromatography, Leics, UK), DEAE Sephacel and Mono Q (Pharmacia, Milton Keynes, UK), PL SAX 1000 (Polymer laboratories, Shropshire, UK), Fractogel EMD TMAE 650 (S) (Merk, Darmstadt, Germany or BDH Chemicals, Poole, Dorset, UK).

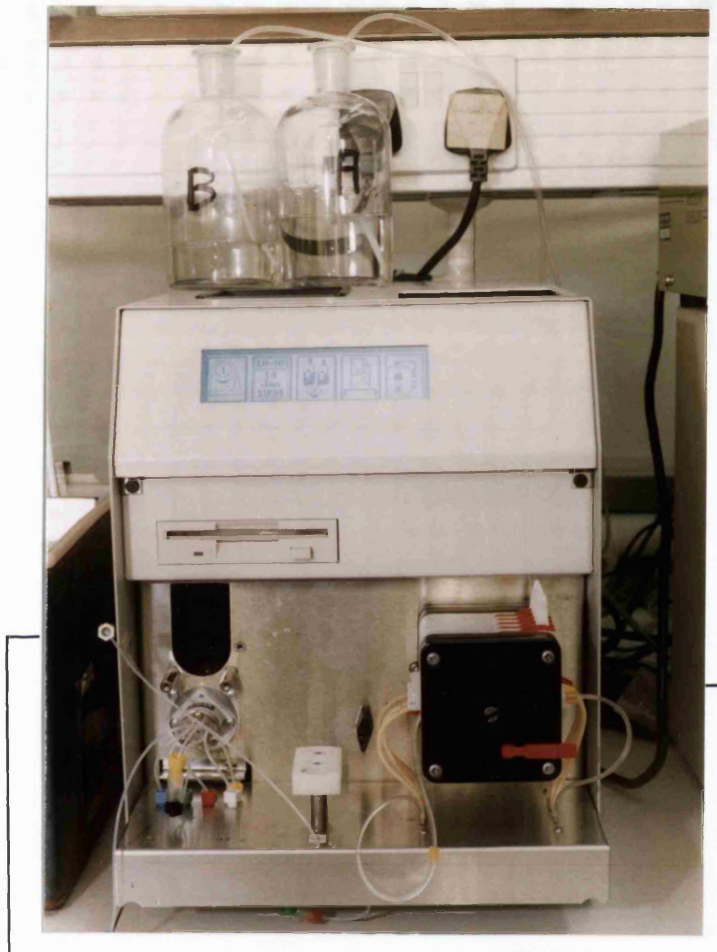


Fig.II-2: *G 15 system*

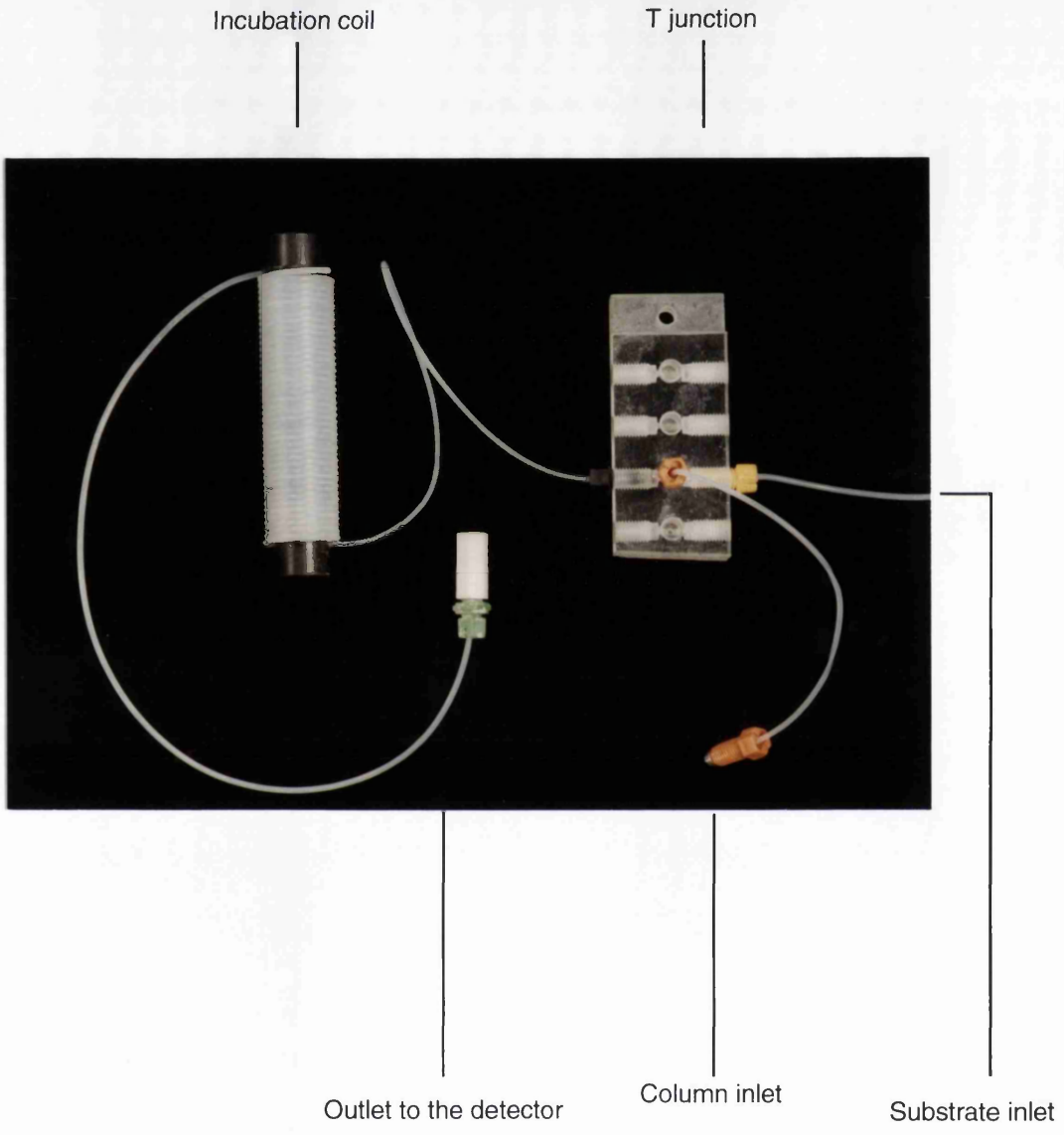


Fig.II-3: *Post-column system*

3–1 General considerations

Resolution from a chromatography column is governed by three main parameters: capacity, efficiency and selectivity of the packing material [Pharmacia News, 1991]. In order to find a packing with the best combination of the three criteria for this study the following questions were addressed.

3–1–1 Anionic or cationic exchanger?

Because proteins exist either as anions or cations, ion-exchange chromatography should provide an 'ideal' system for their separation. Retention depends on the competition between the protein ionic sites and the mobile phase for the ionic sites on the support. The type of ionic sites on the protein vary with the pH and the number of sites with the protein structure.

The pI of the three proteins studied in this work (ALP, hexosaminidase, transferrin) were less than 6, and at pH= pI plus one unit the proteins are still in fairly physiological conditions and in an anionic form. Therefore, anionic exchangers should allow the separation of proteins which retain their catalytic properties.

3–1–2 Weak or strong exchanger?

A strong ionic exchanger has a strong acid or base attached to the ligand and therefore it will be fully dissociated over the whole pH range. A weak exchanger has a weak acid or base attached and will have a dissociation equilibrium depending on the pH conditions. In conditions of a high pH it will cease to work effectively as an anion exchanger.

Although the literature shows a preference for using a weak anion exchanger for working with enzymes, a comparison between the two was carried out to establish the most suitable conditions for Micro column chromatography (MCC) – see Section 3-2.

3–1–3 Cellulose, sllica or synthetic polymer?

In 1958 Sober and Peterson described the use of diethylaminoethyl (DEAE)-derivatised cellulose for the separation of proteins [Regnier and Chicz, 1990]. Cellulose-based ion exchangers are characterised by high protein recovery, good loading capacity and chemical stability when used between pH 2 and 12. Their primary limitation is a poor mechanical strength, and therefore they can not be used at high velocity or small particle size, two important criteria to improve chromatography. They are however commonly used in

protein analysis, especially packings like DEAE-Sephacel which have an improved stability [Kwoh-Gain, 1990].

The application of surface modification to micro-particulate porous silica opened new ways for the synthesis of bonded silica for almost every mode of chromatography while providing high resolution and high recovery. The first separation of proteins on ion exchangers was described in 1976 [Chang et al, 1976] and a large variety of packing materials based on silica or synthetic polymers have now been developed. A limitation of silica is the pH range, as silicate formation impairs the ion-exchange function above pH 8.

3–2 Comparison between different packing materials for MCC analysis

A series of anionic exchangers with different properties were investigated in order to choose the most suitable for analysis on MCC (see Table II-7).

Three main points have to be noted:

(i) A number of chromatograms obtained by MCC analysis of alkaline phosphatase and hexosaminidase have been used to illustrate the various points discussed in this chapter. They are all part of developmental work and may not resemble the chromatograms shown in later chapters once the definitive conditions were used.

(ii) The various peaks are usually not labelled as the main purpose of this exercise was to find the best resolution conditions and as details of the various isoenzymes and isoforms will be presented in later chapters.

(iii) The Resource Q column was not initially included in this part of the work as the packing material itself was not available and a column of suitable size was not obtainable. However, 1 ml cartridges became available during the last few months of this study and some comparison was made using this material for transferrin analysis.

3–2–1 Comparison between DEAE-Sephacei and PL SAX

Because of its long history in the separation of isoenzymes, DEAE-Sephacel was compared with PL-SAX for the separation of hexosaminidase isoenzymes. Fig.II-4 shows the results obtained from separating hexosaminidase isoenzymes in an extract from normal fibroblast and in normal serum. Although PL SAX gives a slightly better resolution, the elution patterns are similar when the same conditions (length of the column, gradient, flow rate) are used. The two main peaks for hexosaminidase are well resolved and a third one is present as a shoulder using DEAE but, almost completely resolved on PL SAX. However, some chemical degradation took place with DEAE which was not observed with PL-SAX.

Table II-7: *Main features of the packing materials studied*

	DEAE-Sephacel	AX 300	PL SAX 1000	Fractogel TMAE 650	Zorbax SAX	Resource Q
Nature of ligand	Weak	Weak Polyethyleneimine (PEI)	Strong fully quaternised PEI	Strong R'=$CH_2CH_2N^+(CH_3)_3$	Strong Aliphatic quaternary amine	Strong Quaternary ammonium
Nature of support	Cellulose cross-linked with epichlorohydrin	Bonded silica	Rigid macroporous polymer matrix	Hydrophylic resin-based –Tentacle type	Bonded silica	Monodisperse polystyrene/bivinyl benzene
Pore size (Å)		300	1000	650	70	200-10 000
Bead shape	Spherical	Lattice	Almost spherical	Acrylamide derivatives tentacles	Spherical	Monosphere
	40-160 µm (wet)	6.5 µm	10 µm	25-40 µm		15 µm
pH stability	2-12	2-8	1-13	1-13	2-8	2-12
Ionic capacity	0.095-0.135 mmol/ml	?	>0.2 mmol/ml	Up to 25 ionic groups per tentacle	?	
Dynamic protein loading	@150 mg/ml HSA	?	>40 mg/ml BSA	100 ± 10 mg/ml BSA	?	Up to 25 mg/ml of proteins
Exclusion limit	1.10^6 Da	>200 000 Da	1.10^6Da	?	?	

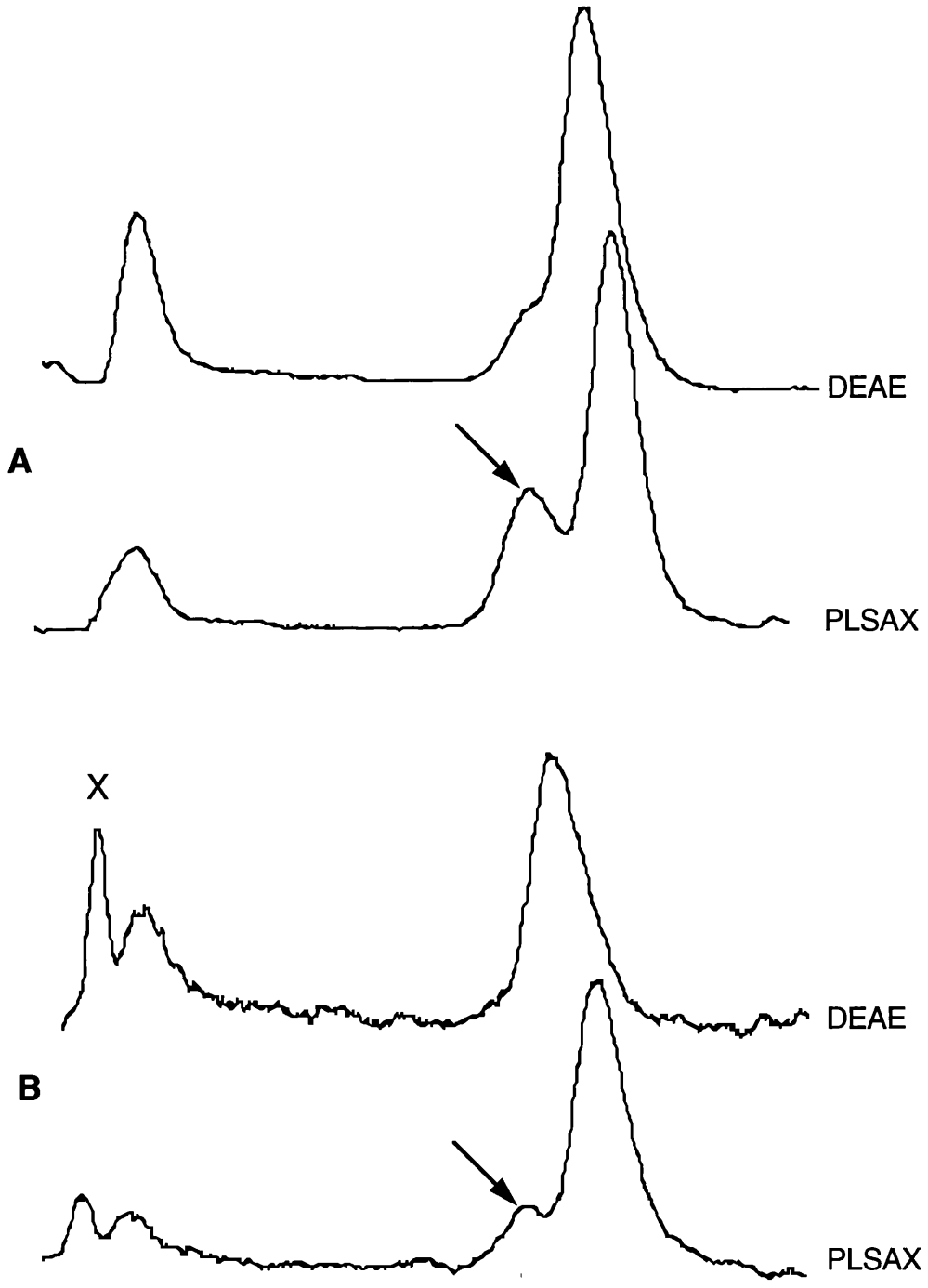


Fig.II-4*: Separation of hexosaminidase isoenzymes on DEAE and PL SAX 1000.*

A= normal fibroblast, **B** = normal serum, X= artefact present despite base-line subtraction. Conditions: 20 mm x 6.4 mm i.d column, sodium phosphate buffer, pH 6.0, 0.8 ml/min. The arrows indicate the additional peak resolved with PL SAX.

As a result the resolution would deteriorate after the analysis of approximately twenty samples and the packing material 'packed down' raising the back pressure to a point that the pump could not handle. Washing the DEAE column by reverse flow with high salt concentration and NaOH improved the situation but only temporarily. On no occasion did the PL SAX material 'pack down'. The filter would typically get blocked by fibrinogen after the analysis of 45-50 samples before degradation of the packing could be seen. Because of the design of the column, the filter could not be changed without repacking the whole column. An in-line filter would have been helpful in improving the life of the column but no suitable practical solution was found during the course of this study.

3–2–2 Comparison of PL SAX, AX 300, Fractogel and Zorbax

Aliquots of the same normal serum were loaded onto columns (20 mm x 6.2 mm i.d), packed with PL SAX, AX 300, Fractogel and Zorbax respectively, and eluted at identical conditions with regard to gradient profile and flow rate. Comparison between the four packing materials were carried out varying the gradient profile to ensure that optimal conditions would be achieved (as documented in Appendix A).

Fig II-5 shows some elution profiles obtained for alkaline phosphatase isoenzymes using the following conditions: stepwise gradient between 0 and 350 mmol/L of NaCl, for a total length of 34 min, at a flow rate of 0.8 ml/min. A minimum number of five isoenzymes (Intestinal, Bone, Liver, High Molecular Weight liver and Placental) were expected as the usual number obtained with HPLC is seven (Intestinal, Bone 1, Bone 2, Liver 1, Liver 2, HMW or Liver 3 and Placental).

3–2–3 Outcome

The general conclusion, when comparing each packing material run in the optimum gradient conditions was as follows:

- AX 300 was found to be too weak as the proteins were not always retained in a reproducible manner.

- Fractogel seemed to give the best resolution, especially in the latter part of the chromatogram (see Fig.II-6) but the bench life of the column was quite short and the base line soon became unstable at the level of sensitivity used for the detection of ALP isoenzymes. This was thought to be due to a limitation in the regeneration ability of the material. The exchanger groups are synthesised as 'tentacles' so that the proteins can keep

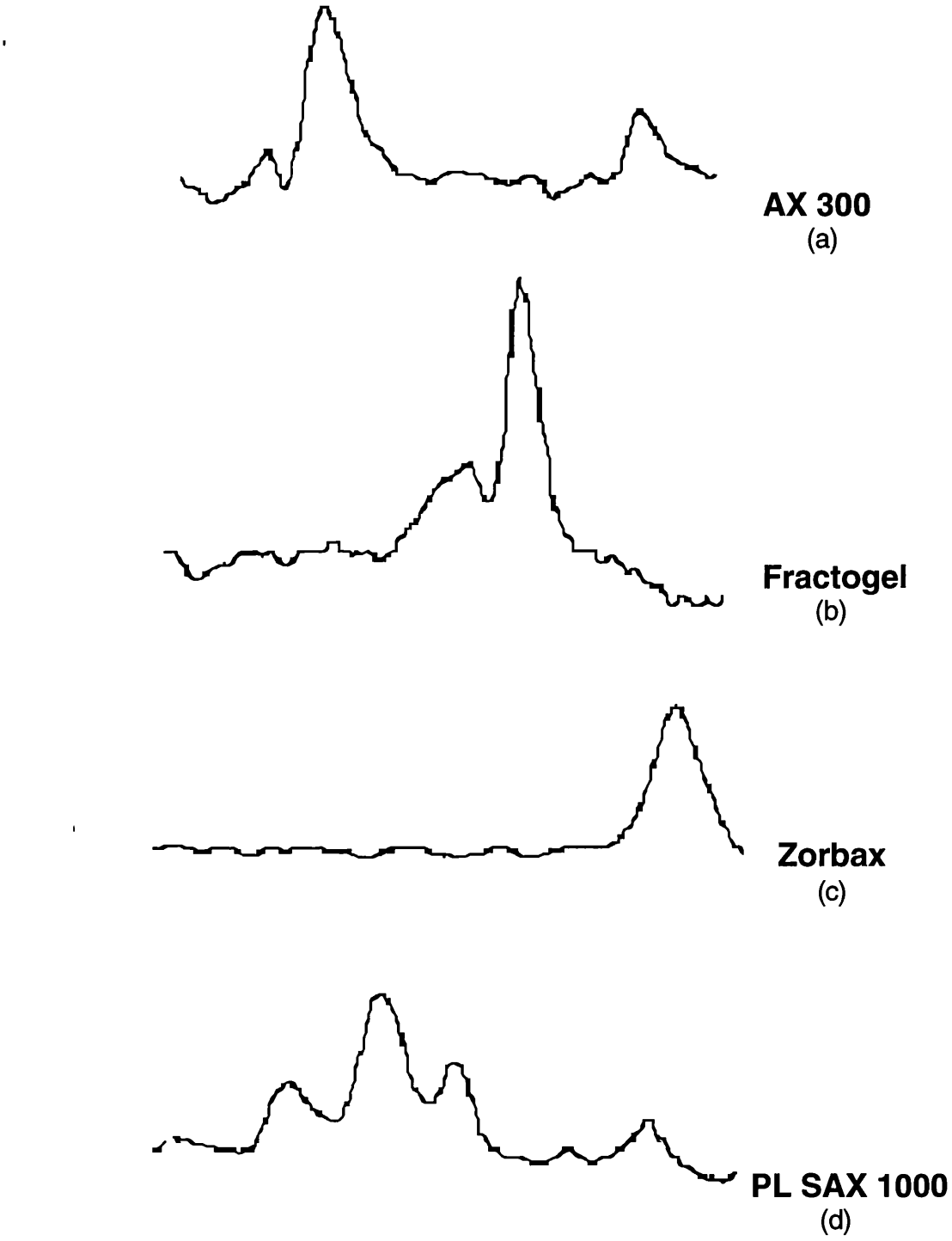


Fig II-5: *Comparison of four packing materials*. Elution of ALP isoenzymes in a normal serum on a 20 mm x 6.4 mm i.d column with a stepwise gradient for 34 min at a flow rate of 0.8 ml/min (for details on the gradient, see Fractogel G5 in Appendix A) with Tris HCl buffers pH 8.2.

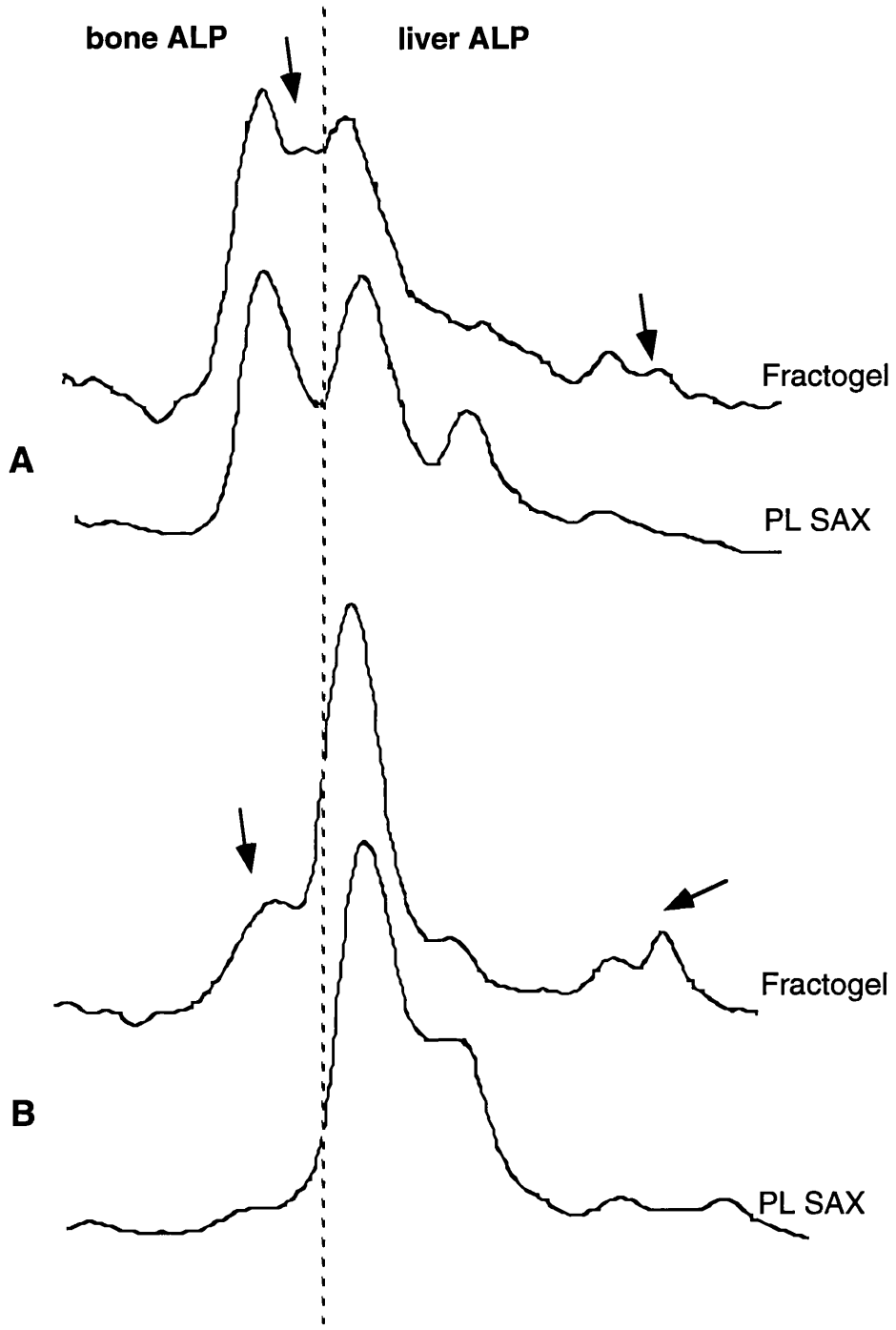


Fig.II-6: *Comparison between Fractogel and PL SAX 1000 on analysis of serum ALP.* **A**= sample known to contain high bone ALP, **B**= sample known to be mainly liver ALP – The arrows show the main differences between patterns. The column, buffer and flow rate conditions were as described in Fig.II-5, the gradients were adapted to individual packing materials.

their conformations, and when salt is added to the eluting buffer, the 'tentacles' retract releasing the molecule. If they do not regenerate properly they can not bind the proteins in a reproducible manner. Thus this packing material had no real advantages over the PL SAX.

• Zorbax, was found to be too strong. The eluting conditions had to be drastic to remove the isoenzymes off the column and the resolution was not good (Fig II-5c).

• PL SAX was found to be robust, easy to handle and the best, as the proteins of interest bound strongly and eluted easily with a salt gradient.

3–3 Conclusions

Based on information in the literature (see Tables II-5), a silica-based anion exchanger would seem to be the best choice for the task of separating isoenzymes and isoforms. However, the comparison carried out indicates that a strong, synthetic polymeric anion exchanger would be more suitable for a short column where the proteins have to bind quickly as a sharp zone.

When trying to understand the reasons for the differences observed in elution patterns with packing materials of similar chemistry, it was noted that the pore size of these packing seemed to be the varying factor as Zorbax has a pore size of 70 Å. It can be noted that it does not obey the rule quoted by Toren and Smith that the pore size should be >100 Å (see Section A-2-4) whereas PL SAX and Fractogel have pore sizes of 1000 Å and 650 Å respectively (see Table II-7), which probably gives the necessary space for the proteins to bind and elute as sharp zones.

PL SAX, the best of the tested packing materials, was chosen for its similarities in chemical composition and bead shape to Mono Q which has successfully been used to separate isoenzymes [Shoenau et al, 1986]. The Resource Q column was shown to give an even better resolution than PL SAX for analysis of transferrin isoforms. This was thought to be due to a larger pore size (200 to 10 000 Å) and a very specific matrix (monospheres). If this packing was available loose and not only as a pre-packed column as at present, a 5 mm column could be sufficient for the analysis of ALP and hexosaminidase, assuming that the material is as robust as PL SAX.

4 – Size of the column

The relationship between the length of a column and chromatographic resolution was addressed as early as 1973 [Ikonne and Ellis, 1973], when working with diethylaminoethyl

cellulose support.

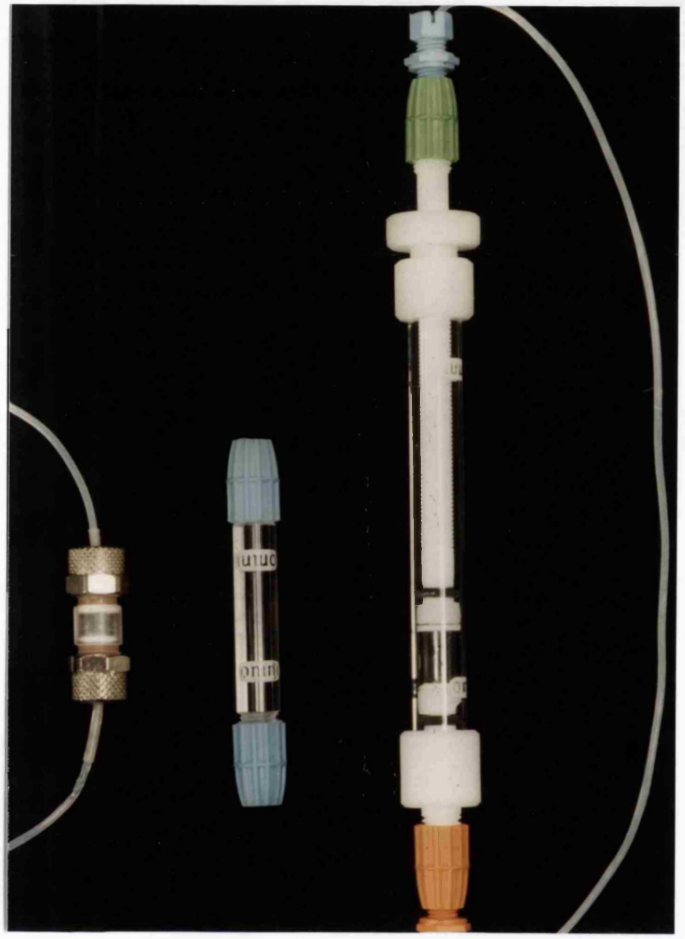
The general purpose of this study was to develop a method which was fast, 'economical' in the amount of sample used and could accept plasma sample directly after dilution with the initial buffer. It was shown that with a column below a critical size there was a risk of saturating the packing material with proteins other than those of interest as at pH>6, many of the human serum proteins are in their anionic form, especially albumin.

Several sizes of column with PL SAX 1000 were tried in this thesis: (i) 5 mm x 4.6 mm i.d, (ii) 50 mm x 3 mm i.d (iii) 20 mm x 6.4 mm id, (see Fig II-7). The results of separating hexosaminidase isoenzymes in a serum from a patient with Tay-Sachs disease on these three columns are shown in Fig.II-8, and for the separation of ALP in Fig.II-9. It is apparent that there are changes in the chromatographic profiles between the columns on Fig.II-8 as (i) resulted in 1 peak and three distinct shoulders, (ii) in three well resolved peaks for the same sample and (iii) two peaks. Although the capacity of (ii) (353.5 µl) is half the capacity of (iii) the resolution was better indicating that both the geometry of the column and its capacity influence the elution pattern. Unfortunately because of back pressure induced by the filters fitted on each side of (ii) the use of this type of column was impractical. The differences between (i) and (iii) was not thought to be substantial as, for the analysis of hexosaminidase (iii) gave a better shaped chromatographic peak but (i) resolved the same peak into two. The differences between (i) and (iii) on Fig.II-9 were consistent with the results in Fig.II-8 as only the end of the chromatogram showed a better resolution. Therefore column (i) 5 mm x 4.6 mm i.d, was chosen for the main study.

5 – Flow rate

The determination of the optimal flow rate was mainly by empirical study. A low flow rate will enhance the ability of the packing material to bind the proteins to be separated, but will increase the analysis time and often decrease the resolution by broadening the bands, especially with the use of labile substrate (e.g. methylumbelliferyl phosphate) at 37°C.

Differences between elution patterns for hexosaminidase isoenzymes from normal fibroblast and serum separated on PL SAX 1000 at two flow rates (0.4 ml/min and 0.6 ml/min) are shown on Fig.II-10. The resolution was better for hexosaminidases isoenzymes (the small peaks are more defined and the artefact at the beginning more detached) at the lower velocity. Therefore, flow rate of 0.4 ml/min on Glycomat and 0.55 ml/min on G15 were chosen for further work.



(i) (ii) (iii)

Fig.II-7: *Column sizes tested in this study.* (i) 5 mm x 4.6 mm, 'home made', (ii) 50 mm x 3 mm Omnifit, (iii) 20 mm x 6.4 mm adjustable Omnifit column.

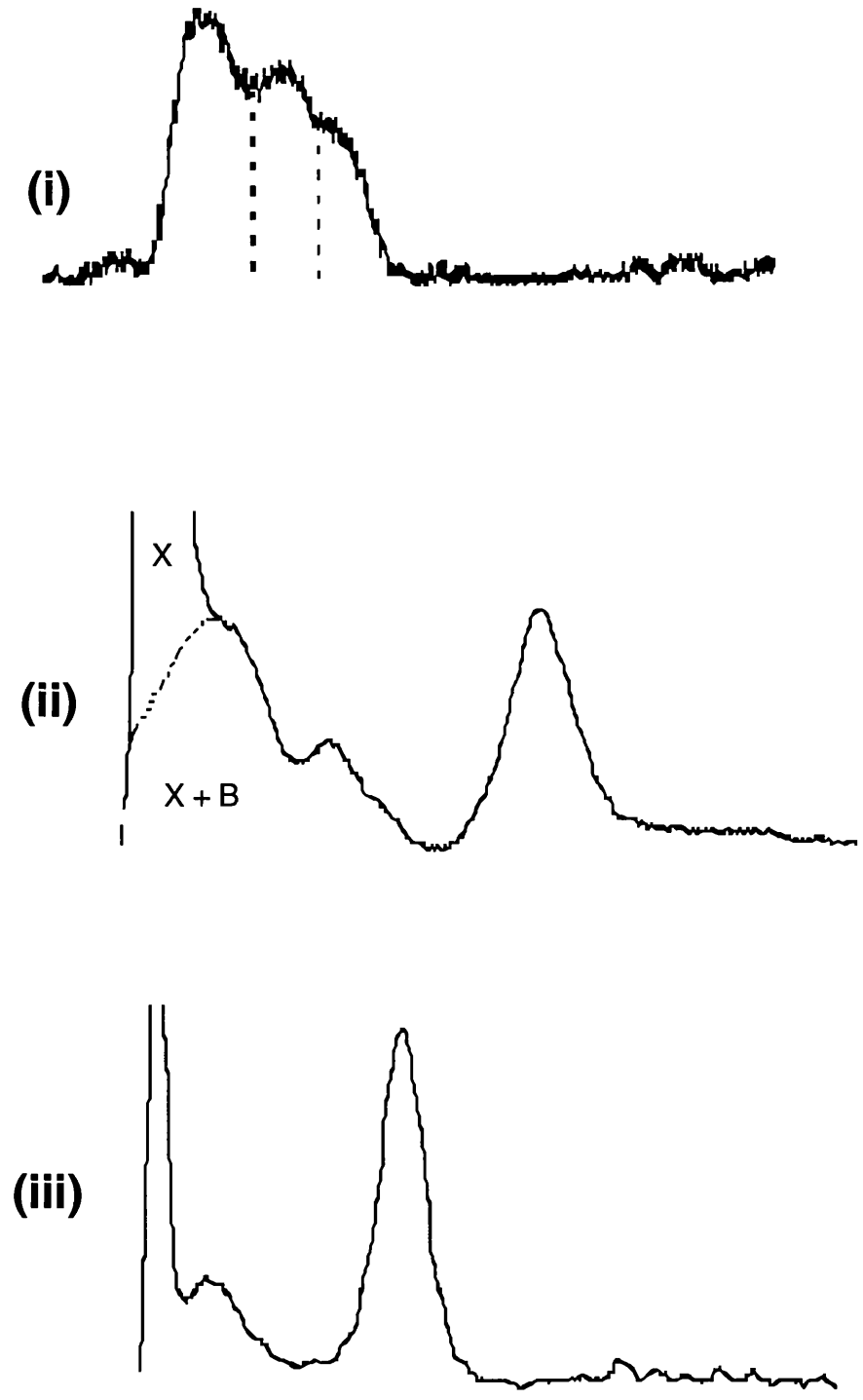


Fig.II-8: *The effect of length and shape of the column on the separation of hexosaminidase isoenzymes* **(i)** 5 mm x 4.6mm i.d **(ii)** 50 mm x 3mm i.d **(iii)** 20 mm x 6.4 mm i.d, **B**= the first hexosaminidase isoenzyme eluting on chromatography, **X**= artefact, the line in **(ii)** has been drawn to shows the peak shape assuming removal of **X**. The packing material was PL SAX 1000 and the rest of conditions as described in Fig.II-4.

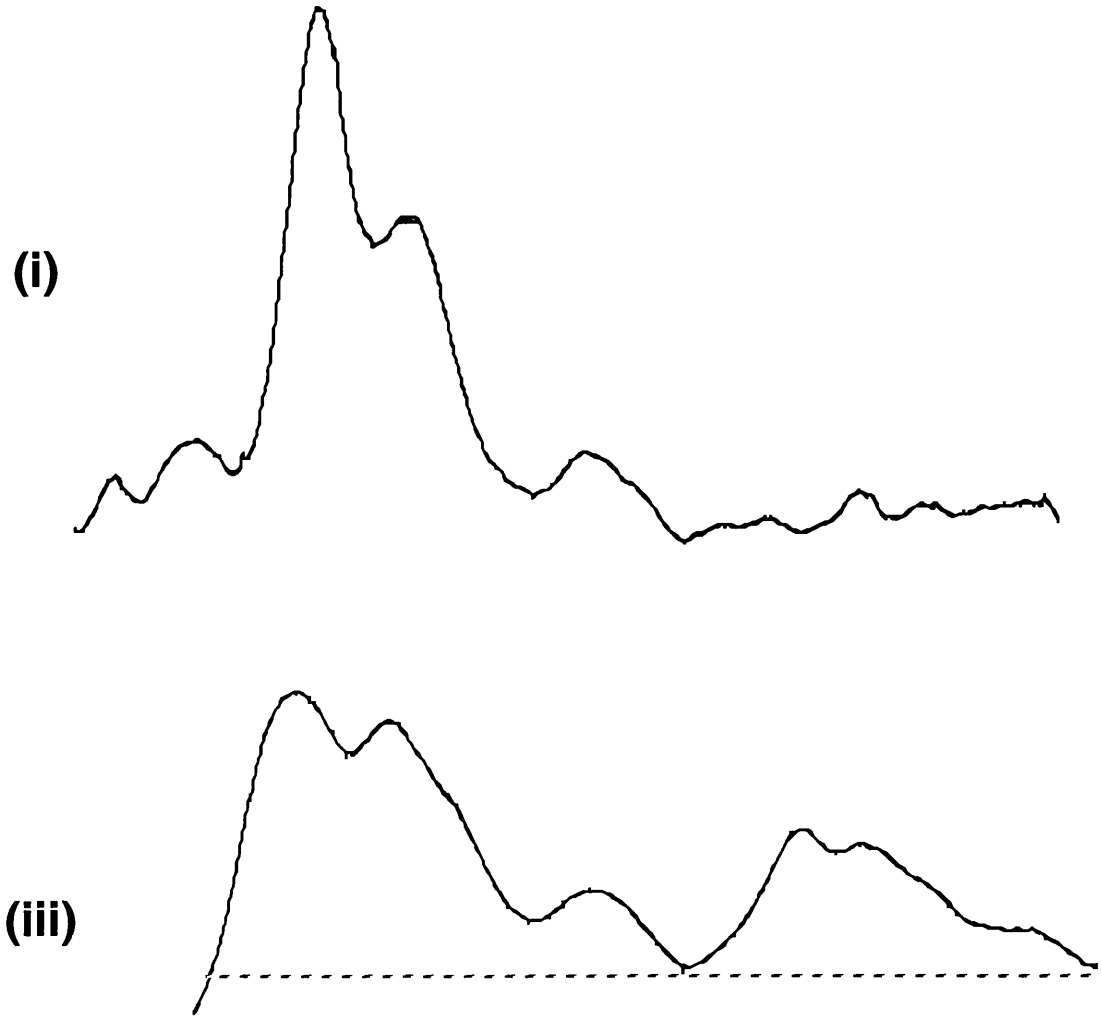


Fig.II-9: *The effect of the length of the column on separation of ALP isoenzymes from a normal serum*. **(i)**-5 mm x 4.6 mm, **(iii)**-20 mm x 6.4 mm. Details of analysis are given in Chapter IV.

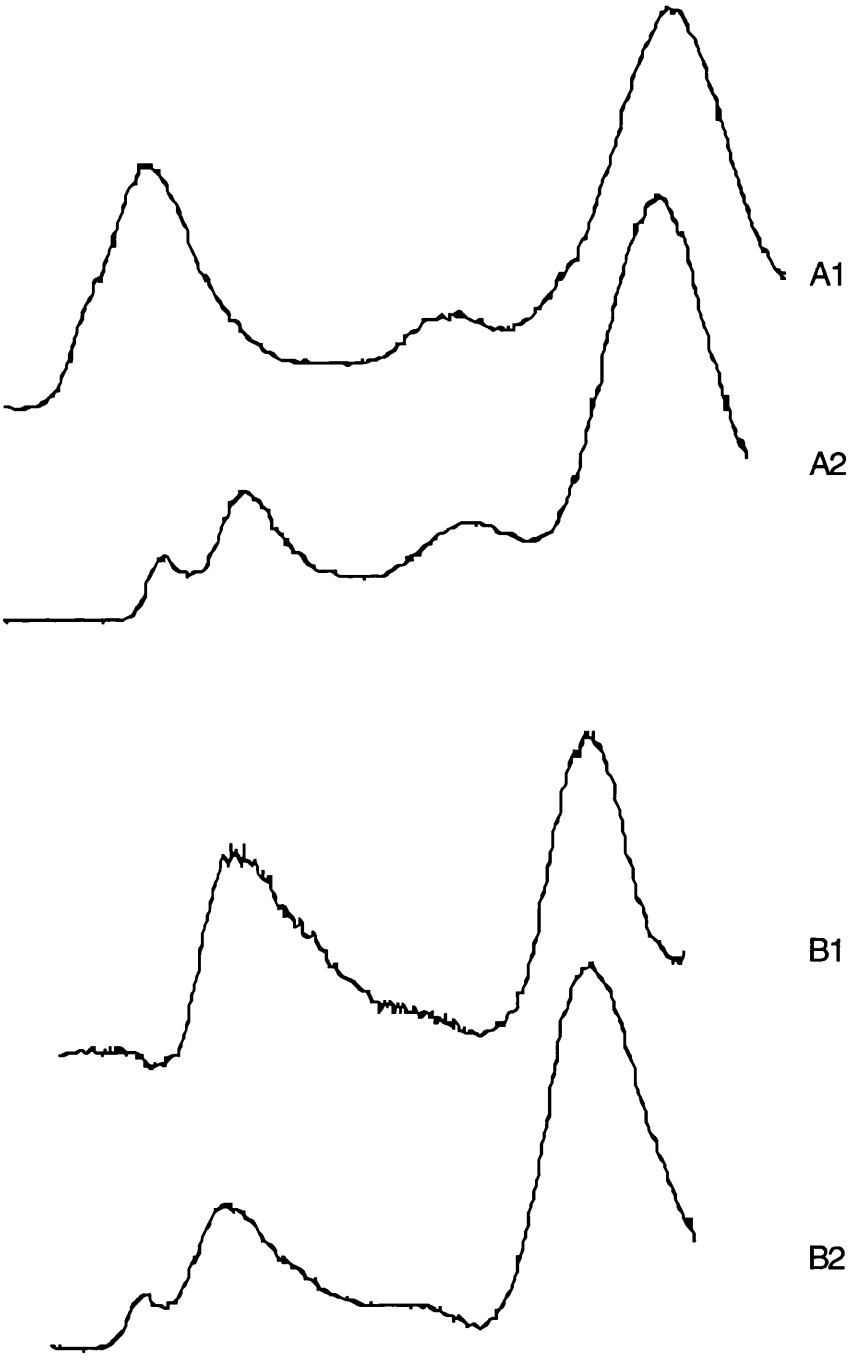


Fig.II-10: *Comparison of profiles of hexosaminidase isoenzymes analysis obtained with different flow rates.* A1= normal serum analysed at 0.6 ml/min, A2= same sample at 0.4 ml/min, B1= normal fibroblast at 0.6 ml/min, B2= same sample at 0.4 ml/min. The packing material was PL SAX 1000, the rest of the conditions as described in Fig.II-1.

6 – Gradient

In general two kinds of gradient can be envisaged based on changing pH or salt concentration. Reproducible pH gradients are traditionally difficult to obtain, as the pH can change by absorption of carbon dioxide from the atmosphere. Such changes would also cause interferences in the post-column detection used in this work which is pH-dependent. A salt gradient was therefore chosen for this study.

Every couple protein/packing material has its own optimal gradient. In this study gradients were established by comparing different step gradients empirically. Some illustrative results are presented in Fig.II-11 for the separation of ALP isoenzymes using AX 300. The same basic studies were repeated with all packing materials and ALP (see Appendix A for more examples). Based on this experience typically three gradients were compared for the separation of the other proteins (hexosaminidase and transferrin) using the most appropriate packing material. The main conclusion was that in most cases a gradient with many short steps gives a better resolution.

7 – Detection

In this work detection methods have been used where the selected isoenzymes or isoform can be detected in the presence of other proteins. This approach was chosen in preference to complicated sample preparation procedures aimed at removing interfering proteins. Sample purification often results in a poor recovery and can include time consuming steps (such as dialysis) without always providing a sufficiently pure sample (cf. transferrin, Chapter V).

ALP, like hexosaminidase and many other enzymes, can convert fluorometric substrates, which allow for specific and sensitive detection superior to colorimetry when small samples volumes are used [Shoenau et al, 1988].

UV detection is non-destructive to the sample and commonly used but not specific, as a number of proteins show absorbance at the typical wavelengths of 280nm or 254nm (see Fig II-12). Transferrin represents 5-10% of all proteins in plasma, and therefore quantification would be difficult at 280 nm. Co-chromatography of isoforms with other protein would also go undetected. In this study the detection of transferrin was based on the fact that iron bound to transferrin absorbs at 460 nm [Jeppsson et al, 1993].

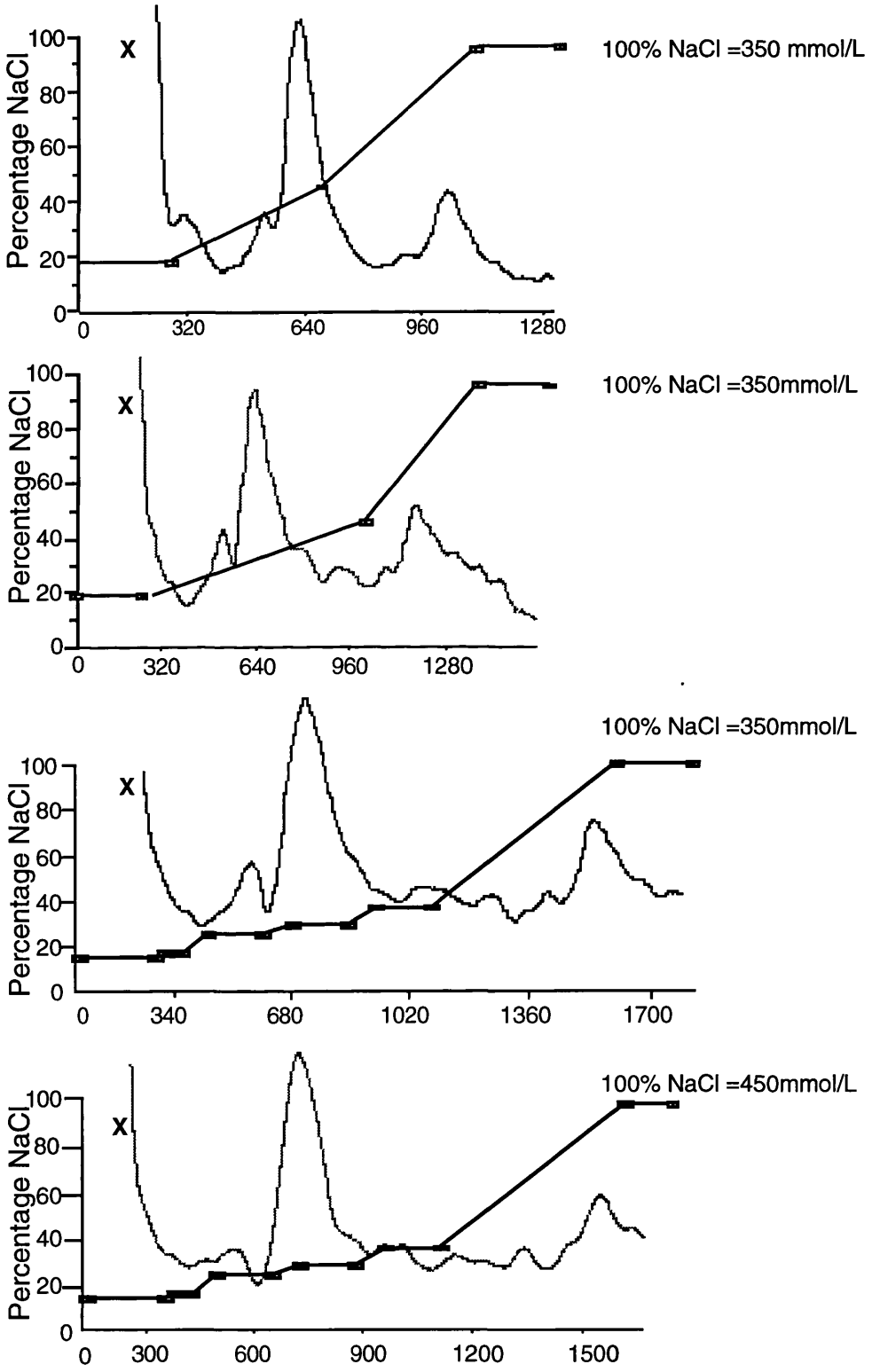


Fig.II-11: *Effect of varying the gradient profile on the separation of ALP isoenzymes on AX 300.* On the gradient profiles, 'x' axis represents Time (sec) and the 'y' axis represents percentage of buffer B, X= artefact.

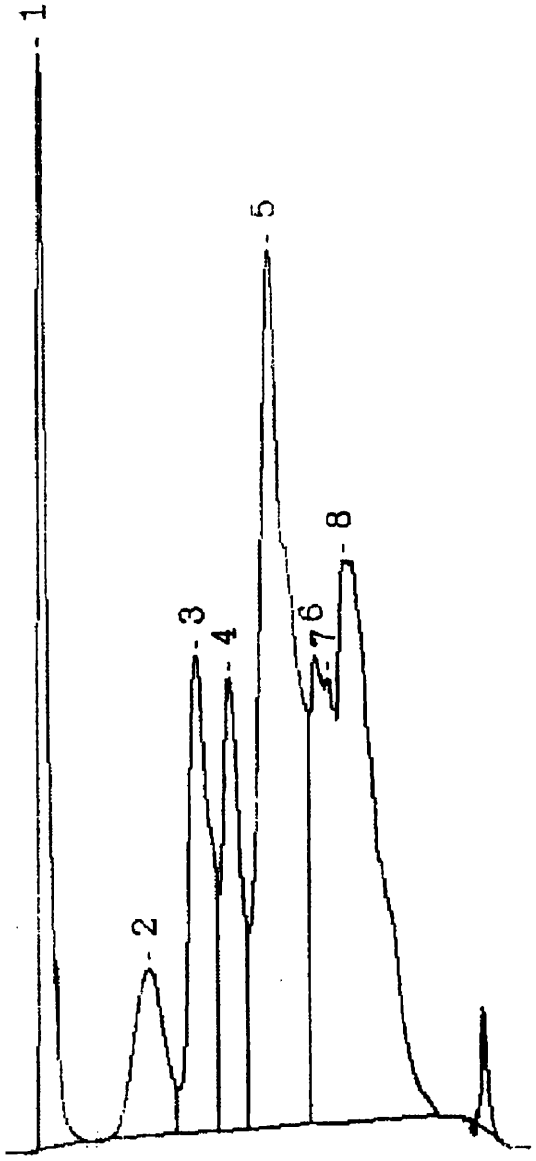


Fig.II-12: *MCC analysis of neat serum onto PL SAX detected at 280 nm.* Conditions as in Chapter V. 1= IgG, 2= transferrin, 3 and 4= haptoglobin and α2 microglobulin, 5-8= albumin. This pattern was deducted from the ones obtained with chromatography of urine protein on Mono Q column [Suzuki et al, 1985].

8 – Conclusion

Some features which are of importance for the use of MCC have been identified in this chapter and are summarised below.

(i) Technical features of GMAT and G15:

The artefactual peaks produced by GMAT may be accentuated by the use of post-column detection, as the substrate stays in the incubation coil at 37°C for about 45 seconds undergoing an endogenous breakdown which results in a raised signal. As will be shown in subsequent chapters this artefact impaired the resolution every time an isoenzyme was eluted in the void volume and this resulted in impaired quantitation. An attempt was made to reduce this effect by the use of base-line subtraction but this approach was not always successful.

It was noted that shallow gradients were not handled as well by the G15 as by the GMAT. This resulted in decreased reproducibility between chromatograms, and was thought to be due to the lack of a mixing chamber. The GMAT forms a gradient by constantly pushing solutions through syringes which work at different speeds to give the correct proportions. The solutions are rapidly mixed in a short wide piece of tubing placed between the outlet of the syringes and the inlet of the column. On the G15, the gradient is formed by a valve rotating between channels A and B, the mixing being made in the pump tubing. This kind of system is not reproducible with steps < 10% as the mixing is not adequate. Thus the choice between the use of GMAT or G15 depended on the type of protein to be analysed.

(ii) Post-column detection: a simple incubation system using a teflon coil was shown to be adequate for detecting isoenzymes activity.

(iii) Choice of a packing material: the Polymer Laboratories PL SAX 1000 and the Pharmacia Resource Q column were found to be the most appropriate material for the applications chosen in this thesis.

(iv) Flow rate: Better resolution was obtained with a slow flow rate and was thought to be related to the size of the proteins studied. Although this experimental work was carried out only with hexosaminidase isoenzymes, the outcome was assumed to be true for alkaline phosphatase and transferrin as well.

Chapter III: Separation of hexosaminidase isoenzymes

A – Introduction

β-hexosaminidase (E.C. 3.2.1.52) is a lysosomal enzyme which hydrolyses terminal non-reducing N-acetylglucosamine and N-acetylgalactosamine residues in gangliosides, oligosaccharides, mucopolysaccharides and glycoproteins. In 1968, Robinson and Stirling identified two isoenzymes, hexosaminidase A (Acid) and hexosaminidase B (Basic), so-called because of differences in pH optima. It was subsequently shown that these are oligomers with a subunit in common (β) and a subunit specific to hexosaminidase A (α). Later another isoenzyme, hexosaminidase S, composed only of α subunits, was found in tissues from patients with a lysosomal storage disorder called Sandhoff disease.

Other isoenzymes I1, I2, C, and P (Intermediate, neutral and Pregnant) are also found in tissues and body fluids but seem to be isoforms of hexosaminidase A and B.

1 – Biochemistry

1–1 Biosynthesis

As already mentioned (Chapter I) the two subunits α and β are coded by different genetic loci. However, the two subunits show substantial homology (57%) in their primary structure.

The biosynthesis of these subunits can be summarised in four steps. It starts with the synthesis in the rough endoplasmic reticulum of a prepropolypeptide containing a signal peptide [Mahuran et al, 1988]. Then post-translational modifications begin in the ER, amino acids are removed, oligosaccharides added, disulphide bonds formed leading to 'pro-subunits' called pro- α and pro-β respectively [Weitz and Proia, 1992]. In the Golgi the pro-sequence are removed from pro-α and pro-β, and pro-β undergoes some internal cleavage (of a tetrapeptide and a tripeptide from hexosaminidase A and B respectively) leading to the formation of the precursor forms of the subunits. Finally, after transport to the lysosomes, some specific proteolytic processing takes place to produce the mature forms: α, a single polypeptide and β composed of two polypeptides, βa and βb (see Table III-1).

Table III-1 : *From precursor to mature subunits*

Subunit	Precursor	Mature form
α	67,000	54,000
β	63,000	2 x 29,000

N.B. The molecular weights of the units are only approximate since glycosylated proteins do not always migrate in SDS electrophoresis as theoretically predicted [Hassilik and Neufeld, 1980].

1–2 Structure of hexosaminidase isoenzymes

Hexosaminidase A can be defined as $\alpha(\beta_a\beta_b)$ and hexosaminidase B as $(\beta_a\beta_b)2$ [Mahuran et al, 1988]. Hexosaminidase I and P have intermediate pI and electrophoretic mobility, the serum form of hexosaminidase I runs close to hexosaminidase B on electrophoresis [Sandhoff et al, 1989], whereas the purified form from liver tissue resembles hexosaminidase A [Dewji et al, 1986]. Although α and β are synthesised in equal amounts they dimerise at different rates. $\beta\beta$ dimerise in the endoplasmic reticulum but the location for $\alpha\beta$ is still unknown [Neufeld, 1989].

1–3 Glycosylation and phosphorylation

Glycosylation is a prerequisite for hexosaminidase activity but is not site-specific. The α subunit usually contains three glycosylated sites at Asn^{115} Asn^{157} Asn^{295}. The total absence of glycosylation alters the folding of the peptide and induces its retention in the lumen of the ER caused by the formation of insoluble disulphide linked complexes [Weitz and Proia, 1992]. No residual activity is found associated with a subunit and its mobility is comparable to that in cells treated with a glycosylation inhibitor. If two N-glycated sites are removed, the decrease in enzyme activity is substantial; if only one site is deficient, the activity is almost normal but the mobility of the subunit is increased.

The β subunit contains four N-linked oligosaccharides at Asn^{84}, Asn^{142}, Asn^{190}, and Asn^{327}. There is a similar relationship between the degree of glycosylation and enzyme activity as for the α subunit.

Phosphorylation usually follows the glycosylation of a protein and requires a specific tertiary conformation. For the hexosaminidase isoenzymes each subunit is phosphorylated on a specific site, at Asn^{295} for the α chain and at Asn^{84} and Asn^{327} for the β chain. If the phosphorylation is abnormal the circulating form of hexosaminidase will increase in parallel with a decreased mature lysosomal form [Sonderfeld and Proia, 1989].

1–4 Kinetics

It is known that hexosaminidase A (α ($\beta a\beta b$)) and hexosaminidase B $(\beta a\beta b)_2$ have different substrate specificities. Although α and β are not active as monomers, they are active as precursor forms and the possibility of two different catalytic sites acting in concert has been suggested [Neufeld, 1989]. In the α subunit Arg^{211} and Arg^{178} have been shown to be 'active' residues, (i.e. they are a part of the catalytic site) but do not contribute to the substrate binding. The subunit is specific for negatively-charged

substrates like oligosaccharides with a penultimate charged residue or with a negative charge on the terminal N-acetylglucosamine [Brown & Mahuran, 1991].

Hexosaminidases are specific for N-Acetylglucosamine and N-Acetylgalactosamine in β anomeric linkages, with the nature of the aglycone being usually of little importance. *In vivo* they catalyse the metabolism of a variety of glycoconjugates such as gangliosides (G_{M2}, G_{A2} and globoside), glycosaminoglycans and protein-derived oligosaccharides. Oligosaccharides with a negative charge on the terminal N-acetylglucosamine are a substrate for hexosaminidase A and S only.

Both hexosaminidase A and B have identical kinetic constants and a pH optimum at 4.5 against water-soluble substrates but when a negatively charged oligosaccharide is used as a substrate hexosaminidase A and S have a pH optimum at 3.8. The Michaelis constant for 4-methylumbelliferyl- and p-nitrophenyl-β–D-N-acetylglucosaminide are the same for the three isoenzymes A, B, and S and are usually in the range of 0.3 to 1 mmol/L [Sandhoff et al, 1989].

2 – Hexosaminidase and lysosomal storage disorders

2–1 Tay-Sachs disease

A mutation in the α subunit results in variant B or Tay-Sachs disease [Sandhoff et al, 1989]. Tay-Sachs disease is panethnic, but is more common within the Ashkenazi Jews. The carrier frequency in this community in the USA (detected by enzyme assay) is 1 in 27 compared with 1 in 250 in other races. The most common mutations are described in Table III-2.

The formation of hexosaminidase A and S is blocked and only the unaffected β subunits dimerise to form hexosaminidase B. In the absence of hexosaminidase A the G_{M2} gangliosides can not be hydrolysed and accumulate in lysosomes, especially in neuronal tissues. This leads to progressive neurological deterioration.

There is a great variability in the clinical expression of Tay-Sachs disease, and it can be classified into four types: infantile, juvenile, adult-onset, and hexosaminidase A deficiency with normal phenotype. The main clinical features of Tay-Sachs disease are described in Table III-3.

In general, a complete deficiency of enzymatic function is associated with the infantile form – characterised by onset of symptoms in infancy, rapid progression and death before the age of 4 years. The juvenile cases, described for the first time in 1968 by Bernheimer

Table III-2: *The main mutations involved in Tay-Sachs disease*

Population	Deletion	Biochemical phenotype
Ashkenazi	*Splice junction mutation at 5' end of intron 12– accounts for 20-30% of infantile cases *4 bp insertion at exon 11 – accounts for 70% of infantile cases *G to A nucleotide substitution on exon 7, induces a reduction of hexosaminidase A – adult onset [Trigg-Raine et al, 1990]	Presumed to affect RNA nuclear-cytoplasmic transport, processing or stability
French-Canadian	7.6 Kb deletion at the 5' end of the α subunit.	idem
Italian families	Mutation affecting the intracellular transport.	α polypeptide not phosphorylated, not associated to β subunits, not secreted and not converted to mature form.
Mutation at the catalytic site	Single base change such as Arg^{178}->Hist	Results in changes of the secondary structure

Table III-3: *Clinical features of the three main types of Tay-Sachs disease*

Infantile	• mild motor weakness 3-5 months • progressive weakness, hypotonia, poor head control and decreasing attentiveness 6-10 months • unusual eye movements, apparent staring episodes, cherry-red spot • macrocephaly by the age of 1.5 to 2 years
Juvenile	• motor ataxia between 2 and 6 years • progressive dementia with loss of speech, increasing spasticity and seizures • loss of vision occurs much later and finding of cherry-red spot is not consistent
Adult onset	• spinocerebellar and lower motor neurone dysfunction are the most prominent features • psychosis, often with episodes of depression • vision and intelligence remain unaffected in most of the cases

and Seitelberger, have very similar features to the infantile form but a high residual activity is generally found leading to a slower accumulation of gangliosides with delayed onset of symptoms. The adult onset is the most clinically variable type and in many cases cannot be distinguished from chronic G_{M2} gangliosidosis (a more indolent form, patients still living in their third or fourth decade). The last case relating to α subunit deficiency, (not described in the table), is hexosaminidase A deficiency with normal phenotype. The 'patients' have a very low hexosaminidase A level when tested in serum and leucocytes with synthetic substrates. They are usually identified in their forties or fifties and have been completely asymptomatic [Sandhoff and al, 1989]

2–2 Sandhoff disease

In contrast to Tay-Sachs disease, Sandhoff disease is rare within the Ashkenazi Jews. The incidence of heterozygotes and the predicted birth rate depends on the community as shown below.

Incidence		*Birth rate*
Jewish	1/500	1/1,000,000
Non Jewish	1/278	1/ 309,000

Sandhoff disease, or variant 0, results from a deficiency of the β subunit which is common to both hexosaminidase A and B. The residual hexosaminidase activity measured in patients with Sandhoff disease is due to the presence of hexosaminidase S.

A partial deletion of the gene, plus a normal or reduced level of mRNA, may be responsible for this deficiency. In the juvenile form of the disease patients have 1-3% of hexosaminidase A activity which is sufficient to delay the onset of the symptoms [Sandhoff et al, 1989].

Clinically the infantile picture is almost identical to that of Tay-Sachs, the distinguishing feature being mainly the ethnic background of the child. Occasional bony deformities, organomegaly, foamy histiocytes in the bone marrow and the presence of N-acetylglucosamine oligosaccharides in urine have been reported. The juvenile form is much rarer and only a few cases have been described between three and ten years old. They usually have a virtual absence of hexosaminidase B and a profound reduction of hexosaminidase A which induces slurred speech, cerebellar ataxia, progressive psychomotor retardation, increasing spasticity, hypertonia and mental deterioration. A few cases of adult Sandhoff disease have also been reported [Thomas et al, 1989].

B – Development and assessment of a micro column chromatography method for the separation of hexosaminidase isoenzymes

1 – Aim

The purpose of the work in this chapter was to develop a MCC method for the separation of hexosaminidase isoenzymes applying the conditions established in Chapter II, and to assess the performance of this method by comparison with electrophoresis and isoelectric focusing.

2 – Materials

2–1 Chemicals

Sodium dihydrogen orthophosphate (NaH_2PO_4), sodium chloride (NaCl), disodium orthophosphate (Na_2HPO_4) and citric acid (BDH Chemicals, Poole Dorset, UK).

Dimethyl sulfoxide (DMSO), D-sorbitol and glycine from (SIGMA, Poole Dorset, UK).

Agar Noble from DIFCO laboratories(USA), agarose ME (SEAKEM, Flowgen, Sittingbourne, UK) IEF agarose (Pharmacia, Milton Keynes, UK) Nitro-cellulose (Sartorius, Epsom, UK) and Resolytes 4-8 (BDH Chemical, Poole, Dorset, UK).

2–2 Substrate

4–Methylumbelliferyl–2–acetamido–2–deoxy–β–D–glucopyranoside, molecular mass 379.4 (Melford Laboratories, Ipswich, UK).

2–3 Packing materials

DEAE SEPHACEL (Pharmacia, Milton Keynes, UK) and PL SAX 1000 (Polymer Laboratories, Shropshire, UK).

2–4 Samples

2–4–1 Origin of the samples

Sera and leucocytes from teenagers – age 14 to 18 years – who had been screened for carrier status of Tay-Sachs disease, were kindly supplied by the Clinical Neurochemistry Laboratory, Institute of Neurology and Neurosurgery, London.

Sera and fibroblasts from cases of adult Sandhoff, infantile Tay-Sachs and Sandhoff disease were kindly provided by the Enzyme Laboratory, Institute of Child Health, London.

2–4–2 Sample preparation

Skin fibroblasts were trypsinised, washed twice with 0.9% saline, condensed as a pellet and frozen at -20°C. The pellet was thawed and sonicated in 50 µl of water prior to analysis. Leucocytes were prepared in two stages: (i) the red cells were lysed with hypotonic buffer and the preparation washed with saline and frozen at -20°C, (ii) the pellet was resuspended in 0.9% saline, sonicated, and 1% Lubrol added to lyse the cell membranes and, after spinning down, the supernatant was used for analysis.

Sera were diluted one in ten, fibroblasts or leucocytes one in twenty, with 10 mmol/L sodium dihydrogen orthophosphate buffer, pH 6.00 (buffer A – see below); initial dialysis against buffer A did not change the elution profile.

3 – Micro column chromatography (MCC)

3–1 Method

3–1–1 Buffers

3–1–1–1 Sodium phosphate buffer

Buffer A was NaH_2PO_4, 10 mmol/L, pH 6.00 ±0.02.

Buffer B was A plus NaCl 0.3 mol/L, pH 6.00 ±0.02.

Care was taken to achieve the same pH for each batch of A and B to avoid the formation of a pH gradient in addition to the salt gradient. A stock of NaH_2PO_4 0.5 mol/L was prepared as this proved more stable, and diluted to 10 mmol/L for daily batches. After checking the pH the buffer was passed through a 45 µm filter.

3–1–1–2 McIlvaine buffer

Prepared according to McIlvaine, 1921.

Stock solutions, 0.1 mol/L citric acid and 0.2 mol/L dibasic sodium phosphate were prepared in advance and stored at 4°C. 540 ml of citric acid were mixed with 460 ml of dibasic sodium phosphate and the pH adjusted to 4.5. 50 ml aliquots were stored at -20°C until required.

3–1–1–3 Glycine NaOH

1 mol/L Glycine NaOH pH 10.4 was prepared, kept at room temperature and diluted to 0.5 mol/L as required.

3–1–2 Substrate

A 2 mg/ml solution of 4–Methylumbelliferyl–2–acetamido–2–deoxi–β–D-glucopyranoside in McIlvaine buffer was prepared in batches of 50 to 100 ml.

3–1–3 System

A 'continuous' flow system was set up according to the principle shown in Figure III-1. This system is a modification of the one developed by Ellis et al in 1975. The column used by Ellis et al was 45 mm x 3 mm i.d (capacity of 318 µl) packed with a DEAE slurry and the post-column was an air-segmented incubation coil. The method developed during this work is based on a smaller column (capacity of 83 µ l) and presents improvement in two respects:

(i) The nature of the packing material used, i.e. PL SAX 1000. Ellis (and others) had to have two columns ready in order to get a continuous analysis as the DEAE has to be discarded after the analysis of a single sample.

(ii) Automation: because of the improved packing material, time-consuming sample preparation (dialysis against loading buffer to equilibrate the sample) can be avoided and the column can be regenerated automatically between each sample.

The amount of sample injected was determined by the length of the loading loop and was found to be 60 µl.

3–1–4 Equipment

Two different systems were used, both of which were modified versions of commercial instruments (Drew Scientific, Chiswick, London) described in details in Chapter II. They were adapted for the analysis of hexosaminidase isoenzymes by adding an extra multi-channel peristaltic pump in order to run the enzymatic reaction at pH 4.5, followed by addition of Glycine NaOH pH 10.4 to increase the pH rapidly for the fluorometric detection (see Fig. III-1).

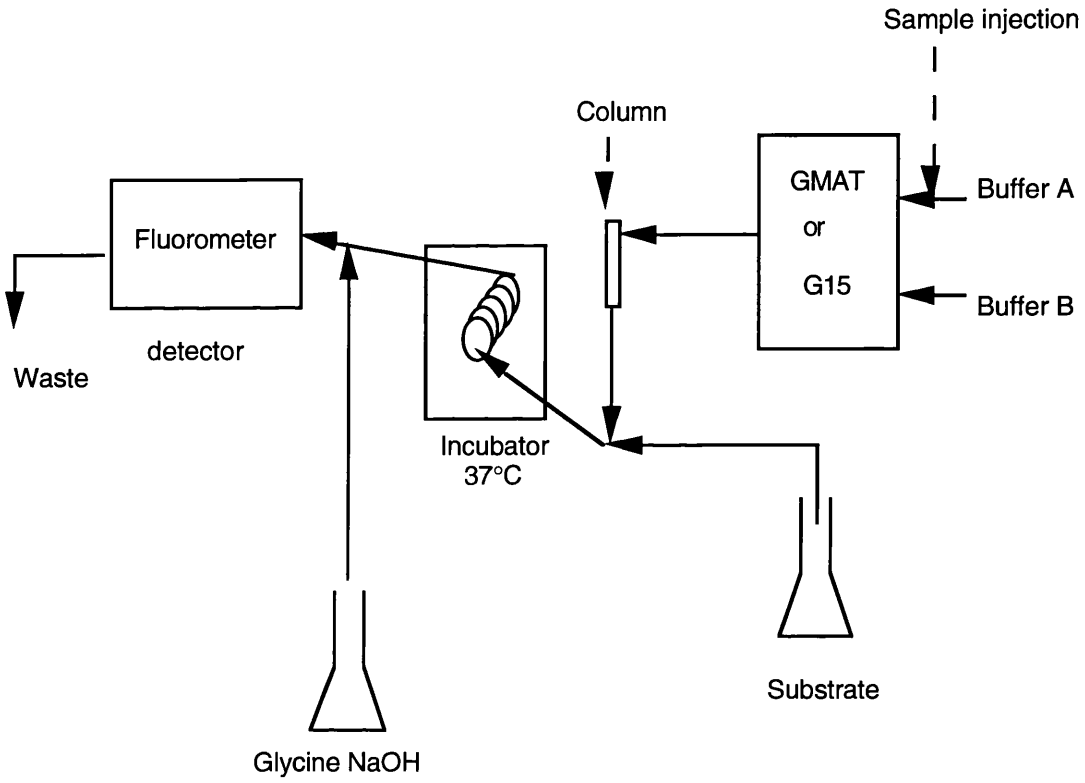


Fig.III-1: *The equipment used for the separation of hexosaminidase isoenzymes.*
Buffer A= NaH_2PO_4 10 mmol/L pH= 6.0, Buffer B= buffer A plus 0.3 mol/L NaCl pH 6.0. Substrate= 4-methylumbelliferyl-2-acetamido-2-deoxi-β-D-glucopyranoside. Fluorometer settings: λexc= 365 nm, λem= 455 nm.

The general procedures used on GMAT and G15 were as similar as possible in order to compare the performances of the two systems. However, some modifications had to be made which are specified and discussed in this chapter.

3–1–5 Gradient

The final gradient used for the analysis of hexosaminidase isoenzymes on Glycomat (GMAT) was as follows:

Time (sec)	% buffer B	flow rate (ml/min)
0	15 (45 mmol/L)	0.4
150	15	0.4
300	26.66 (80 mmol/L)	0.4
1100	55 (165 mmol/L)	0.4
1350	100 (300 mmol/L)	0.4
1800	100	0.4

The final gradient used for the analysis of hexosaminidase isoenzymes on G15 was as follows:

0	10 (30 mmol/L)	0.55
90	10	0.55
240	26.60 (80 mmol/L)	0.55
600	55 (165 mmol/L	0.55
700	100 (300 mmol/L)	0.55
1000	100	0.55

3–1–6 Post-column detection

The substrate solution was added to the eluate from the column at a flow rate of 0.1 ml/min for the GMAT and at 0.15 ml/min for the G15 system. Eluate from the column plus substrate were incubated for 4 min at 37°C in the incubation coil before adding Glycine NaOH (0.2 ml/min) for 10 sec in order to make the product from the enzyme reaction fluorescent.

A fluorescent detector (Shimadzu, RF 535) with the following settings, λ_{exc} 365 nm and λ_{em} 455 nm, was used to monitor the reaction.

3–1–7 Quantification of peaks

A software package (DS 4000, Drew Scientific) was used for the quantification of the peaks. This programme allowed base line subtraction.

3–1–8 Heat denaturation

A modification of a previously described method [O'Brien et al, 1970], was used for identification of hexosaminidase isoenzymes:

Sera diluted 1:10 in McIlvaine buffer (see below) were incubated at 50°C for 3 hours, and then dialysed overnight against phosphate buffer, 10 mmol/L pH 6 ± 0.02.

The dialysate was centrifuged at 2000g and the supernatant analysed manually or by micro column chromatography.

3–2 Results

3–2–1 Analytical performance of the MCC method

3–2–1–1 Optimising the gradient

Initially a linear gradient was used on the GMAT system separating only hexosaminidase A and B in normal sera and fibroblasts (see Fig.III-2). Thereafter the elution profile was modified to increase the resolution. The outcome is shown in Fig.III-3, where a gradient as described under Methods has been used. Three peaks in the 'region' of hexosaminidase A plus a peak for I2 were resolved whereas I1 and B were thought to be merged as one peak because of problems in subtracting the initial artefactual peak, labelled X on the figure, which appeared with all gradients. As already mentioned in Chapter II, this peak was inherent to the mechanics of the GMAT (syringes stopping and restarting) and unfortunately was not sufficiently reproducible to be eliminated by base-line subtraction, which resulted in problems identifying the first peaks eluted.

This gradient, considered to be optimal for the GMAT system, was also tried on the G15 system but was found to give non-reproducible chromatographic patterns. Fig III-4 shows two chromatograms from the same sample run consecutively. The patterns for the 'A type' are different and a reproducible integration of the chromatogram would be impossible. This was thought to be due to a too shallow gradient to be handled in a reproducible manner by the valves of the system (see Chapter II). Thus a steeper gradient was developed (described in the method section) and the outcome is shown in Fig III-5.

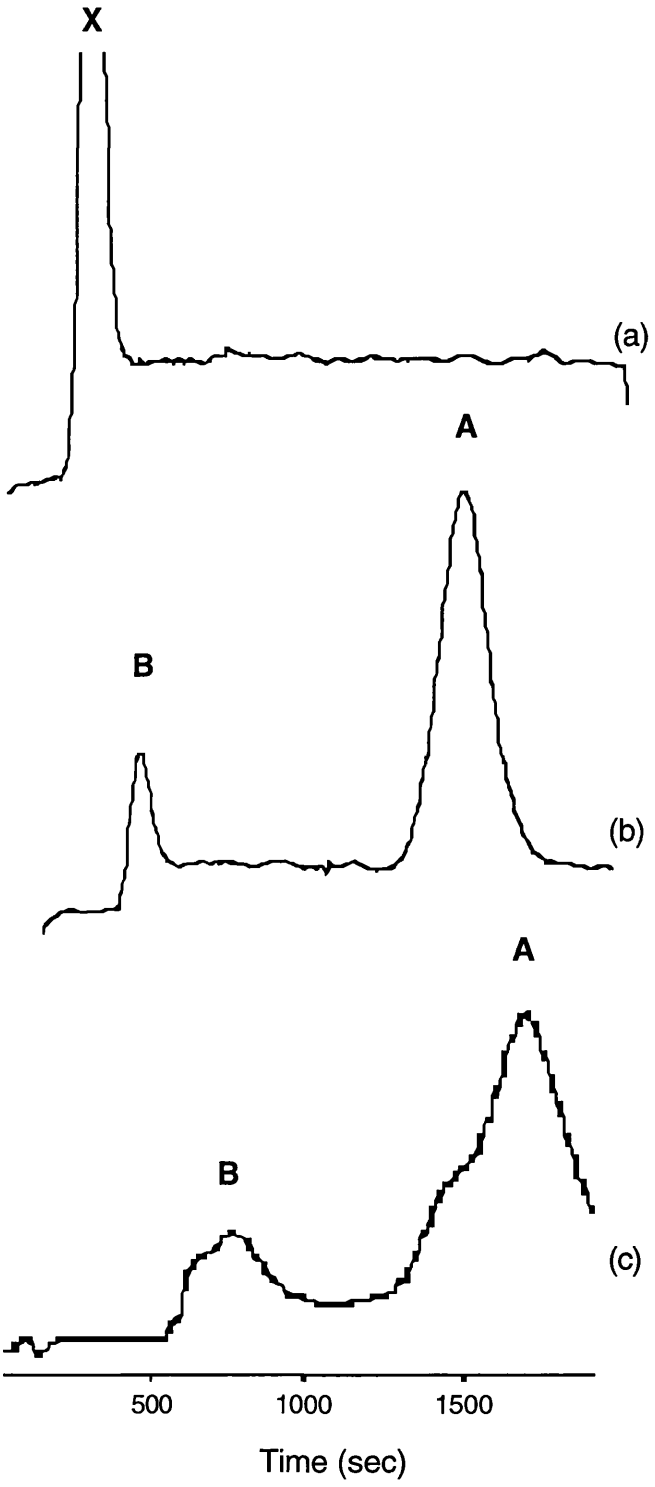


Fig.III-2: *Typical elution profile with a linear gradient on GMAT.* a-= Base line obtained by injecting buffer A, b= normal serum, c= normal fibroblast– traces b and c are shown with base line subtraction. **A**= Hexosaminidase A, **B**= Hexosaminidase B, **X**= artefact.

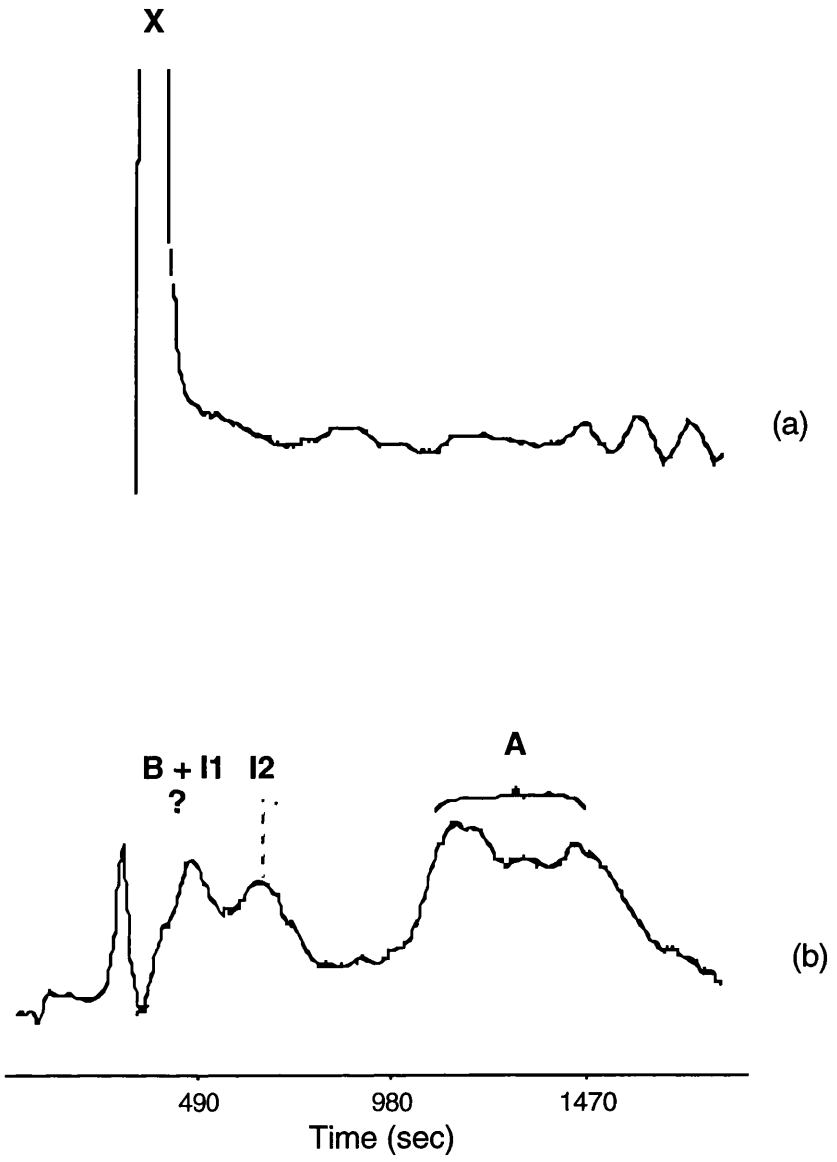


Fig.III-3: *Elution profile with a step gradient on GMAT.* a = Base line, b= normal serum. **X**= artefact, **A**= hexosaminidase A, **B**= hexosaminidase B, **I1** and **I2** hexosaminidase I1 and I2.

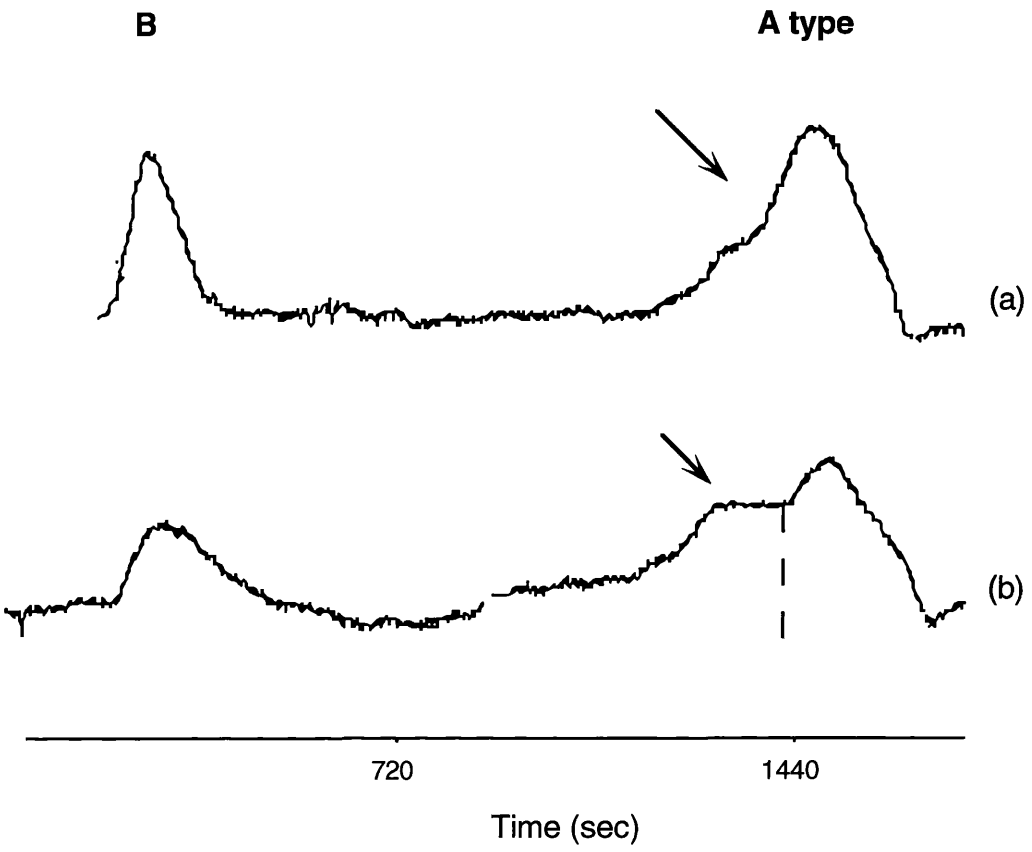


Fig.III-4: *Elution from G15 using the GMAT gradient*. a and b= same normal serum run consecutively. The arrows indicate the differences within the A type hexosaminidase.

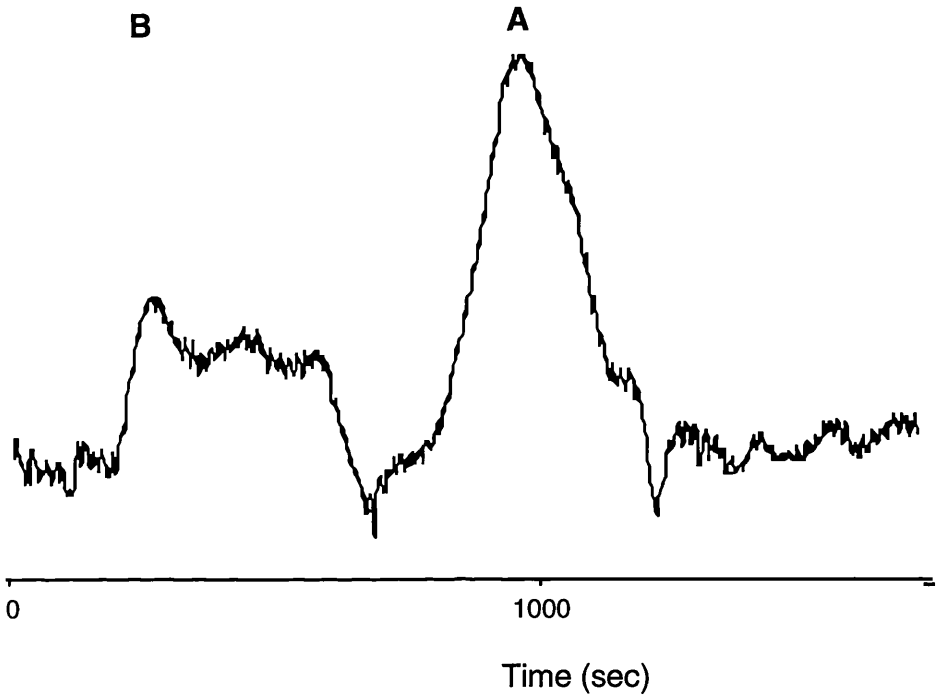


Fig.III-5: *Elution profile on G15 after modifications of the gradient corresponding to that under methods.* **A**= hexosaminidase A, **B**= hexosaminidase B.

A comparison with Fig.III-3 shows that hexosaminidase A is resolved as one peak instead of three, but B, I1 and I2 are now properly separated. This pattern was considered to give adequate information for the purpose of this study (relative percentage of hexosaminidase A to detect carrier status of Tay-Sachs or Sandhoff disease).

3–2–1–2 Specificity

The nature of each peak was determined by running heat denatured serum (hexosaminidase A is labile, B and I heat resistant under the experimental conditions used), and by comparing the chromatogram with the one of a serum from a woman in the last trimester of her pregnancy. The latter is known to have an elevated hexosaminidase I (called hexosaminidase P) helping to confirm the position of the intermediate peaks (see Fig.III-6).

3–2–1–3 Retention times (RT) for hexosaminidase isoenzymes

The separation of hexosaminidase isoenzymes offered an opportunity to compare the reproducibility of retention times between GMAT and G15 and to evaluate their respective imprecision. Hexosaminidase B and A only, were studied with G15 as the integration software could not always resolve the isoenzymes I1 and I2.

(i) Long-term variability: Two series of random samples, (n= 18 for GMAT and n= 26 for G15) were used to study the variability of the RTs for the peaks over a period of five days. The results are summarised in Table III-4 for the two sets of equipment.

On the GMAT, the standard deviations for the RTs were found to increase with the run time (see Table III-4 and Fig.III-7). However, SDs for the relative RTs (RT for hexosaminidase A divided by other respective RTs) were found to be small (from 0.03 to 0.06 sec – see Table III-5) corresponding to CVs of approximately 2% (see tables III-4 and III-5).
On the G15, the two SDs for the RTs of Hex.B and Hex.A were found to be similar (14.09 and 13.51 sec) but the CV for Hex.B was 3.6 times larger than for Hex.A.

(ii) Variation of retention times using a single column: Fig.III-8 shows the variations of the retention times in one batch of 17 samples. Table III-6 shows the mean RT, the SDs and CVs for three batches of samples (respectively 11, 17, and 11 samples) run on a single column over three days.

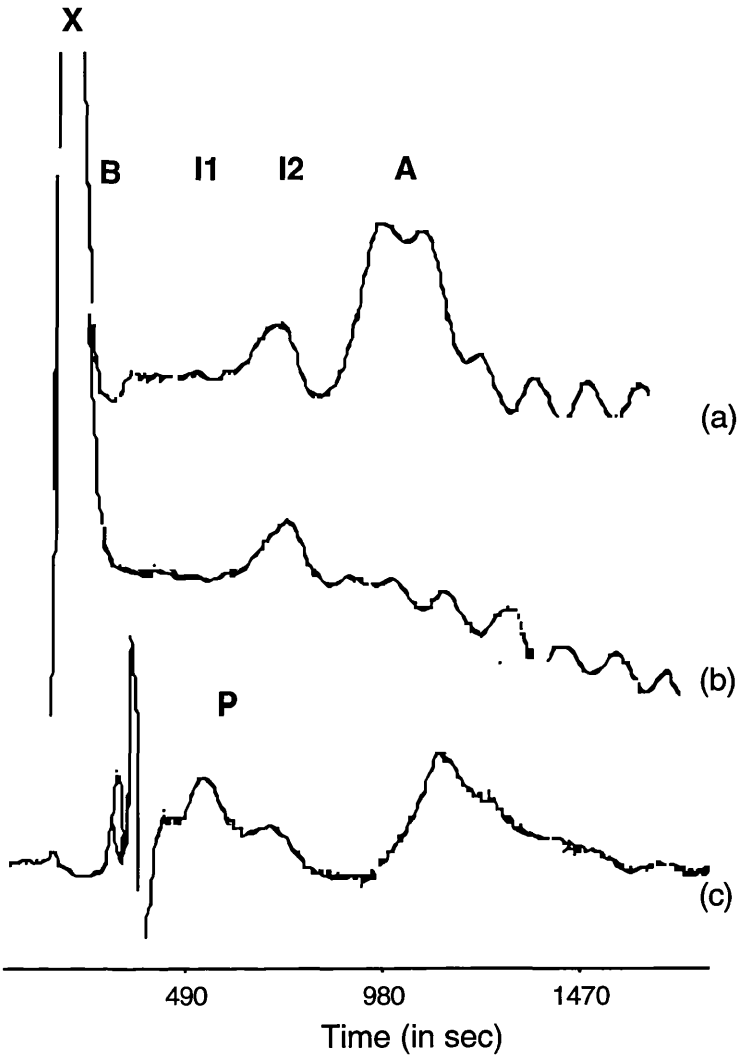


Fig.III-6: *Identification of the hexosaminidase isoenzymes.* a= Normal serum, b= heat denatured serum, c= serum from a pregnant woman. **X** = artefact, **A** = hexosaminidase A, **B**= hexosaminidase B, **I1**= hexosaminidase I1, **I2**= hexosaminidase I2, **P**= hexosaminidase I in pregnant women.

Table III-4: *Retention times (in seconds) for hexosaminidase isoenzymes on the GMAT and the G15.*

	Hex B	Hex I1	Hex. I2	Hex.A
GMAT (n=18)				
Mean	495.6	661.3	974.9	1214.2
SD	5.7	6.3	14.6	22.4
CV	1.85	1.5	0.95	1.15
G15 (n=26)				
Mean	282.3	-	-	957.7
SD	14.09	-	-	13.51
CV	4.99	-	-	1.41

Table III-5: *Variations of relative retention times on the GMAT.*

	Hex A/Hex B	Hex A/Hex I1	Hex A/Hex I2
Mean	2.45	1.83	1.25
SD	0.06	0.04	0.03
CV(%)	2.25	2.16	2.32

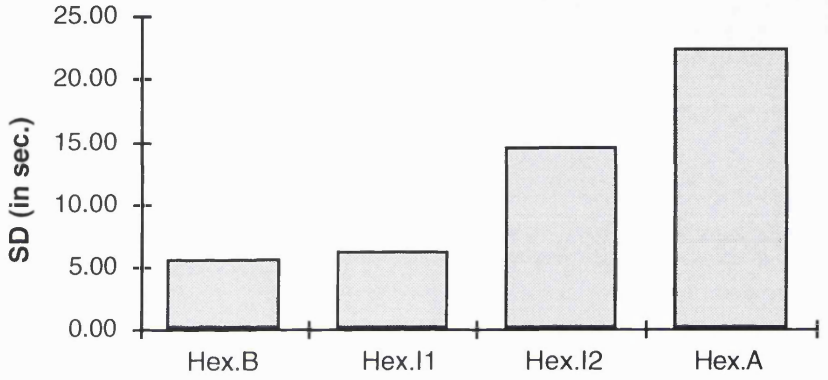


Fig.III-7: *Variations of the retention times for hexosaminidase isoenzymes on the GMAT.*

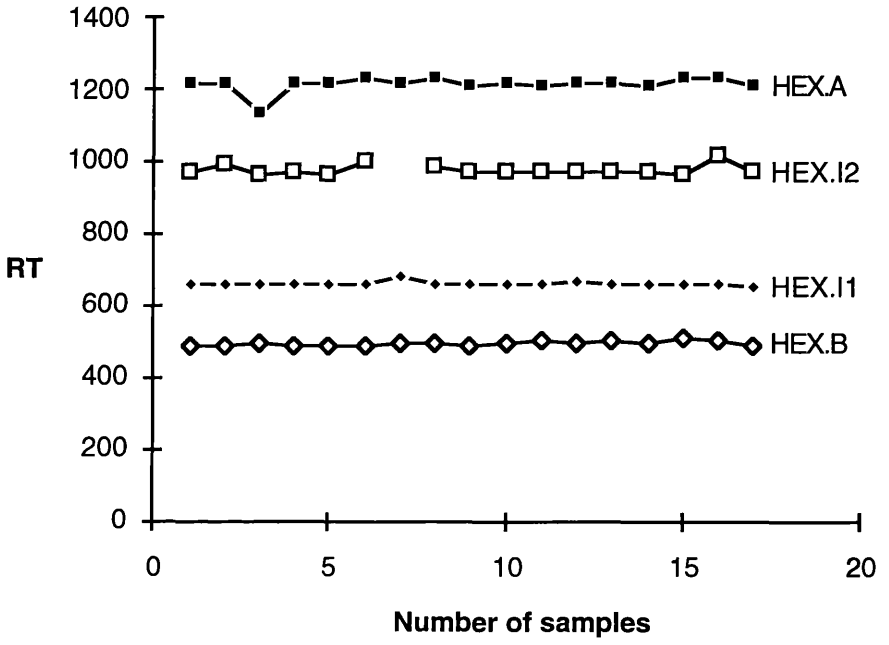


Fig.III-8: *Reproducibility of RT (in sec) on 17 samples within one batch on GMAT.*

Table III-6: *Imprecision on GMAT calculated from three batches of samples (n=11, n=17, n=11), using one column.*

	Hex A	Hex I2	Hex I1	Hex B
Mean (sec)	1252.67	853.33	613.00	464.67
SD (sec)	43.39	145.61	66.57	38.76
CV (%)	3.46	17.06	10.86	8.34

The CVs were found to be 3.46%, 17.06%, 10.86% and 8.34% for Hex. A, I2, I1, and B respectively. A test of the variation of retention times for hexosaminidase A comparing day 1, 2 and 3 showed some significant differences as the probability for two samples to be from the same population was less than 0.02, (for data see Appendix B1). Fig.III-9 clearly shows a number of outliers for hexosaminidase A (RT= 1447 sec, 1458 sec and 1461 sec) and a general decrease in retention time for hexosaminidase I1, I2 and B for day three. The ageing of the column was therefore thought to be one of the reasons for the imprecision obtained for the retention times of hexosaminidase isoenzymes. However this should affect the analysis on both GMAT and G15.

(iii) Conclusion: A few reasons were envisaged to explain the variations of RTs. Firstly, the sampling rate of the computer was the same during the total length of the run and it is known that the ability to measure the point of inflexion of a peak in a reproducible manner decreases with the run time. Secondly, it is known that a step gradient produces less reproducible results than an isocratic run and the gradient used for the separation of hexosaminidase isoenzymes in this study has many steps.

When the two system were examined individually, it was noticed that with the G15 the CV of the first peak was 3.6 times larger than the second peak suggesting that the first peak was not eluted in a repeatable way. Because of the peristaltic pump, the G15 system would take longer to equilibrate than GMAT and is more sensitive to any pressure changes during the loading phase; the tubes tend to dilate inducing non-reproducible changes in the flow rate and mixing likely to interrupt the steady flow. Another possible explanation is that the first peak seen on the GMAT is mainly a 'system peak', producing a falsely good reproducibility. Hexosaminidase B is the first peak to elute and therefore does not bind very strongly to the column, and these conditions are known to be the most difficult to obtain reproducible results. Answer to these questions could only be obtained from a detailed study of the mechanic and electronic parts of the systems used.

3–2–1–4 Imprecision (Data in Appendix B2)

Data characterising the analytical imprecision of the GMAT and the G15 are summarised in Table III-7. Data for the GMAT are limited because of the difficulties in quantitation of hexosaminidase B due to the artefact. At the time the work was done I anticipated that G15 would provide a better system for analysis and thus, limited time was spent on imprecision work using GMAT. A CV of 2.2% was however obtained for within-batch variations.

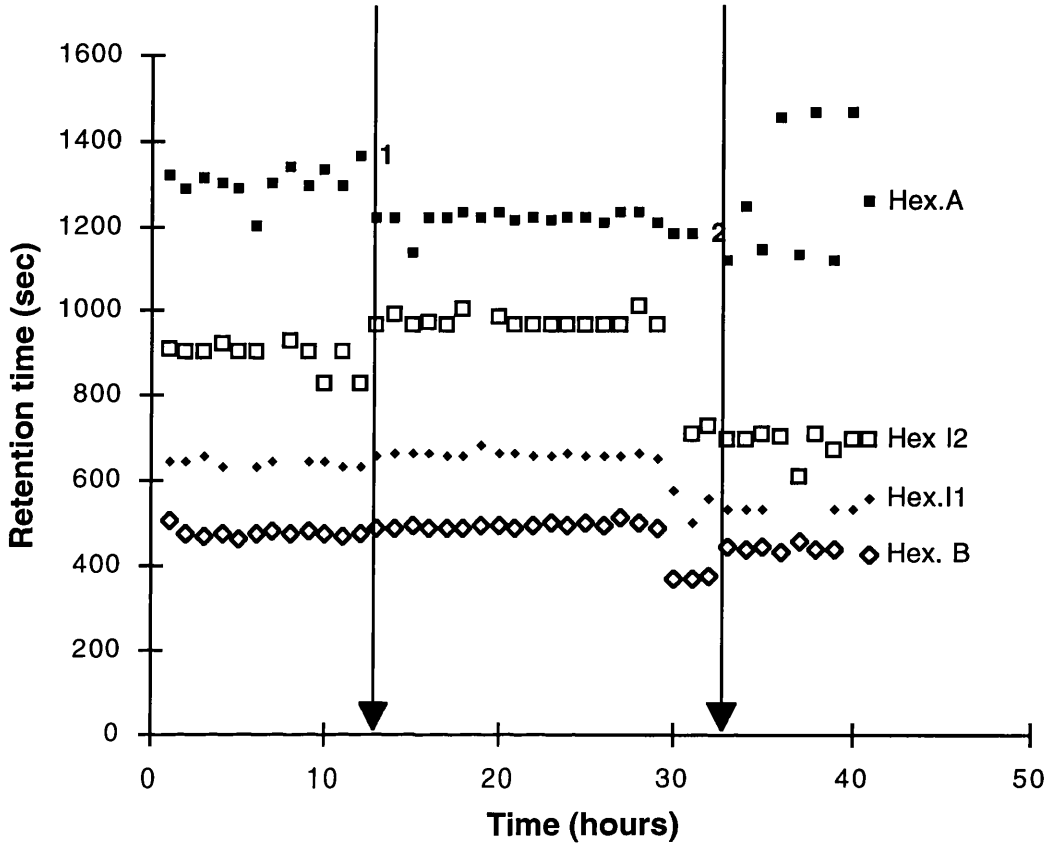


Fig.III-9: *Reproducibility of retention time for hexosaminidase isoenzymes on one column.* The arrows 1 and 2 indicate the end of days 1 and 2 respectively. Hex A= hexosaminidase A, Hex I1= hexosaminidase I1, Hex I2= hexosaminidase I1 and Hex B= hexosaminidase B.

Table III-7: *Imprecision of percentages of hexosaminidase A activity after GMAT and G15 analysis*

		n	Mean	SD	CV(%)
GMAT	Serum	14	89	2	2.2
Within day					
G15					
Within day	Serum	6	61.56	1.48	2.41
Normal	Leucocytes	10	71.64	4.12	5.75
Within day	Serum	9	49.39	4.65	9.41
Tay-Sachs carrier	Leucocytes	9	33.79	2.66	7.87
Between day	Normal serum	6	61.66	0.74	1.20

For G15, within-batch imprecision was obtained by running several sample in a consecutive manner, every cycle lasting 45 min (including regeneration of the column), no more than one batch was run at a time. The between day data were obtained by running the same serum (aliquots kept at -20°C) every day in the third position of a batch. Generally the imprecision found (CV= 2.41% for within-batch and 1.20% for between-batch) was comparable with many assays for routine analysis (e.g. total proteins), and with the electrophoresis values [Morris, 1994].

3–2–2 Analysis of clinical samples by MCC

Further validation of MCC was carried out by analysing a series of clinical samples.

3–2–2–1 Normal plasma and leucocytes

As can be seen on Fig.III-3 and III-5, normal plasma resolve in up to 5 peaks. The first one, hexosaminidase B, comes out in the void volume and can be seen only with proper base line subtraction. The second and third are hexosaminidase I1 and I2, and the last two peaks have been identified as 'A type'. The two A peaks are not resolved on electrophoresis. Some work would need to be done to clarify their meaning and their importance for carrier status detection.

3–2–2–2 Sample from patients with Tay-Sachs disease

Carriers of Tay-Sachs disease are detected because of the different ratio between hexosaminidase A and hexosaminidase B but the general chromatographic pattern is similar to that of normals.

In homozygotes for Tay-Sachs disease, hexosaminidase A has disappeared and the isoenzymes B, I1 and I2 constitute the enzyme activity as is demonstrated in Fig.III-10b. In fibroblasts and leucocytes, the relative proportion of hexosaminidase B seems increased compared to the pattern in normal subjects. In the two fibroblasts samples from patients with Tay-Sachs disease run on MCC there was also a peak in position of I1 absent in fibroblasts from heterozygotes for the disease (see Fig.III-11a).

3–2–2–3 Sample from patients with Sandhoff disease

Carriers of Sandhoff disease have low total activity and an abnormally high percentage of hexosaminidase A (over 80%). A typical pattern is shown in Fig.III-12a where hexosaminidase B or I were not detected.

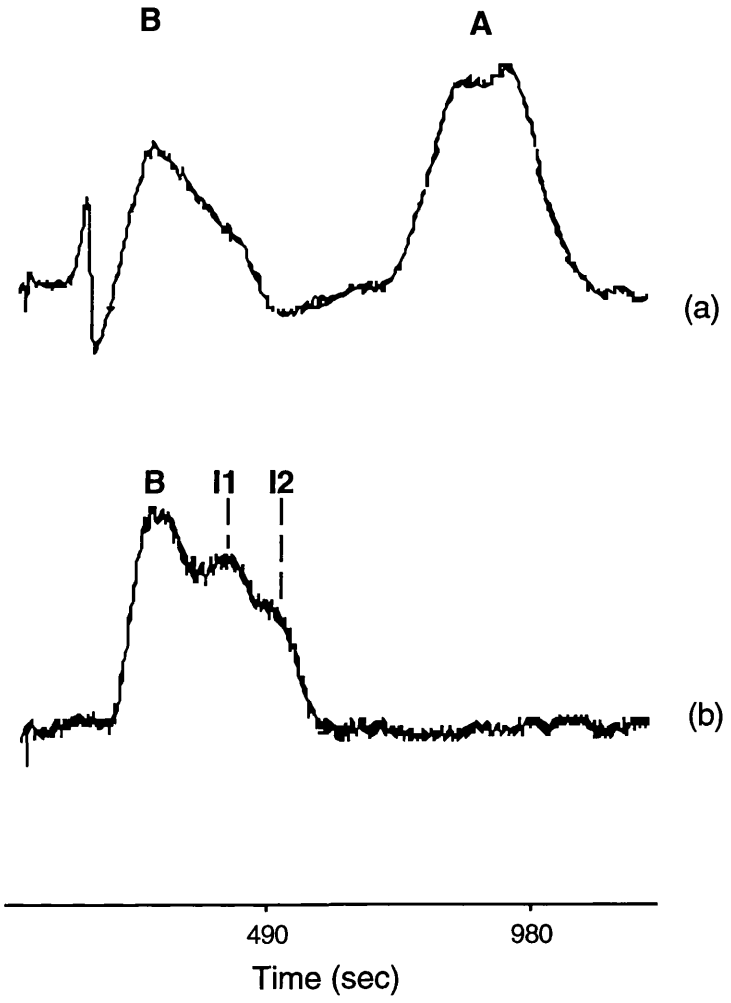


Fig.III-10: *Representative chromatograms of serum from a Tay-Sachs carrier and a patient homozygous for Tay-Sachs disease.* a= serum from a carrier of Tay-Sachs disease, b = serum from a child affected by Tay-Sachs disease. **A** = hexosaminidase A, **B**= hexosaminidase B, **I1** and **I2** = hexosaminidases I1 and I2.

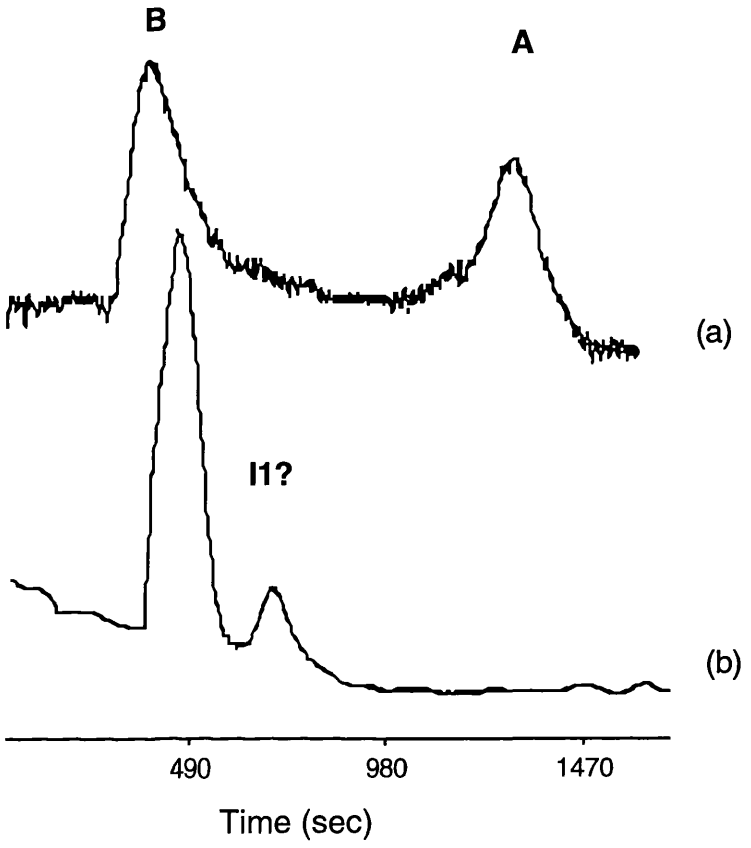


Fig.III-11: *Representatives chromatograms of Hexosaminidase isoenzymes from fibroblasts from a carrier and a patient homozygous for Tay-Sachs disease.*

a = fibroblasts from Tay-Sachs heterozygote, b = fibroblast from a child with Tay-Sachs disease.

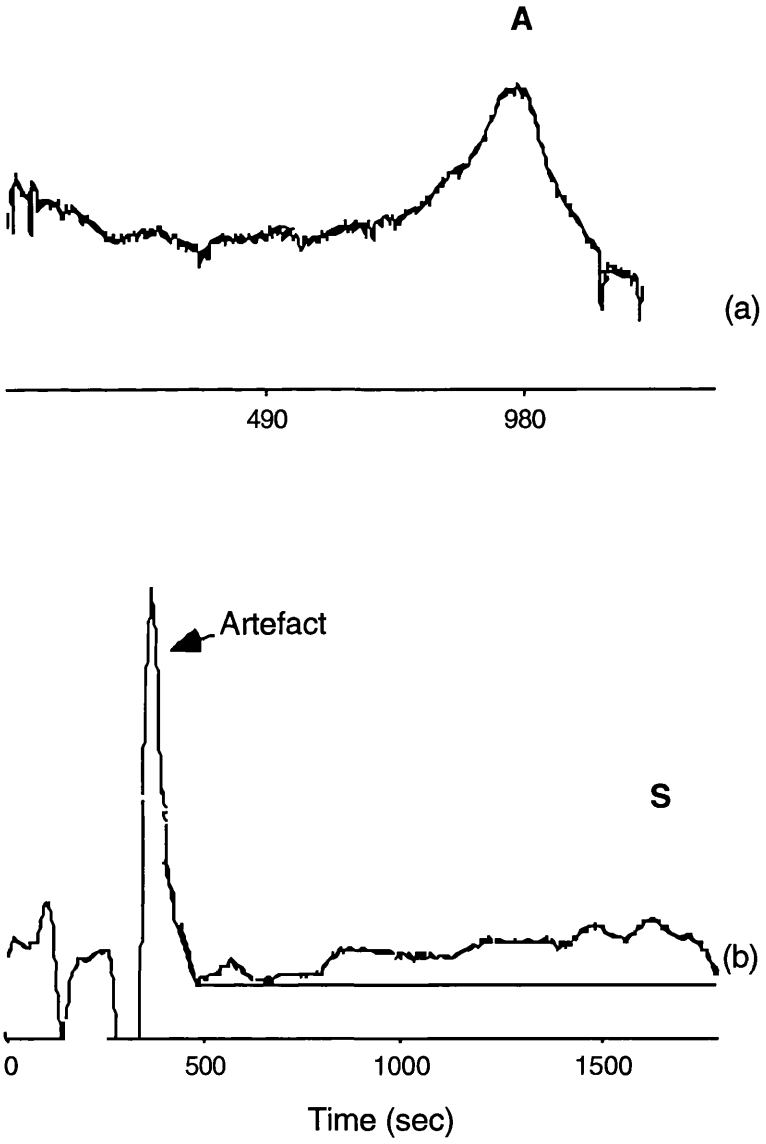


Fig.III-12: *Isoenzymes of hexosaminidase from the serum of a carrier and an infant homozygous for Sandhoff disease.* a= serum from an adult heterozygote for Sandhoff disease, b= serum from a case of infantile Sandhoff.

It was difficult to obtain a good chromatogram from plasma or fibroblasts from patients with Sandhoff disease because of the low enzyme activity (see Fig.III-12b). Better results were obtained in two cases of adult Sandhoff where the total activity was higher than in infantile cases. Hexosaminidase 'type A' and hexosaminidase S could be detected in fibroblasts (Fig. III-13a) but not in serum (Fig.III-13b).

4 – Electrophoresis of hexosaminidase isoenzymes

Electrophoretic separation of hexosaminidase isoenzymes was used as the reference methods as this procedure is the most commonly used for the separation of isoenzymes. A method developed by [Morris et al, 1994] was used.

4–1 Method

The samples used were as described in Section 2–4–1, and the method was as follows:

4–1–1 Solutions

Gel and running buffers were sodium barbitone, pH 8.6, 0.05 mol/L and 0.06 mol/L respectively. The substrate was 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-gluco-pyranoside 6 mmol/L in McIlvaine buffer, pH 4.5 (see Section 3–1–1–2). The fluorescent enhancement solution was Glycine NaOH/DMSO 50/50 (v/v) to reveal the fluorescence on the nitro-cellulose membrane.

4–1–2 Preparation of gels

Analytical gel: Agarose (1%) and sucrose (5%) in barbitone buffer, poured into a plate 220 x 125 x 0.5 mm.

Substrate gel: Mix 5 ml of boiled Agar Noble 2% with 5 ml of substrate, quickly pour a plate on a flat bed (220 x 125 mm) not earlier than 10 minutes before use.

4–1–3 Running conditions

Electrophoresis was run at 300 V constant voltage (about 70–80 mA) until marker (bromo phenol blue added to pooled normal sera) had migrated 4 cm from the application point.

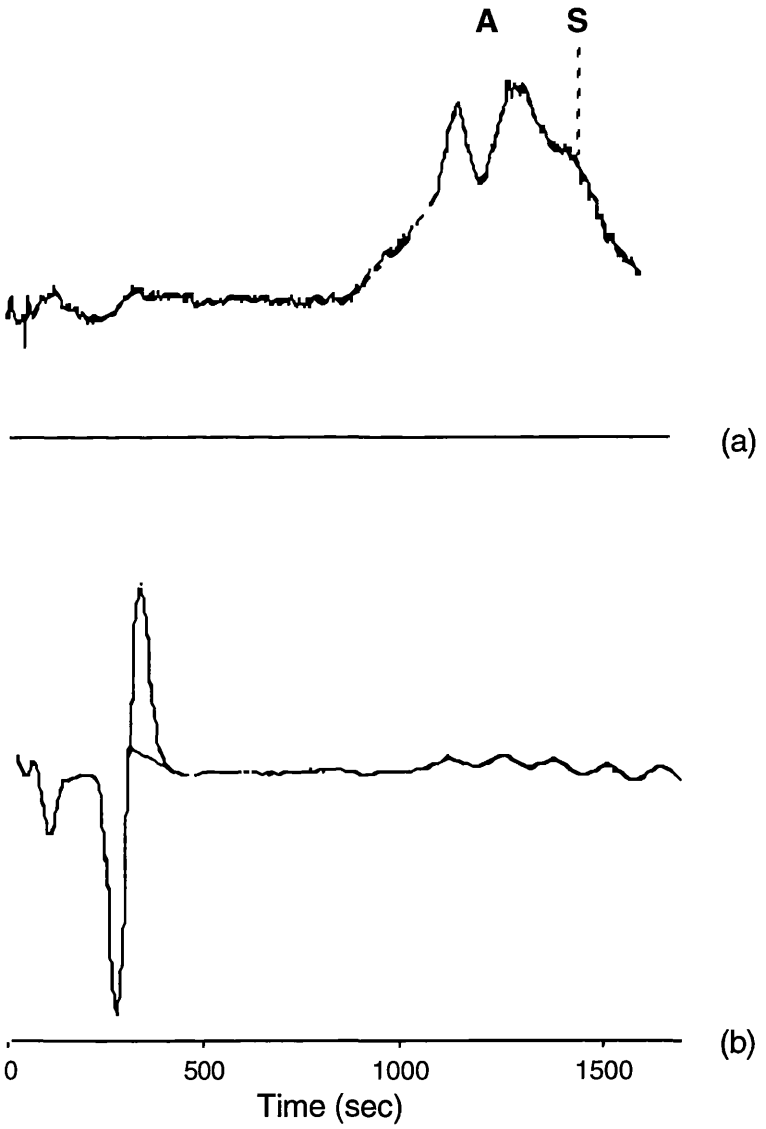


Fig.III-13: *Hexosaminidase isoenzymes from an adult affected by Sandhoff disease analysed by MCC.* a= fibroblast, b= serum, **S**= hexosaminidase S, **A**= hexosaminidase A. The negative inflexion at the beginning of the chromatogram is due to a shift in time of the artefact.

4–1–4 Procedure

Neat serum (2 µl) or leucocyte preparation(1 µl) were applied to the gel. After the marker had reached the 4 cm point, the gel was dried with a nitro-cellulose membrane and blotted onto another membrane for 30 minutes.

The protein blot was carefully layered on the top of the substrate gel and left in a damp box to incubate at 37°C for 1 hour.

A solution of Glycine NaOH/DMSO was prepared immediately before scanning (or photographing). The nitro-cellulose membrane was dipped into the solution, the excess of fluid blotted with some filter paper and the membrane rapidly exposed under a transluminescent U.V lamp for visual inspection of the bands or scanned with E.D.C Scanner (Helena Laboratories).

4–2 Results

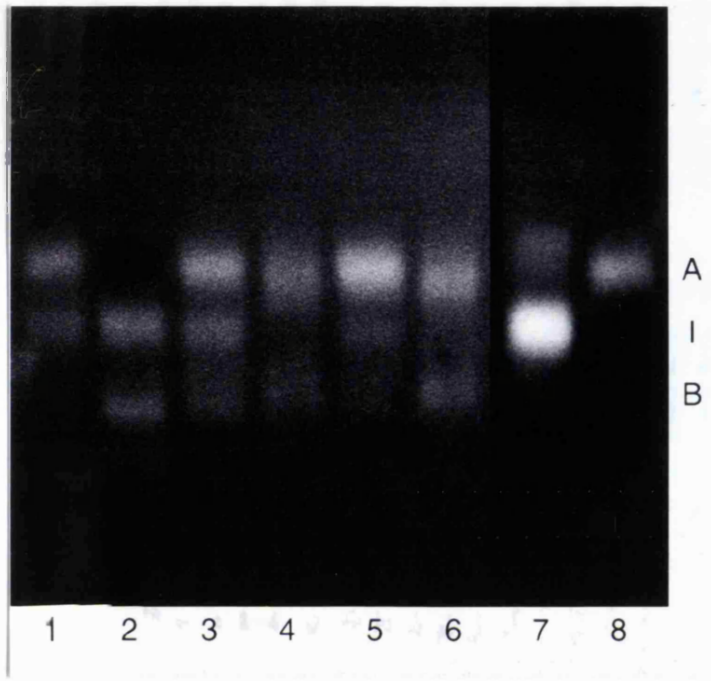
4–2–1 Analysis of clinical samples

The electrophoretic pattern contains three bands representing hexosaminidase A, I and B (Fig III-14). The identity of each band was established by using the sulphated substrate (which reacts preferably with α subunits) or heat denaturation [Morris et al, 1994]. The order of migration results from differences in charge between the isoenzymes and is therefore expected to be similar to the order of elution after chromatography The plates were scanned and the relative percentage of each isoenzymes calculated.

4–2–2 Analytical performance

The performance of this method was evaluated by participation in the International Tay-Sachs Disease Testing Scheme (San Diego, USA) which indicated that the method measures hexosaminidase A percentage adequately. Data in Table III-8 [from Morris, 1994] illustrate the analytical performance of the electrophoretic method. Both within and between day imprecision (CVs of 6.1% and 10.5% for serum from normals and Tay-Sachs carriers respectively), were considered to be acceptable for an electrophoretic method. The reference range for isoenzymes established by this method was:

Normal subjects	58–85% isoenzyme A
Carriers of Tay-Sachs disease	33–58% isoenzyme A
Inconclusive results	53–59% isoenzyme A
(where a definite diagnosis could not be made)	
Carriers of Sandhoff disease	71–86% isoenzyme A
(in combination with a low total activity)	



1-Normal serum;
2-Serum from patient with Tay-Sachs disease;
3-Serum from a carrier of Tay-Sachs disease
4-Leucocytes from a carrier of Tay-Sachs disease
5-Normal serum
6-Normal leucocytes
7-Serum from a pregnant lady
8-Serum from a carrier of Sandhoff disease

Fig.III-14: *Electrophoresis of hexosaminidase isoenzymes.* A= hexosaminidase A, I= hexosaminidase I, B= hexosaminidase B. The anode is at the top of the figure.

Table III-8: Coefficients *of variation obtained for the between-batch variations of values for hexosaminidase A analysed by electrophoresis*. [Morris et al, 1994].

	Type of sample	N	CV (%)
Normal	serum	51	6.1
Tay-Sachs heterozygote	serum	67	10.5

5 – Isoelectric Focusing (IEF)

An isoelectric focusing method was developed in an attempt to gain qualitative information for carriers of Tay-Sachs disease.

5–1 Method

5–1–1 Gels

0.3 g of IEF agarose and 3.6 g of D–Sorbitol were added to 27 ml of a 10% glycerol solution. This was boiled and equilibrated at 65°C. 2.5 ml of ampholytes solution (Resolyte 4–8) was then added and the mixture poured rapidly on an horizontal plate.

5–1–2 Running conditions

Electrolytes : 10% solution of Resolyte 4–8.

Electrical setting : 1250 V, 20 W, 150 mA, run for 1300 V/H.

5–1–3 Procedure

The same procedure as described earlier for electrophoresis was followed except that the samples were loaded on alternative lanes to avoid cross-contamination caused by diffusion of the fluorescent product.

5–2 Results

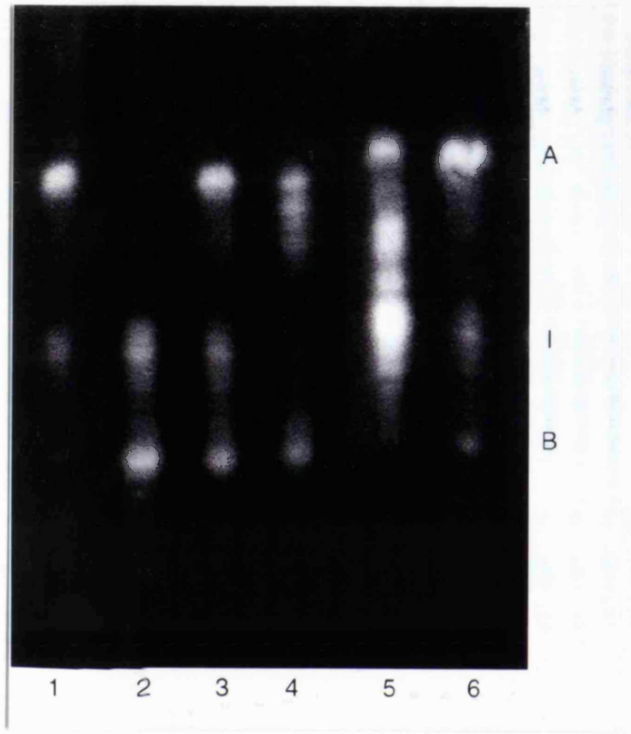
Isoelectric focusing was shown to resolve hexosaminidase isoenzymes in normal serum as follows (see Fig. III-15) :

2 bands for the A type

3 bands for the Intermediate type

1 tight band for the B type

The scanning and interpretation of such plates turned out to be difficult as the number of bands was so important. The main hope from focusing had been to develop an easier and more reliable way of detecting Tay–Sachs carriers by relying on qualitative information rather than a quantitation, which leads in some cases to inconclusive results. Unfortunately this could not be achieved during this study.



1-Serum from a normal adult

2-Serum from a patient with Tay-Sachs disease

3-Serum from a carrier of Tay-Sachs disease

4-Normal leucocyte

5-Serum from a pregnant lady

6-Normal serum

Fig.III-15: *Isoelectric Focusing of hexosaminidase isoenzymes.* A= hexosaminidase A, I= hexosaminidase I, B= hexosaminidase B.The anode is at the top of the figure.

6 – Method comparison between MCC and electrophoresis

6–1 The GMAT system

The percentage of hexosaminidase A in samples from healthy subjects and Tay-Sachs carriers was analysed by the GMAT system and electrophoresis. For MCC each sample was run once over a period of two days using the same buffers, gradient and column. Duplicate runs were carried out on the electrophoretic system and the average value was used for the method comparison. It was assumed that the sum of the 'A type' peaks obtained by MCC corresponds to the individual band observed on electrophoresis.

Because none of the two methods produces error-free results, Deming's regression was used for the comparison (the errors were assumed to be similar for the two methods). A scatter diagram is shown in Fig. III-16 and the regression line obtained was:

$$MCC = 6.6307 + 0.8907\ Electrophoresis \qquad (r=0.85, n=32, p<0.001)$$

The correlation coefficient of 0.85 was significant implying that MCC measures hexosaminidase A percentage with a positive bias compared to the electrophoretic method.

6–2 The G15 system

A similar comparison was carried out for the G15 system; a scatter diagram is presented in Fig.III-16. The regression line is:

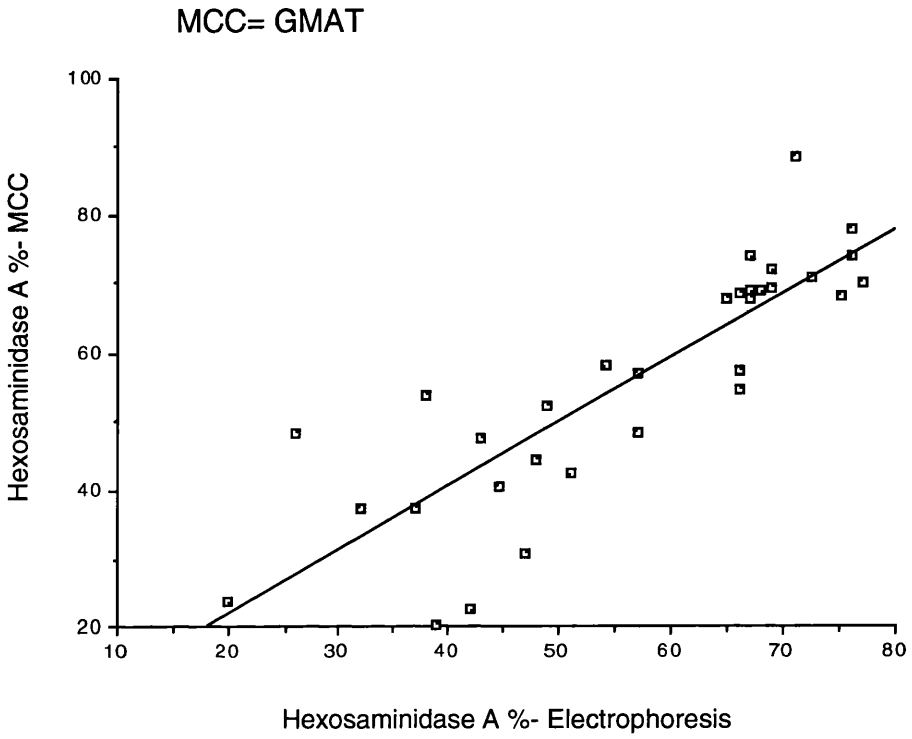
$$MCC = 1.7562 + 0.9140\ Electrophoresis \qquad (r=0.79, n=24, p<0.001)$$

The general outcome was similar to that for the GMAT system.

Samples from the International Tay-Sachs Disease Testing Scheme were received from the USA for blind assays by chromatography and electrophoresis. The percentage of hexosaminidase A obtained by both methods differed in most of the cases (see Appendix B3). However the clinical conclusions reached were the same in 14 of the 16 samples analysed using the electrophoresis range, which gives 87.5% success rate, and a similar correlation as previously described was obtained between electrophoresis and MCC (see Fig.III-17).

6–3 Qualitative comparison between MCC and electrophoresis

Electrophoresis gives three bands whereas the chromatography seems able to resolve more isoforms. Therefore the quantitation for each isoenzyme and isoform might be better and more informative using chromatography. With the equipment provided some mechanical



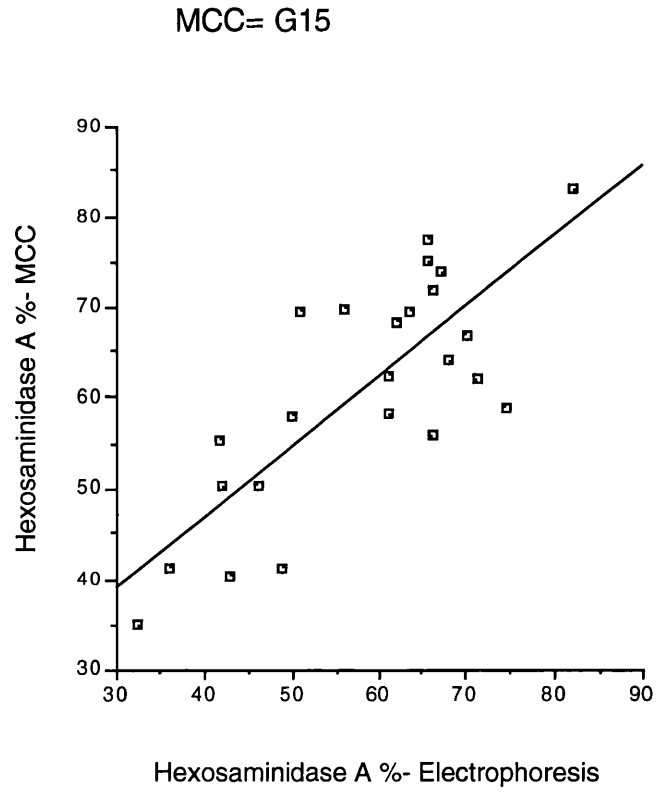


FIG. III-16 : *Deming's regression line obtained when comparing electrophoresis with GMAT and G15.*

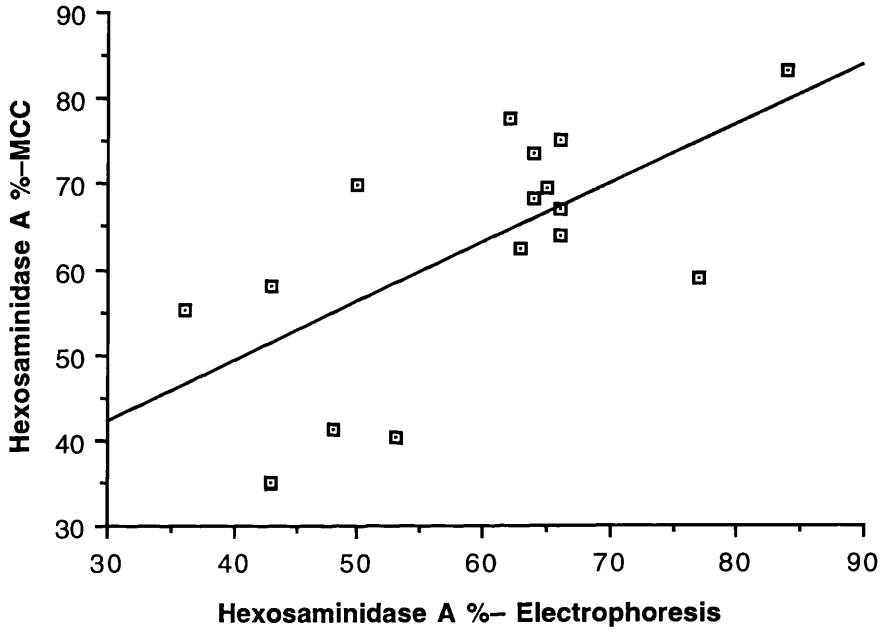


Fig.III-17: *Comparison between Electrophoresis and MCC on QC samples.*
MCC = 3.56 + 0.89x Electrophoresis, r=0.65, n=16.

problems prevented the verification of this hypothesis. The three peaks of the A type could not always be resolved and the first peak which should represent hexosaminidase B only contained the artefact.

7 – Conclusions

Micro column chromatography used under the conditions established in this study was shown to resolve and quantitate isoenzymes of hexosaminidase.

The correlation coefficient between electrophoresis and chromatography on Glycomat indicates that both methods measure the same components although there is a difference in resolution of the isoenzymes. The chromatographic system seems to produce more peaks, e.g. two peaks can be observed for the I isoenzyme and three peaks for the A type. A comparison between individual isoenzymes quantified by both methods may show a better correlation as, although the two methods are based on a charge differentiation, the 'physico-chemical' conditions are not exactly the same. The resolution could differ in subtle ways. Further work should be done in establishing a reference range for MCC.

The principal advantages of MCC is that it offers a completely automatic system, fairly robust and mainly independent of technician's skill. However, improvements would have to be made. The quantification system available is not suitable for the chosen applications, and, a choice would need to be made between GMAT and G15. GMAT offers a better quality of chromatography (A, I1, I2, B resolved in a reproducible manner) but would need some further work to solve the artefact problem in order to get a reliable quantitation. G15, gives reliable quantitation but with a lack of qualitative information which may be overcome by spending time on finding a better balance flow rate – slope of the gradient and, perhaps through a mechanical improvement by installing a mixing chamber.

Although none of the combinations used gave a 'perfect' result, it was evident that MCC could be used for separating hexosaminidase isoenzymes. The different forms could be resolved and quantified adequately for clinical use. The results were very similar to those obtained by Ellis et al in 1975 but in this study, the system was completely automatic using a shorter column and a more robust packing material.

Chapter IV – Separation of alkaline phosphatase isoenzymes

A – Introduction

Alkaline phosphatase isoenzymes (ALP, E.C.3.1.3.1) have occupied an important position in enzymology for more than sixty years. In 1923 Robison & Soames discovered that ossifying cartilage from young rats was rich in phosphatase activity. In 1924 they reported that this phosphatase had an alkaline optimum *in vitro*. In 1937 Oscar Bodansky raised the question "are phosphatase of bone, kidney, and serum identical?". In 1954 it became possible to separate liver and bone ALP in the sera from patients with breast cancer. It was then recognised that liver and bone isoenzymes were valuable tools to monitor hepatobiliary and bone diseases. This methodological breakthrough also resulted in the discovery of several enzyme inhibitors (e.g. L-Phe for intestinal ALP) and the demonstration of a high polymorphism of this enzyme. Two of the isoenzymes (intestinal and placental), are expressed in tissue-specific manner whereas the third (liver/bone/ kidney) is classified as tissue non-specific (TNS).

1 – Biosynthesis of ALP

The endoplasmic reticulum is considered to be the primary site of ALP synthesis. The biosynthesis and post-translational modifications of ALP have been extensively studied in human choriocarcinoma cells, (JEG–3) by Takami et al, 1988. By means of pulse and chase experiments with [^{35}S] they identified the major steps in the synthesis of mature ALP and the post-translational modifications involving N-linked oligosaccharides and glycolipid components. Their results suggest that a primary precursor (MW 64,000 Da) is immediately processed to an intermediate form (MW 63,000 Da) by a simultaneous process of proteolysis of the C terminus and the replacement with a glycolipid residue [Ferguson, 1988]. This is then transformed into the mature form (MW 66,000 Da).

2 – Glycosylation

The glycosylation of ALP has been investigated to some extent. It has been demonstrated, using serial lectin binding, that the major precursor in placental ALP contains a high mannose type oligosaccharide and the mature form a complex type [Komoda et al, 1989]. For the tissue non-specific ALP, Koyama [Koyama et al, 1987] showed that both liver and bone extracted from tissue, have bi-antennary complex-type and hybrid-type asparagine-linked sugar chains with the bone isoform having an increased number of hybrid-type

sugar with fucose residues. When serum TNS-ALP is examined, the only difference is the presence of internal fucose residue in the bone isoform [Kuwana et al, 1991]. It seems therefore that serum ALP differs in its oligosaccharide chain structure from the organ-specific one.

It is still not known whether the differences in glycosylation are targeted to the enzyme for a purpose, or whether they are related to the site of synthesis.

3 – Structure of the ALP molecule

The crystal structure of ALP from *E.Coli* has been determined and is used as a model for the mammalian type (25–30% strict homology, with the conserved residues mainly in the central α / β portion) [Kim and Wickoff, 1989]. The mammalian type is larger, contains carbohydrates, and is attached to membranes through phosphatidylinositol moieties. Specific differences in secondary ligands to bind phosphate and the magnesium metal have been established. The primary structure of the human ALP contains a sequence of 36 amino acids, close to the active centre which is absent from the *E.Coli* enzyme [Moss, 1992].

In *E.Coli* the enzyme is a two-fold symmetrical dimer (97 x 47 x 52 Å) with two active sites located about 30 Å from each other, each consisting in Asp–101, Ser–102, Ala–103, a metal triplet (two zinc, one magnesium and their ligands) plus Arg–166 and others amino acids in the vicinity [Kim and Wickoff, 1989]. The mammalian enzyme is 20 to 30 fold more active at its pH optimum and at substrate saturation.

It should, however, be remembered that all this information was obtained from *in vitro* studies of 'free' enzymes and may not be relevant to the ALP *in vivo* which is membrane-bound [Moss, 1992].

4 – Anchor

ALP belongs to a group of proteins linked to cells membranes by glycosylated phosphatidyl inositol (GPI), a so-called anchor. The anchor was extensively studied on *Trypanosome* organism which is known to have a very similar complex named Variant Surface Glycoprotein (VSG). It was shown to contain:

- 1 glucosamine
- 2 -3 mannose
- 0- 8 galactose
- and a micro-heterogeneity in the α–galactose branch has been shown.

[Ferguson, 1988].

This pattern has been shown to be consistent with the anchor of other proteins [Low, 1987].

In 1988, Ogota et al carried out a detailed study of the ALP anchor. Selectively prepared membrane ALP (mALP) and solubilized ALP (sALP) were used, the latter being the former treated with Butanol at pH 5.5 or Phosphatidylinositol phospholipase C (PI–PLC) (see Table IV-1). Their results suggest that the 's' form is derived from the 'm' form by the removal of diacylglycerol. The purified fragment at the anchor domain is composed of Gly–Thr–Thr–Asp, ethanolamine, mannose, glucosamine, inositol and phosphate. This sequence was identified at the position 481–484, and it was noted that the mature form lacks the carboxyl terminal extension at position 513 as predicted by cDNA.

Another group [Micanovic et al, 1988] has demonstrated that the inositol glycan is located on Asp–484 of the pro-enzyme, indicating that a 29 residues peptide is cleaved from the nascent protein during the post-transitional condensation with the phosphatidylinositol glycan. This 29 residues peptide is highly hydrophobic, and therefore its purpose may be to temporarily anchor the pro-ALP to the endoplasmic reticulum and to enable the cotranslational processing to occur.

5 – Dimer/ tetramer/polymer forms of ALP

ALP appears to exist in its native form as a tetramer bound to a membrane and as a mixture of tetramers and dimers, or solely dimers in fluids and secretions. ALP released from any mammalian tissues by PI PLC appears as a dimer [Hamilton et al, 1989], suggesting that endogenous PI PLC may release ALP from cells. The activity of the phospholipase complex varies depending on the type of tissue, cells, or fluid examined [Hamilton et al, 1989].

5–1 Dimer/ tetramer

Varying the experimental conditions for solubilising ALP has elucidated mechanisms for release of the enzyme from its membrane and the conditions required for maintaining its native form (see Table IV-2).

- Butanol at acid pH has the same effect as PI PLC, which would suggest that butanol pH 5.5 activates an endogenous PI PLC-like enzyme [Ogota et al, 1988].
- Butanol at alkaline pH disrupts the membrane, and releases ALP in its native form as a tetramer.

Table IV-1: *Differences in composition between membrane bound and solubilized ALP.*

mALP	**sALP**
Palmitate stearate glycerol all 1 mol/subunit diacylglycerol moiety	no lipid components glycerol
1 mol of inositol + 2 mol of ethanolamine	
differences in phosphate content	

Table IV-2: *Effect of different procedures for isolating ALP from its membrane.*

Solubilization process	**Structure obtained**
Butanol pH = 5.5	Dimer
Bromelain	Dimer
Ficin	Dimer
Proteolitic enzyme	Dimer
Phospholipase C Phospholipase D	Dimer
Butanol pH = 8.5	Tetramer
Triton-X-100 Nonidet P-40	Tetramer

- Ficin and Bromelain are relatively non-specific in their choice of cleavage site, ficin being the more efficient of the two.
- ALP solubilized in detergent (tetramer) is hydrophobic, retaining the fatty acyl chain of the PI; the dimer is hydrophilic implying that the lipid portion has been lost. SDS can also be used to convert the tetramers to dimers indicating that the dimers are also held together by simple protein interaction [Hawrylack et al, 1989].

These observations have led to the conclusion that both the interaction between the fatty acyl of the PI anchor and the ionic interactions between the subunits are necessary to maintain the tetramer. The disruption of one of them is enough for converting the tetramer into a dimer. It was shown that a tetramer with anchorage to the PI seems to be essential for human ALP function [Hawrylak and Stinson, 1988].

5–2 Polymer

In addition to a dimer (140,000 Da) a polymer of the enzyme (450,000 Da) has also been found in human kidney extracts and urine [Verpooten et al, 1989 (a)].

6 – Metal ions and molecular structure

The following findings were obtained by studying ALP from *E.Coli*.

There are three classes of metal binding sites on ALP: structural, catalytic and regulatory. Two metal ions, bind to the catalytic site and are required for the dimeric enzyme to be active when only one subunit can be phosphorylated; further addition of metal ions to the remaining sites prevents negative co-operative regulation and permits both units to be phosphorylated.

The monomeric subunit is able to bind Zn^{2+} in the absence of any subunit interactions and can reassociate with any nascent subunit to form a dimer. It has been suggested that a rapid equilibrium between Zn^{2+} and the folded subunit precedes the dimerisation. In *E.Coli* Zn^{2+} protects 3 histidyl groups at the catalytic site from denaturation.

A pathway for renaturation of ALP was proposed consisting in refolding of the subunit to form a Zn^{2+} binding site, then binding of Zn^{2+} followed by dimerisation and formation of the functional enzyme. On each unit of the dimeric enzyme, Zn^{2+} is bound to the catalytic and structural sites and $Mg2+$ to the regulatory site [McCraken and Meighen, 1981].

7 – Distribution in cells and organs

Alkaline phosphatase is an ectopic enzyme, attached to the cell membrane by a PI anchor as a dimer or tetramer. Generally the TNS-ALP is expressed at low level in many tissues but the main sites are osteoblasts for bone ALP and hepatocytes for liver ALP. The intestinal form is present mainly in enterocytes but has recently been shown in kidney, at the distal (S3) segment of the proximal tubule by [Verpooten et al, 1989 (b)]. The placental form is increasingly present during pregnancy.

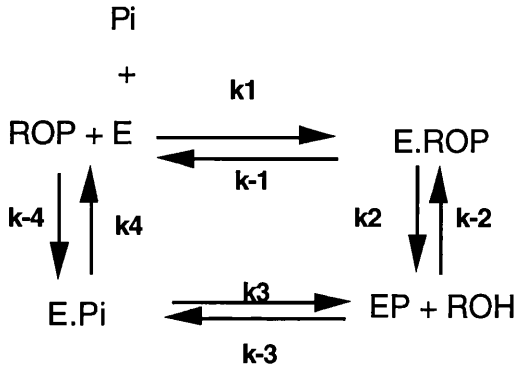
8 – Biological function

Interestingly, and despite the long history of research into ALP, the function(s) of the enzyme *in vivo* remains unknown. Its main function has been as a marker for tissue damage. Recently a hypothesis has been put forward linking the uptake of immunoglobulin from maternal blood to placental ALP in order to transport functional antibodies to the fetus [Makiay and Stigbrand, 1992].

9 – *In vitro* enzymatic activity

In vitro, ALP catalyses the hydrolysis of a variety of phosphate esters with the structure R – O–P, via the formation and break-down of a phosphoryl enzyme intermediate. There is little dependence of the rate of reaction on the nature of R–groups. Thus it has been postulated that it was a common rate limiting step for hydrolysis of the different phosphate esters.

Overall reaction :



A number of factors can influence the reaction rate. Tris buffers are known to give a sharp increase in enzyme activity. Inorganic phosphate (Pi) is a competitive inhibitor; it forms an intermediate, which is indistinguishable from the intermediate formed during hydrolysis of

phosphate esters – a lower concentration of Pi than substrate is required to half-saturate the enzyme (Ki / Km = 0.3). Arsenate, phosphate, and metal chelating agents are also known to inhibit the reaction.

10 – ALP in human diseases

10–1 Hyperphosphatasemia

Usually an increase of ALP in circulation is a sign of pathological process, but in childhood this may not be a sign of disease. In 1954 Bach reported the first case of an infant with a transient increase of ALP, more than five times the upper reference limit without obvious pathological cause, and normalised within three months [Stein et al, 1987]. Since then, many more cases have been investigated but the cause is still unknown.

The second form of hyperphosphatasemia is 'benign inherited' and of intestinal origin. It has been reported in two families where it seems to be inherited in an autosomal-dominant manner without any associated pathological condition [Panteghini, 1991].

10–2 Hypophosphatasia

Hypophosphatasia occurs in all races with an incidence of 1 in 100,000 live births [Whyte, 1989]. It was first described in 1948 by Rathbun, and in 1950 Schneider and Corcoran provided evidence for an inherited disorder. Clinically the syndrome is characterised by a defective mineralization of bone and teeth and has a variable severity from death *in utero* to presentation of the first symptoms (pathological fractures) in early adulthood. Biochemically, an increase of phosphoethanolamine in urine is used as a marker.

The disease has been shown to be autosomal-recessive for the severe forms but the inheritance is still uncertain for the mild forms. Recently a missense mutation was found in the TNS ALP gene locus [Henthorn and Whyte, 1992].

10–3 Miscellaneous

Increase in plasma ALP activity may have a variety of causes. The bone isoform is a marker of increased osteoblastic activity. The liver isoform is a marker of hepatobiliary disease. The proportion of the biliary form correlates with the degree of biliary obstruction [Crofton et al, 1979]. An increase of the intestinal isoform is also a marker of liver disorder, especially cirrhosis. This is because the intestinal isoform is asialylated and is

normally cleared quickly from the circulation by the hepatocytes, thus a plasma increased level usually indicates impaired hepatocyte function and impaired clearance [Price, 1993].

Tumours may induce the appearance of different types of ALP, such as placental, germ cell, or intestinal forms. Some of these are so specific that they have been named from the first patient in whom they were discovered: Regan, (placental-like type) appears in lung cancer [Hirano et al, 1989]; Nagao (also placental-like); Kasahara (fetal intestinal type); and a placental type associated with endometriosis [Kang et al, 1989], or breast cancer [Karmen et al, 1984]. There are in fact too many conditions associated with abnormality of ALP activity to give an exhaustive list in this study.

B – Development of a micro column chromatography method

1 – Aim

The aim of the work described in this chapter was to develop a MCC method for the separation and detection of isoenzymes and isoforms of alkaline phosphatase. In particular attention was focused on achieving a proper resolution and quantitation of the bone and liver isoforms as they are considered to be clinical parameters of most interest in paediatrics.

2 – Materials

2–1 Chemicals

Tris (hydroxymethyl)methylamine $(NH_2C(CH_2OH))_3$ and lithium chloride (LiCl), both AnalaR quality (BDH Chemicals, Poole Dorset, UK).

Triton X100, diethanolamine ($C_4H_{11}NO_2$), magnesium chloride ($MgCl_2$) and *Vibrio cholerae* type II (SIGMA, Poole Dorset, UK).

Agarose ME (SEAKEM, Flowgen, Sittingbourne, UK), nitro-cellulose (Sartorius, Epsom, UK).

Alkaline phosphatase isoenzymes controls (Calzyme, Birmingham, UK).

2–2 Substrates

4–Methylumbelliferyl–phosphate (MUP), MW = 256.15, (Melford Laboratories, Ipswich, UK).

SIGMA FAST^{TM} 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium tablets, (SIGMA, Poole Dorset, UK).

2–3 Packing material

PL SAX 1000 (Polymer Laboratories, Shropshire, UK).

2–4 Samples

Sera from teenagers – aged 16 to 20 years – who were screened for carrier status of Tay–Sachs disease, were kindly supplied by the Clinical Neurochemistry Laboratory, Institute

of Neurology and Neurosurgery (London), to be used as reference sera for this age interval (referred to as 'school population').

A series of paediatric samples were obtained from the Blood Bank, Hospital for Sick Children (HSC), (London) and used to established reference values for ages 1 month to 15 years. These samples were all from children admitted for surgery with no history of liver, bone, intestinal or renal disorders prior to admission. This group will be referred to as 'hospital population' in the following of this study.

Pathological samples chosen for specific clinical conditions (independent of the total activity), were collected from the routine laboratory at HSC. A few samples were kindly provided by the Chemical Pathology Department, Hammersmith Hospital, London.

3 – Sample preparation, analysis of total ALP activity

3–1 Heat denaturation

Heat denaturation of the bone isoenzyme was carried out at 56°C for 15 min. It has been shown that quantitative heat-stability characteristics differ from one sample to another and that at least two measurements of residual activity are needed to provide 'true' quantitation although a multiple-point inactivation curve is even better [Moss and Whitby, 1975]. In this study heat denaturation was only used for identification purposes therefore the above conditions were considered to be sufficient.

3–2 Neuraminidase digestion

5 µl of neuraminidase from *Vibrio Cholerae* was added to 25 µl serum and incubated for 15 min at 37°C. The reaction was stopped by putting the tube in iced water [Crofton, 1987].

The bone isoenzyme is known to be more sensitive than the liver isoenzyme to digestion by neuraminic acid [Moss, 1982]. Therefore a partial digest increases the differences in surface charge between these two isoforms enhancing the resolution when methods like electrophoresis or chromatography with an anionic exchanger are used.

3–3 Total alkaline phosphatase activity

The total activity (TALP) was measured on a Kodak EKTAKEM by the routine laboratory, their age-related reference range was used for the classification of the reference populations.

0 – 6 months	65 to 265 U/L
7 months – 2 years	110 to 440 U/L
3 – 10 years	110 to 350 U/L
11 – 17 years	110 to 440 U/L
Adults	35 to 120 U/L

The Kodak system uses p-Nitrophenyl phosphate as substrate for ALP, one unit per litre (U/L) corresponding to 1 µmol of p-nitro-phenyl phosphate hydrolysed per minute at 37 °C. Although the substrate used for the MCC analysis is different (MUP), alkaline phosphatase isoenzymes do not have known substrate specificity and the combination of the two substrates has been used by other workers [Crofton, 1992]. The relative activity of each peak detected was calculated by multiplying the individual percentage obtained after MCC analysis by the TALP.

4 – Micro column chromatography

4–1 Buffers

Tris HCl

Buffer A was Tris HCl 20 mmol/L + Triton X100 2 g/l, pH 8.2 ±0.02.

Buffer B was A plus LiCl 0.35 mol/L, pH 8.2 ±0.02.

Diethanolamine (DEA)

DEA 0.5 mol/L plus $MgCl_2$ 0.5 mmol/L and Triton X100 6 g/l, pH =10.4 was used as the substrate buffer.

4–2 Substrate

Methyl umbelliferyl phosphate (MUP) in DEA, 1 mmol/L, was prepared fresh in batches of 50 or 100 ml. MUP was noticed to be light and heat sensitive, thus the vial was kept in the dark and on ice during analysis to avoid spontaneous release of umbelliferone.

4–3 System

A 'continuous' flow system was set up according to the principle shown in Fig.IV-1. The G15 system was chosen as the intestinal isoform is known to elute almost in the void

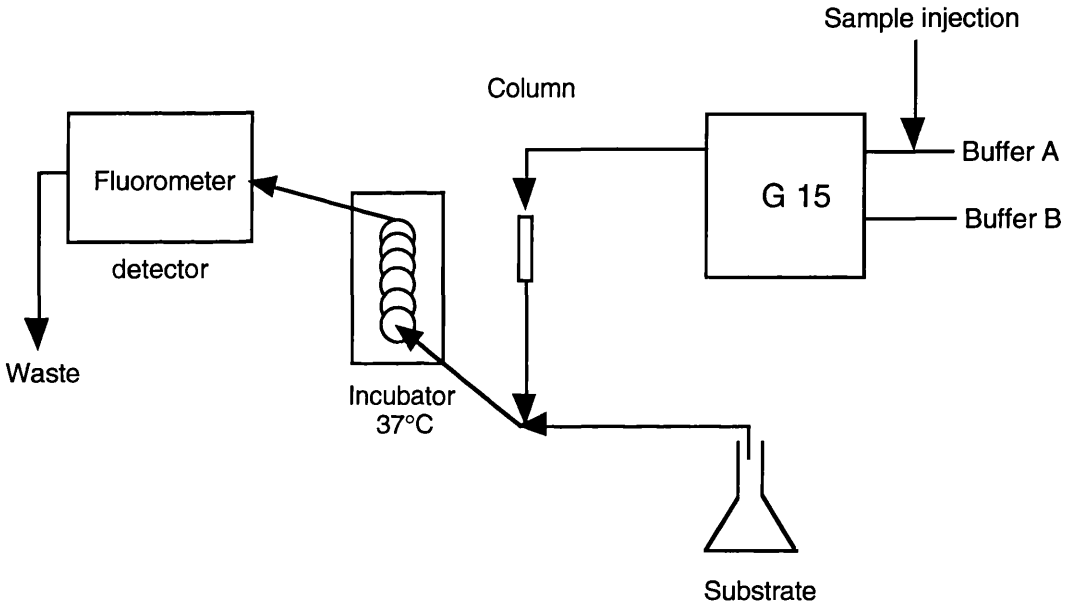


Fig.IV-1: *The equipment used for the separation of alkaline phosphatase isoenzymes.* Substrate: MUP, 1 mmol/L, at 0.10 ml/min. Buffer A: Tris-HCl 20 mmol/L, pH 8.2, at 0.55 ml/min, Buffer B: Tris-HCl 20 mmol/L plus LiCl 350 mmol/L, pH 8.2 at 0.55 ml/min. Fluorometer settings: λexc = 365 nm, λem = 455 nm.

volume and the artefact created by the GMAT system would impair the resolution (see Chapter II-Section 2–1).

The column packing material used in this work was PL SAX 1000.

The column used was 5 mm x 4.6 mm i.d. The injection volume was 60 µl with dilution according to the total activity (one in ten for up to 350 U/L).

4–4 Gradient

The final gradient used for the analysis of ALP on G15 was :

Time (sec)	% buffer B	flow rate (ml/min)
0	0	0.55
180	0	0.55
300	17	0.55
480	17	0.55
600	26	0.55
720	26	0.55
840	30	0.55
1080	30	0.55
1260	37.5	0.55
1380	37.5	0.55
1680	100	0.55
2140	100	0.55

followed by a six-minute wash.

4–5 Post-column detection

The substrate flow rate was 0.1 ml/min leading to a three-minute incubation period in the mixing coil at 37°C.

A fluorescent detector (Shimadzu, RF 535) was used to monitor the reaction with the following settings: λ_{exc} 365 nm and λ_{em} 455 nm.

4–6 Quantification of peaks

The peaks for alkaline phosphatase isoenzymes could not be evaluated like those of hexosaminidase (see Chapter III, Section 3–1–8). The software DS 4000 was unable to integrate every peak in a reproducible manner, and therefore manual integration was adopted. The peak area was calculated after base line subtraction (in order to reduce any systematic artefact) by the following method :

Area = Height of the peak x width of the peak at half height.

and the relative percentage of each peak calculated.

5 – Results

5–1 Analytical performance

5–1–1 Specificity

Fig.IV-2 shows a typical chromatogram obtained after MCC analysis. As no reference material is known to exist for alkaline phosphatase isoenzymes, the specificity of the five peaks commonly resolved was identified by a combination of three procedures: (i) information from the literature, (ii) the use of commercial controls available from Calzyme and (iii) the comparison of chromatograms of sera from patients with known clinical conditions.

(i) Information from the literature:

Different packing materials give a slightly different order of elution for the isoenzymes (see Table II-5). However the system used in this study was similar to that of Magnusson et al [1992] and Shoenau et al [1988]. These two groups obtained similar results and their conclusions were assumed to be valid for this study. Their pattern was:

Peak 1 = Bone 1 (B1) Peak 2 = Bone 2 (B2)

Peak 3= Liver 1 (L1) Peak 4 = Liver 2 (L2) Peak 5 = Liver 3 (L3)

A sixth peak, running at the front of the chromatogram was thought to be the adult intestinal isoenzyme as it was rarely present.

(ii) Use of control materials:

Controls from Calzymes were used for identification of the isoenzymes. They are said to be pure isoenzymes but no information was available about the method used to purify

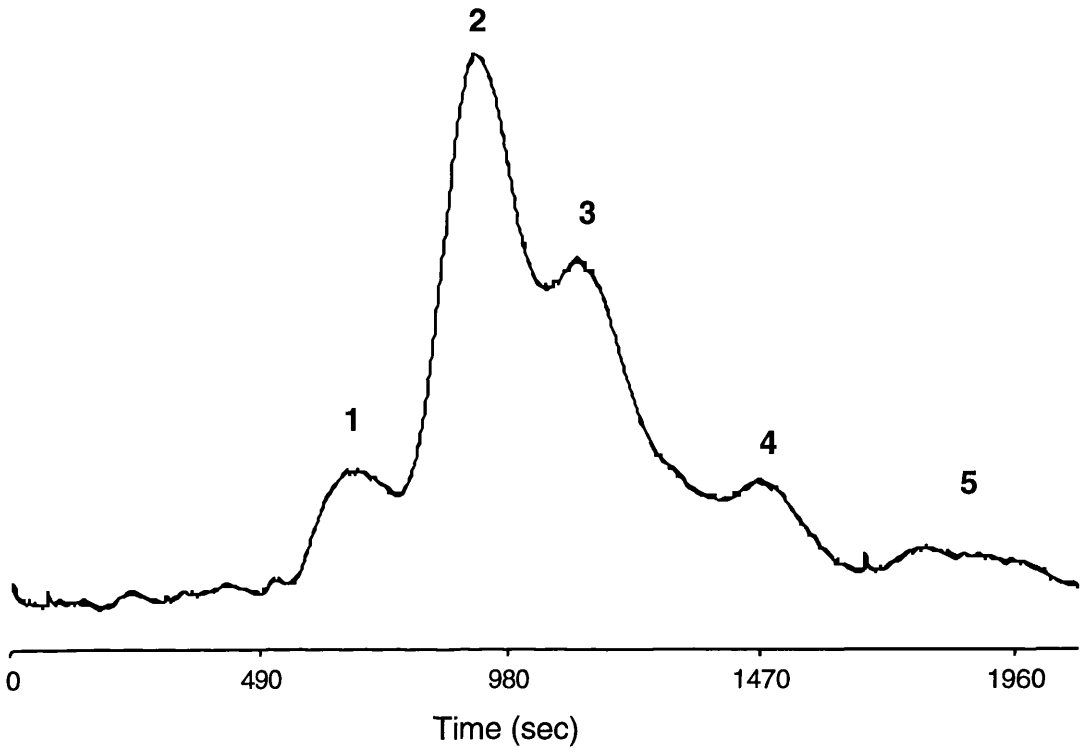


Fig.IV-2: *MCC analysis of a serum from an healthy 18 years old man.* Showing a typical pattern of 5 peaks.

the samples or the nature of the sample matrix before they were freeze dried. This introduced a question mark over their comparability with human serum ALP. The samples were reconstituted in water before use for MCC analysis as recommended by the manufacturer.

When separated by MCC the bone control gave two peaks eluting in the early part of the chromatogram in similar places to those called B1 and B2 (see Fig.IV-3a). The intestinal control showed four peaks, two of them eluting almost in the void volume plus two additional peaks with similar retention times to those seen in the bone control (see Fig.IV-3b). These results are not in agreement with the results obtained by Magnusson et al as they found three peaks for the bone isoenzymes and only one for the intestinal form. This could be due to a difference in gradient profile. These differences could also be due to impurities in the batch of controls used in this study. The liver control resolved in four peaks (see Fig.IV-3c) of which the first had a retention time very close to the peak B2. It could not be established during this work if this peak represented contamination, or a true liver peak poorly resolved under the chromatographic conditions. Heat denaturation was attempted to identify contamination but the results were inconsistent compared with the information in the literature (the liver control was completely inactivated whereas the bone control still showed some activity).

The placental control eluted half-way through the chromatogram implying that it would not be resolved properly and would not be identified in a serum sample using MCC. Because of the minor importance of the placental isoenzyme in paediatrics no further work was carried out on this isoenzyme.

Fig.IV-4 shows a chromatogram of pooled controls (Intestinal, bone, liver). It can be seen that the intestinal form and B1 run together and only three peaks of the liver type were resolved.

(iii) Using patients samples:

Fig.IV-5a shows the chromatogram of a serum from an adult patient with a liver disorder. The major peaks were those with retention times similar to L1, L2 and L3 in the liver control material, no B1 peak was detected. After heat-inactivation of the serum, the peaks changed shape but the retention times stayed the same (see Fig.IV-5b) confirming their 'liver nature' (see section II.2.1) When examining the chromatogram from an adult patient with Paget's disease (Fig.IV-6a), the two peaks corresponding to B1 and B2 in the control material are the major ones. This is compared with a chromatogram from a serum from an hospital patient of similar age with increased ALP activity for unknown reasons where, as

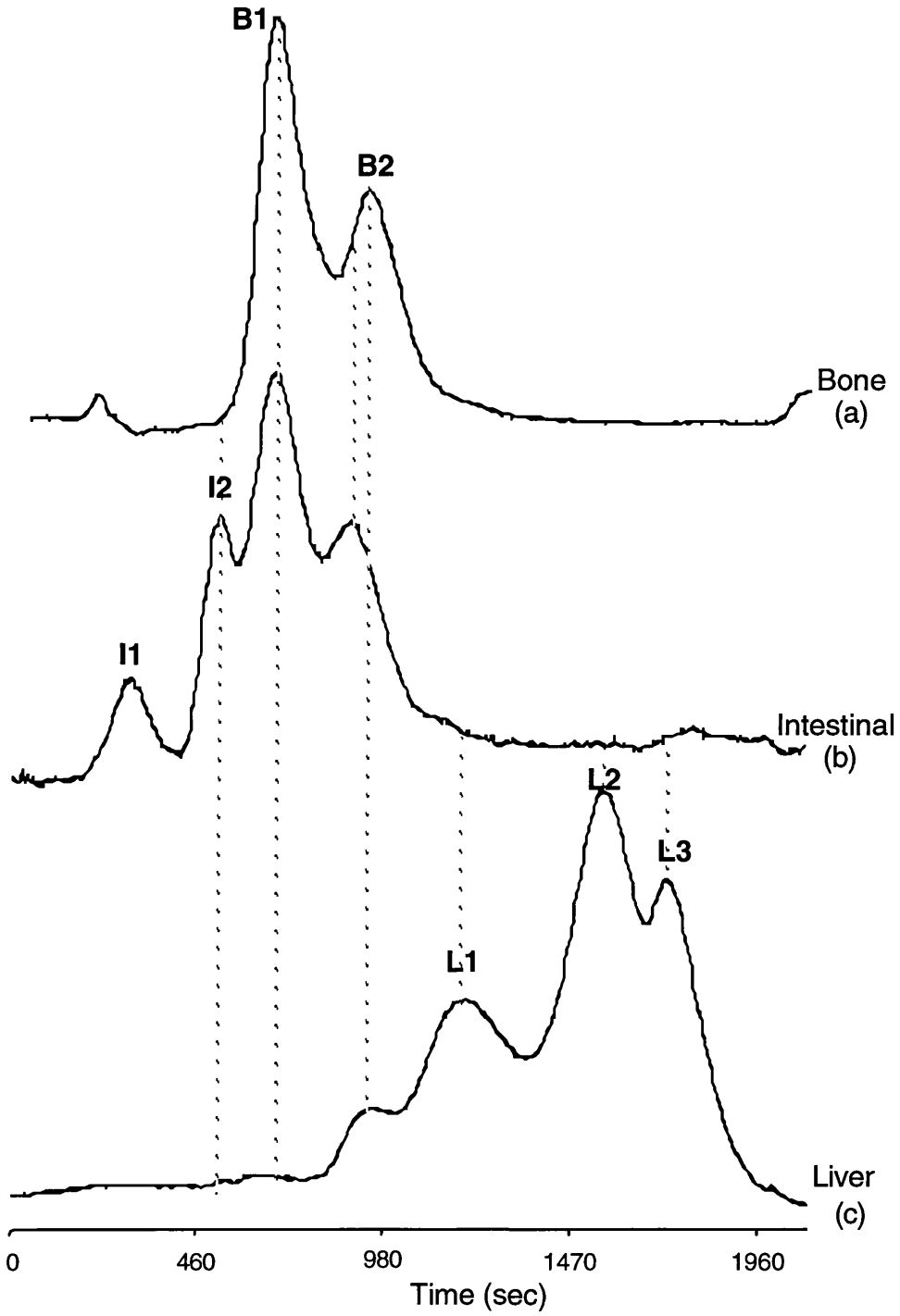


Fig.IV-3: *MCC analysis of Calzyme controls.* I1 and I2= intestinal isoenzymes, B1= bone 1, B2= bone 2, L1= liver 1, L2= liver 2 and L3= liver 3.

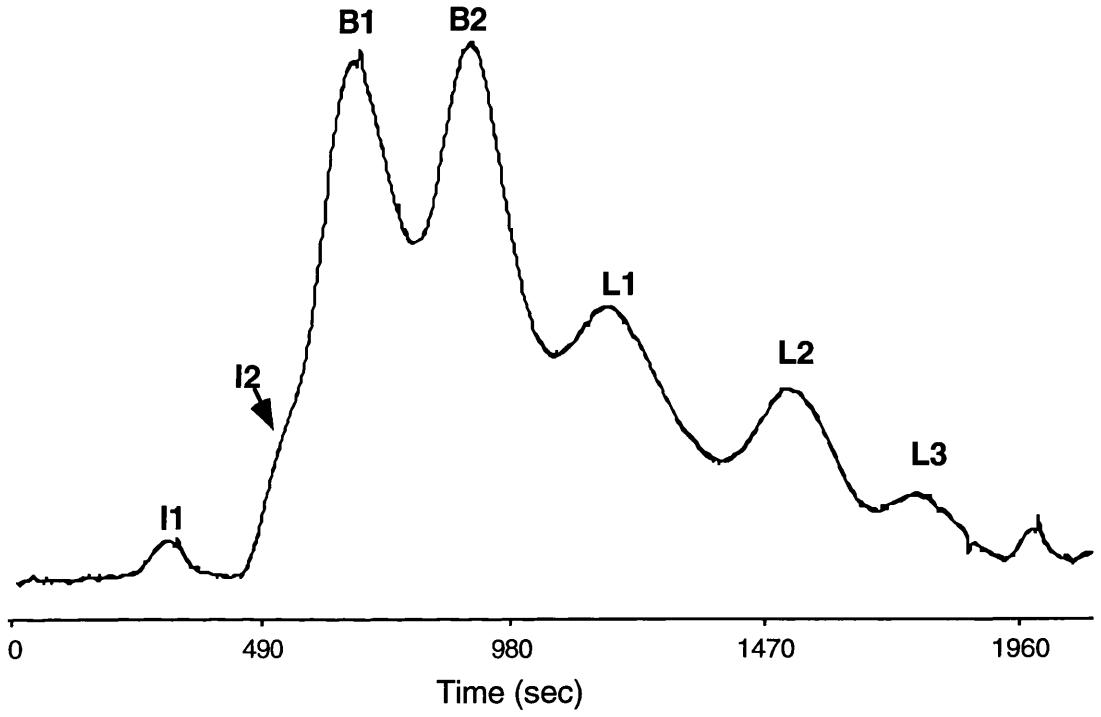


Fig.IV-4: *MCC analysis of Calzymes' controls mixture.* I1, I2, B1, B2, L1, L2 and L3 are as described in Fig.IV-3.

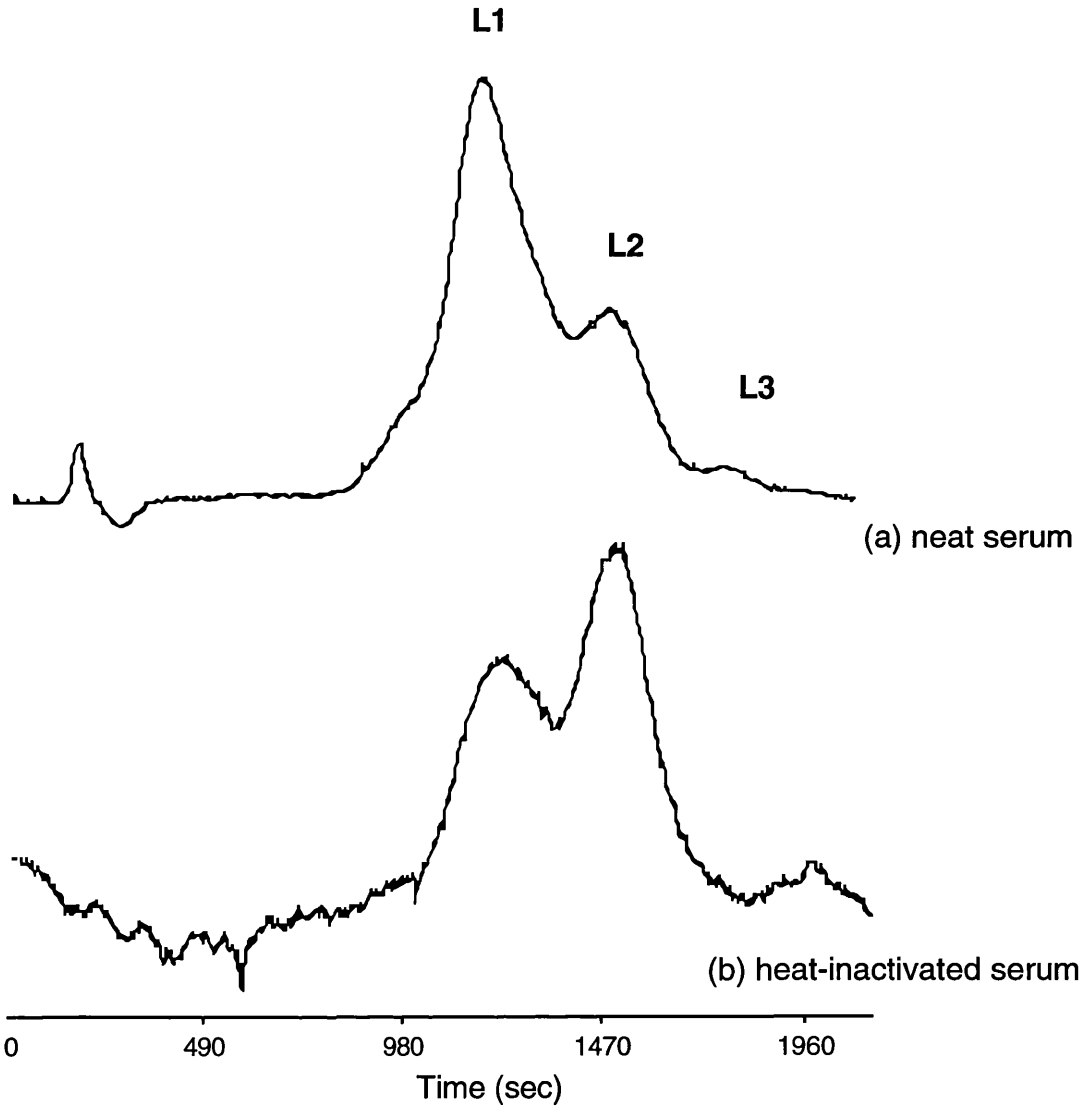


Fig.IV-5: *MCC analysis of a serum from a patient with liver disorder.* I, B1, B2, L1, L2 and L3 are as described in Fig.IV-3.

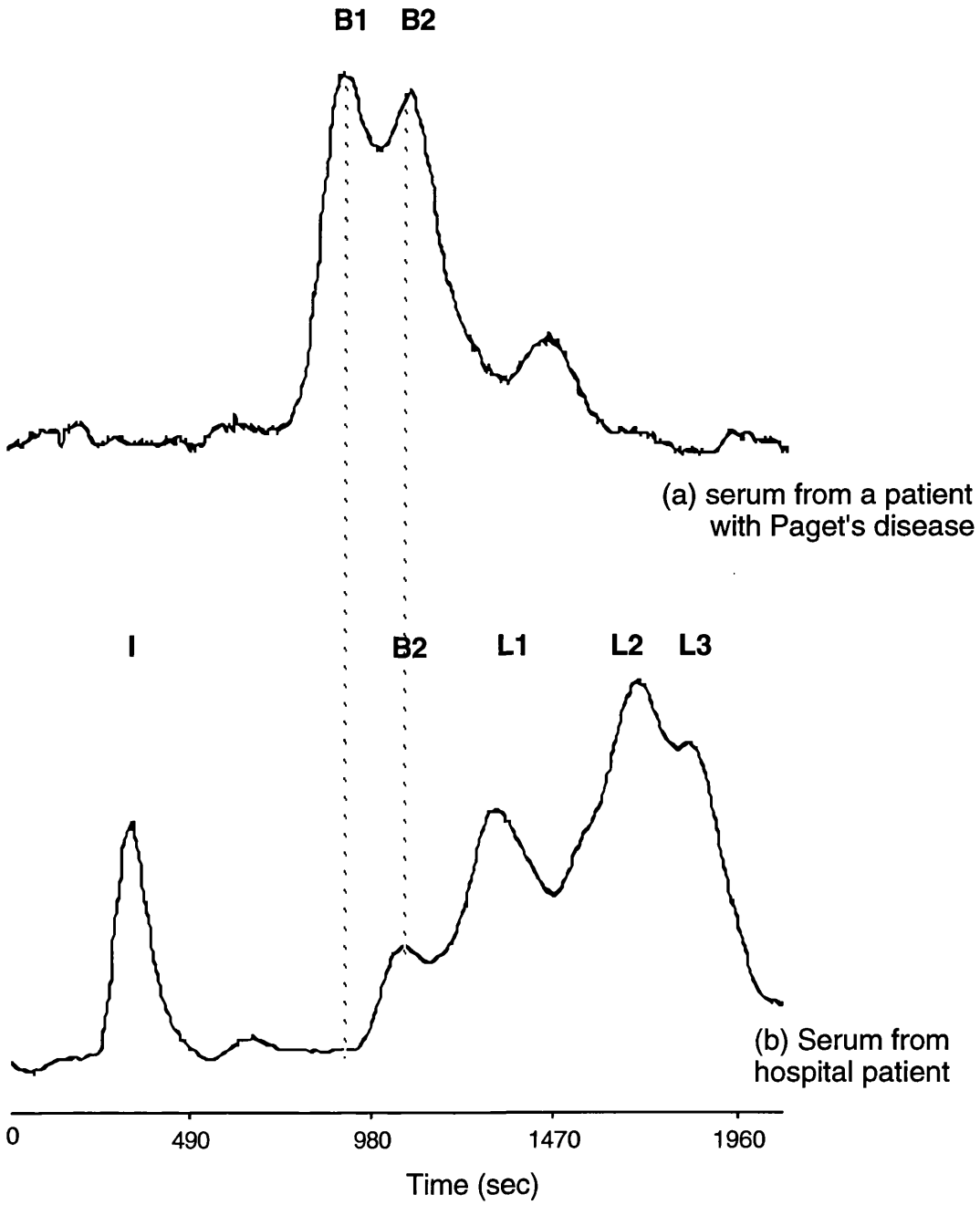


Fig.IV-6: *Comparison of chromatograms from patients with different clinical conditions.* I, B1, B2, L1, L2 and L3 are as described in Fig.IV-3.

expected in adults, the bone isoenzymes are present in very small percentage compared to the liver isoenzyme (Fig.IV-16b). In conclusion, a pattern of five peaks namely B1, B2, L1, L2 and L3 was used as the basis for further work. The intestinal isoenzyme was not normally present in paediatric samples.

5–1–2 Chromatographic performance (data in Appendix C1)

The separation of ALP isoforms on MCC, resulting in five main distinct peaks, offered an opportunity to study the reproducibility of the retention times and to assess the performance of a 5 x 4.6 mm column packed with PL SAX 1000 in some detail.

(i) **Within-batch variability**: A batch is defined as 14 consecutive injections starting with three blank runs, two to equilibrate the column and the third used for establishing a base-line. The system would be switched off between-batches. Two samples with different total activities were chosen (582 U/L and 36 U/L). Table IV-3 and IV-4 and Fig-IV-7 and IV-8 show that the RTs are very stable within one batch independent of the total activity of the sample with CVs (%) of 2.1 for B1 (both for low and high activity), 0.95 and 0.88 for B2, 3.08 and 2.5 for L1, 0.88 and 0.81 for L2 and 0.53 for L3, respectively.

(ii) **Between-batch variability**: Two samples were analysed on consecutive days (8 days for a sample from a healthy new born baby and 6 days for a pathological sample). Two different columns and several batches of eluting buffers and substrate were used. CVs for the retention times varied between 0.6% and 2.4% (see Table IV-5) indicating the robustness of the system.

The largest variation in the retention times for the isoenzymes in the two samples was found for B2 (Fig.IV-9 and Table IV-5), where mean values of 896 sec and 982 sec were obtained. This difference seems too big to be explained by minor variations in the elution conditions. The most likely explanation is that two isoforms run closely together in the B2 area. The relative proportions of these isoforms will then influence the retention time for the 'joint' peak.

(iii) **Long-term variability** : Hundred samples representing a random population of normal and pathological samples from subjects aged from a few days to 18 years old were analysed over a 5 working days period. This involved using three columns as each column would safely analyse 35 to 45 samples before having to be changed. A graphic

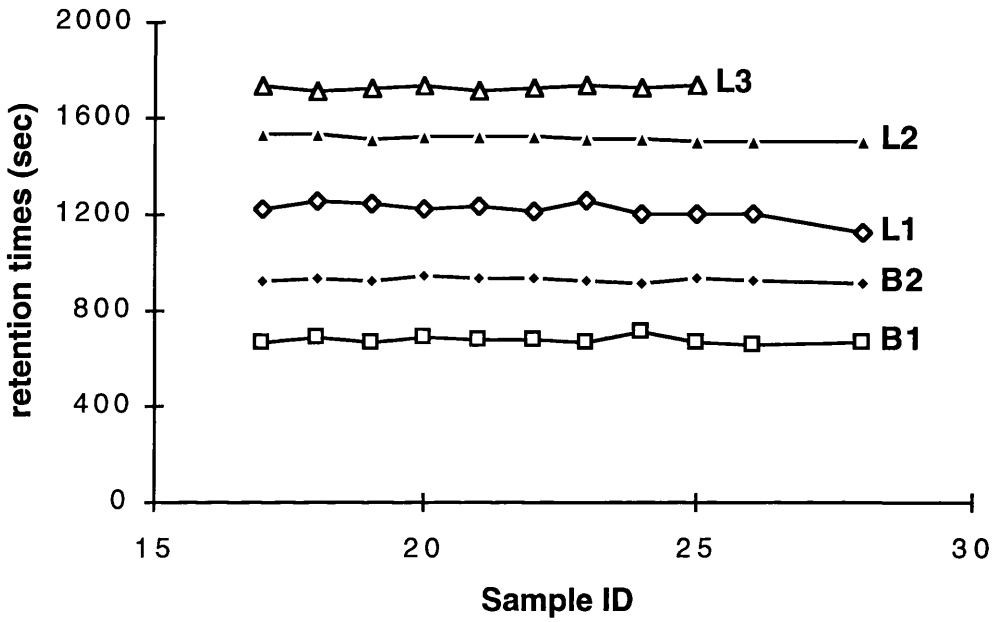


Fig.IV-7: *Within-batch variability of RTs for a sample with a low ALP activity (36 U/L).* B1, B2, L1, L2 and L3 are as described in Fig.IV-3.

Table IV-3: *Statistical data for within-batch variability of RTs in a sample with a low total ALP activity (36 U/L).*

	B1	B2	L1	L2	L3
Mean (sec)	676.73	928.55	1213.55	1515.45	1721
SD (sec)	14.19	8.86	37.42	13.26	9.11
CV (%)	2.1	0.95	3.08	0.88	0.53
Range	658-708	916-948	1120-1251	1497-1537	1705-1731

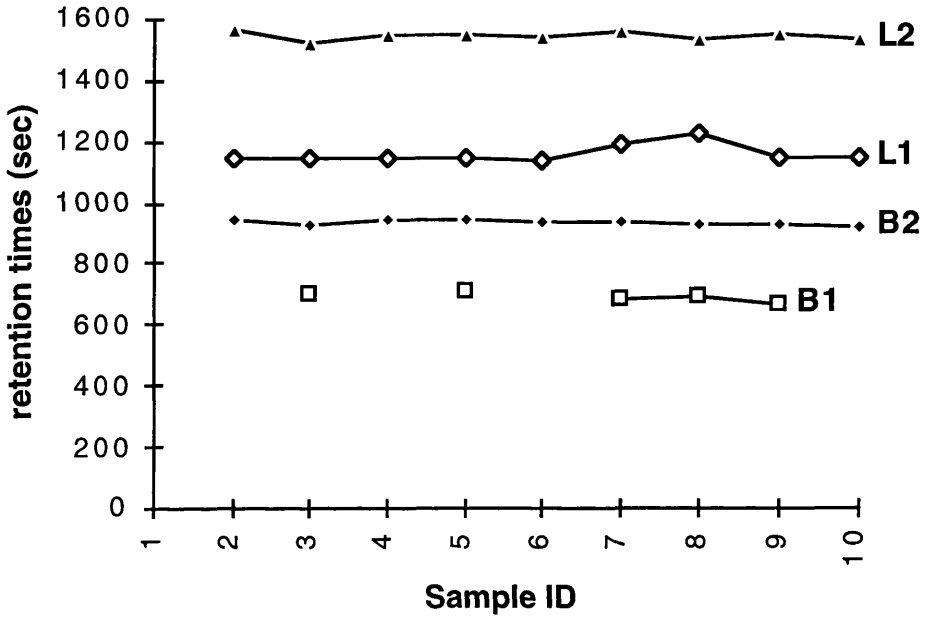


Fig.IV-8: *Within-batch variability of RTs for a serum with a high total ALP activity (582 U/L).* B1, B2, L1 and L2 are as described in Fig.IV-3.

Table IV-4: *Statistical data for within-batch variations of RTs for a sample with a high total ALP activity (582 U/L).*

	B1	B2	L1	L2	L3
Mean (sec)	687.6	933.4	1158.1	1541.7	'
SD (sec)	14.47	8.17	29.08	12.46	'
CV (%)	2.10	0.88	2.51	0.81	'
Range	668-704	917-944	1133-1222	1524-1561	'

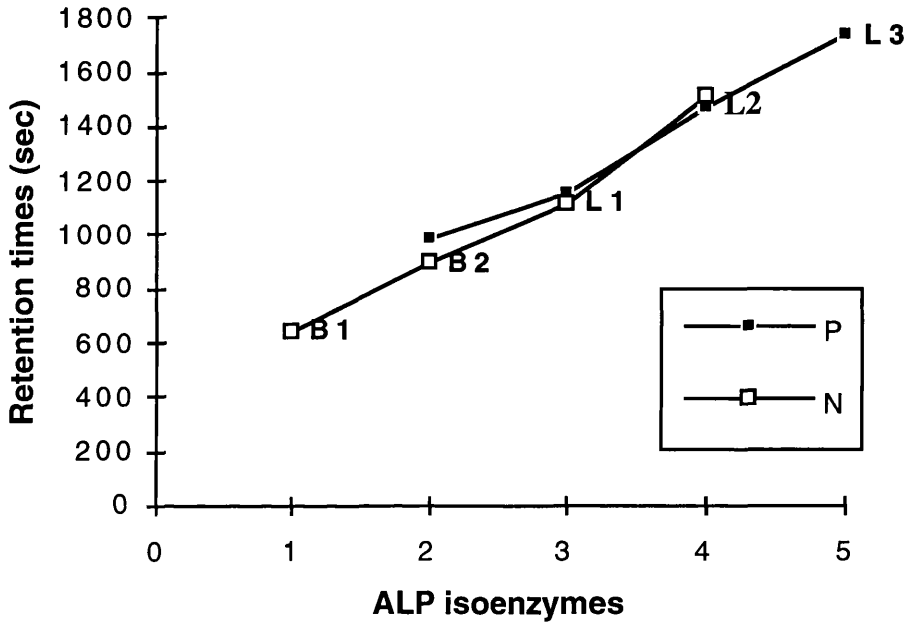


Fig.IV-9: *Graphic representation of the between-batch variations of RTs for two samples with different isoenzymes patterns.* N= Normal, sample from healthy new born baby and P= Patient, sample from adult with a liver disease; each point represent the mean (n=8 for N, and 6 for P) of the between-batch analysis. B1, B2, L1, L2 and L3 are as described in Fig.IV-3.

Table IV-5: *Statistical data for the between-batch variation of RTs for two samples with different isoenzymes patterns* (n=8 for NORMAL, n=6 for PATIENT).

	B1	B2	L1	L2	L3
NORMAL					‘
Mean (sec)	643.9	895.9	1116.9	1505.6	
SD (sec)	15.4	13.8	7.3	14.4	‘
CV (%)	2.4	1.5	0.6	1	‘
Range	618-663	872-910	1108-1132	1483-1523	‘
PATIENT					
Mean (sec)	‘	981.6	1147.5	1467.7	1733.3
SD (sec)	‘	19	17.7	16.7	39.7
CV (%)	‘	1.9	1.5	1.1	2.3
Range	‘	981-999	1132-1176	1444-1491	1660-1781

representation of the variations for each isoform (see Fig.IV-10) shows a narrow time window and a good reproducibility from column to column. The CVs, between 4.5% and 2.4% (see Table IV-6), are slightly higher than would usually be expected for chromatography (1 to 2%). This was thought to be due to variability of the gradient as indicated by a CV for the relative retention times of 4.5% (data in Appendix C1).

5–1–3 Imprecision (data in Appendix C2)

Imprecision for measures of ALP isoenzymes activities was determined by analysing the same sample several times within-batch and between-batch. The results are shown in Table IV-7 and IV-8, in both cases the SDs and CVs show a similar scatter among the fractions with CVs ranging from 5.6% to 48%. In each case the bone fractions B1, B2 and the liver fractions L1, L2, L3 were respectively pooled in order to see if the results became more comparable to those obtained following electrophoresis. Although still higher than the values found in the literature for electrophoresis (9.1% for liver and 2.3% for bone within the same run for a sample from a normal child n=8 [Crofton, 1992]), the CVs were in the range 1.08% to 24.94%, the smallest being for the liver fraction.

This level of imprecision would need to be improved in order to use this method routinely as low level fractions could be misinterpreted. As already mentioned in the previous chapter, an improved method for quantitation of peak areas would be expected to make a major contribution.

5–1–4 Conclusion

Data are presented in this section indicating that MCC has a potential for separating ALP isoenzymes. However some additional work would be required for the system to reach the standard of an established routine method.

(i) As was shown in the introduction of Chapter II, a number of methods exist to differentiate ALP isoenzymes and isoforms. Time did not permit the investigation of all the available procedures, but this would have helped to clarify the identity of each peak.

(ii) The reproducibility of the gradient was shown not to be optimal and the CVs were too high to be acceptable for a routine method . Some improvement of the mechanics of the equipment (a better resistance to back pressure) and of its software will no doubt result in a more reliable and reproducible integration of the peaks.

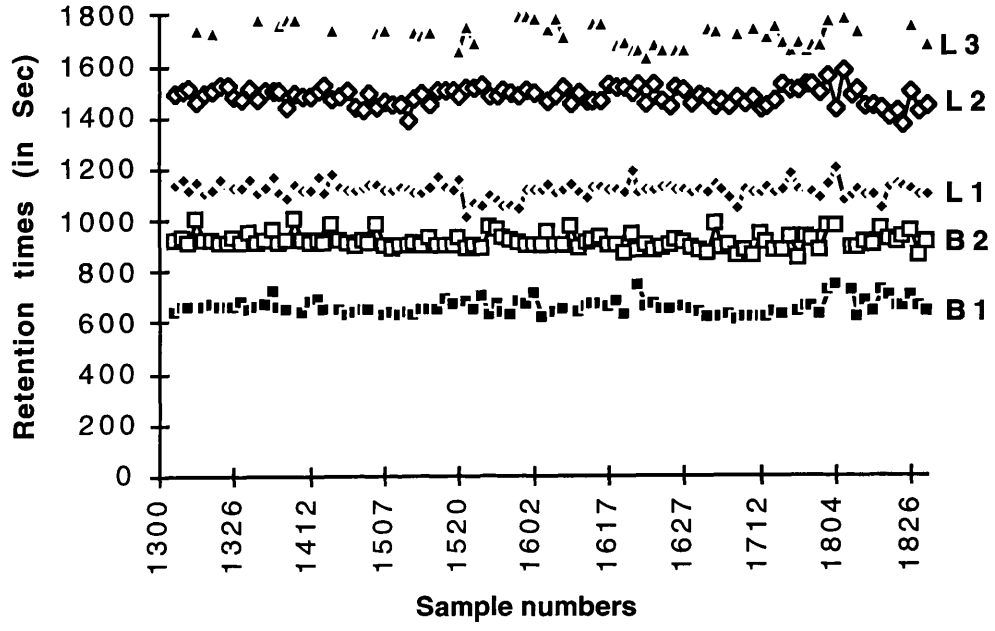


Fig.IV-10: *Variability of the RTs for ALP isoenzymes and isoforms during the analysis of 100 samples on MCC.* B1, B2, L1, L2 and L3 are as described in Fig.IV-3.

Table IV-6: *Statistical data for the variability of RTs during the analysis of 100 samples on MCC.*

	B1	B2	L1	L2	L3
Mean (sec)	653.7	911.0	1114.5	1478.7	1721.7
SD (sec)	29.3	31.0	30.7	35.2	45.3
CV (%)	4.5	3.4	2.8	2.4	2.6
Range	605-744	849-999	1011-1203	1367-1578	1630-1798

Table IV-7: *Within-batch imprecision for ALP isoenzymes after MCC analysis.*

	B1	B2	L1	L2	L3	B1 +B2	L1+L2 +L3
Normal (n=9)							
Mean (%)	5.01	30.00	32.78	29.16	15.79	32.78	67.21
SD (%)	2.43	7.02	7.84	2.49	2.46	7.49	7.49
CV (%)	48.56	23.40	23.93	8.54	15.56	22.85	11.15
Pathological (n=10)							
Mean (%)	1.513	7.41	29.269	47.885	13.989	8.923	91.143
SD (%)	0.44	0.67	4.98	2.68	2.86	0.90	0.98
CV (%)	29.28	9.10	17.00	5.60	20.45	10.04	1.08

%=% of total alkaline phosphatase activity, Normal= healthy young adult (18 years), pathological= child with elevated total alkaline phosphatase because of liver disease.

Table IV-8: *Between-batch imprecision for ALP isoenzymes after MCC analysis.*

	B1	B2	L1	L2	L3	B1+B2	L1+L2+ L3
Normal (n=7)							
Mean (%)	3.99	29.20	45.49	20.11		33.19	66.81
SD (%)	1.04	3.40	3.87	7.39		3.80	3.80
CV (%)	26.03	11.64	8.50	36.74		11.43	5.69
Pathological (n=6)							
Mean (%)		12.80	60.93	22.43	3.91	12.80	87.28
SD (%)		3.19	7.74	8.51	1.50	3.19	3.13
CV (%)		24.94	12.71	37.93	38.24	24.94	3.59

%=% total alkaline phosphatase activity, Normal= young healthy adult (18 years), Pathological= adult with liver disease.

With these problems solved, MCC offers the following advantages: an adequate resolution without sample preparation, a full automation of the system (the ALP would be sufficiently stable with a cooled auto-sampler) and easy interpretation of the chromatogram.

The quality of the present MCC method was, however, considered acceptable for comparison with other methods and for investigation of clinical samples.

5–2 Comparison of MCC with electrophoresis

The purpose of this study was not to develop an 'ideal' electrophoretic method and, as none of the commercial kits could be obtained, it was decided to use a simple electrophoretic method to separate the bone from the liver isoform.

5–2–1 Method

Gels were made from agarose (1%) and sucrose (5%) in Tris-barbital buffer, and the plates poured and prepared as described in Chapter III, Section 4-1.

The serum samples were applied neat (2 µl) and the Calzyme controls diluted 1/1000.

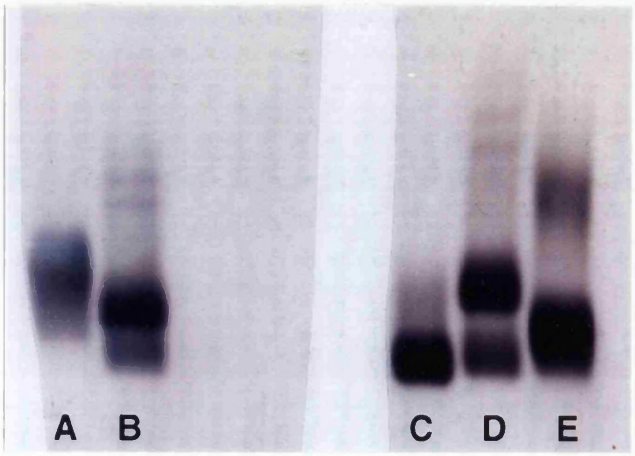
Electrophoresis was run with the Tris-Barbital buffer system at 350 V constant voltage (about 70–80 mA) until the marker (bromophenol blue added to pooled normal sera) had migrated 5 cm from the application point.

The plate was then stained for alkaline phosphatase activity by soaking the gel with the SIGMA FAST™ BCIP/NB tablets, dissolved as recommended by the manufacturer, overnight at room temperature.

As already mentioned in Chapter II, there are many different electrophoretic methods for the analysis of ALP isoenzymes but few of them give a suitable resolution of bone and liver without sample preparation. Neuraminidase digestion, as described in Section 3–2 of this chapter, was chosen for the study.

5–2–2 Results

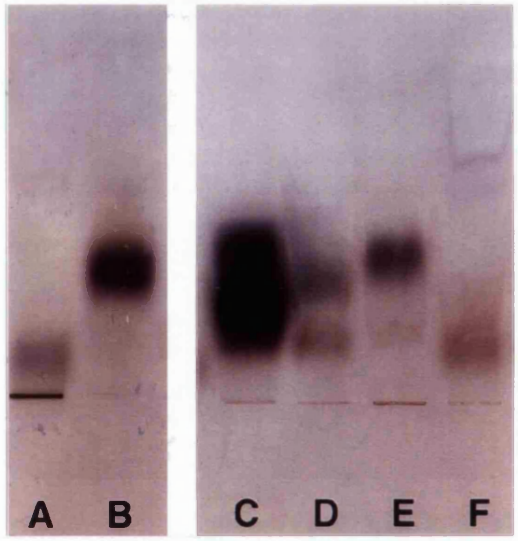
Fig.IV-11 shows the electrophoretic separation of the controls from Calzymes undigested (Lane A and B) and digested with neuraminidase (Lane C, D and E). As might be expected on such a system the bone controls (Lanes A and C) produced only one band. The liver however showed some heterogeneity (Lane E) as after digestion with neuraminidase from *Vibrio cholerae,* a strong band could be seen in the 'bone region'. The question of bone contamination had



Lane A= bone isoenzyme
Lane B= intestinal isoenzyme

Lane C= bone
Lane D= intestinal
Lane E= liver

Fig.IV-11: *Electrophoresis of Calzyme controls.* Lane A and B= samples diluted in water. In Lane C, D and E, the samples have been digested with *vibrio cholerae* according to the procedure described in Section 3-2 prior to analysis. Anode at the top of the figure.



Lane A= neonate
Lane B= patient with a liver disorder

Lane C= Transient
hyperphosphatasaemia
Lane D= patient with Paget's disease
Lane E= child on TPN
Lane F= child with neuroblastoma

Fig.IV-12: *Electrophoresis of alkaline phosphatase isoenzymes.* All the samples have been digested with *vibrio cholerae* according to the procedure described in Section 3–2 prior to analysis.TPN= trans parenteral nutrition.– Anode at the top of the figure.

already been raised but the percentage calculated after scanning was much higher than the percentage obtained by MCC. Therefore the question of whether the controls behaved as human serum could still not be answered. The intestinal controls (Lanes B and D) were definitely contaminated with bone isoenzyme as the pattern should not change after neuraminidase digest in the way seen in lane D.

Fig.IV-12 shows examples of electrophoretic separation of ALP isoenzymes in various type of sample following neuraminidase treatment. As can be observed in lane A, serum from neonate produces only one band at the bone position, in Lane B a sample from an adult patient with a liver disorder demonstrates only one band which runs faster despite neuraminidase digestion. Lanes C, D, E and F represent examples of electrophoretic patterns from patients with transient hyperphosphatasemia, Paget's disease, a child (2 years old) with Acute Lymphoblastic Leukaemia and a child (3 years old) with neuroblastoma. Very little information could be draw from these patterns as the resolution was inadequate, but as in MCC (see Section 5–3–2) the transient hyperphosphatasemic sample showed elevated bone and liver isoenzymes without any detectable specific bands.

5–2–3 Method comparison (Data in Appendix C3)

As electrophoresis provided only one bone fraction whereas MCC resolved at least two fractions (B1 and B2), a comparison was made of the percentage of the bone isoform from electrophoresis against B1 and B2 both independently and combined (see Fig.IV-13, raw data are shown in Appendix C3). As the identity of the peak labelled L1 was questionable and could contain some bone 'contamination', a comparison was also made by pooling the fractions B1, B2 and L1 The results (Fig.IV-13) showed a great amount of scatter with a lack of points near the origin of the axes. A statistical analysis established
weak correlations for this comparison ($p<0.05$) ($r= 0.41$ for B2, $r= 0.45$ for B1 + B2 and $r= 0.57$ for B1 + B2 + L1, $n= 23$). Therefore it was concluded that no correlation existed between the two methods. Explanations for this discrepancy could be:

(i) The samples were treated differently prior to analysis by the two methods. The ones used for electrophoresis were digested with neuraminidase in order to improve the resolution but neuraminidase, although to different degrees, affects all the isoforms. This might be sufficient to modify the surface charge of the protein and therefore change its migration.

(ii) There are different electrophoretic methods and perhaps a comparison with a method such as the one used by van Hoof et al, which gives a high resolution of the ALP isoenzymes without sample preparation using neuraminidase, would give a better

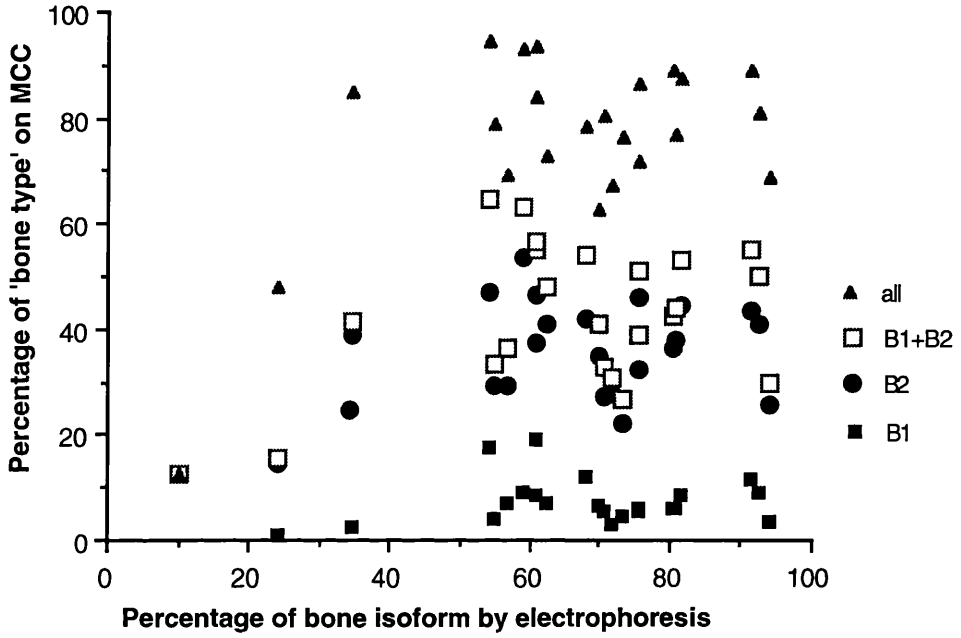


Fig.IV-13: *Plot of the 'bone fraction' percentages analysed by electrophoresis and MCC.* All= B1 + B2 + L1.

correlation. However, electrophoresis and MCC may have different specificities toward the various isoenzymes and isoforms of ALP, in which case correlation does not have to be expected [Moss, 1994].

5–3 Clinical application

5–3–1 Reference range for the individual fractions of bone and liver ALP isoenzymes (Data in Appendix C4)

An initial analysis of data, based on comparison of the distribution of total ALP, indicated that the hospital population and the school population could be pooled, providing a reference population of 62 individuals aged from neonate to 20 years old.

Individual values for the isoenzymes related to age are shown in Fig.IV-14. A statistically significant decrease with age was found for B2 and L1 ($r= -0.412$, $p<0.01$, and $r= -0.322$ $p<0.05$, respectively), with a considerable scatter around the trend line. Examination of the data in Table IV-9 again indicates a considerable scatter of values within the age groups. The data for L3 were less reliable than for the other isoenzymes because of the smaller number ($n< 8$ in all four age groups) of individual values obtained.

Further statistical analysis was carried out by testing for possible correlations between levels of individual isoenzymes. The results, presented in Table V-10, indicate that activity of individual isoenzymes were correlated to the activity of TALP and that there were many correlations between individual isoenzymes. Significant correlations were found for two thirds of the possible combinations and in about 25 per cent the correlations were >0.6. This results indicates that the isoenzymes change in parallel with TALP within the normal range in children.

5–3–2 Selected samples

It is not possible to identify a specific disease from a chromatogram of ALP isoenzymes obtained by MCC analysis in paediatric samples as was the case for hexosaminidase where specific lysosomal disorders could be detected. However, as was shown in Fig.IV-5 and IV-6, liver and bone disorders in adult patients can provide fairly distinct patterns indicating a degree of dysfunction in a specific organ.

In transient hyperphosphatasemia (see Fig.IV-15) peaks corresponding to B2, L1, L2 and L3 were observed. B2 and L1(13.26% and 32% respectively) fell within the range obtained for the hospital population where mean ± 2 SD was 36.16 ± 18% and 32.93 ± 14.2%

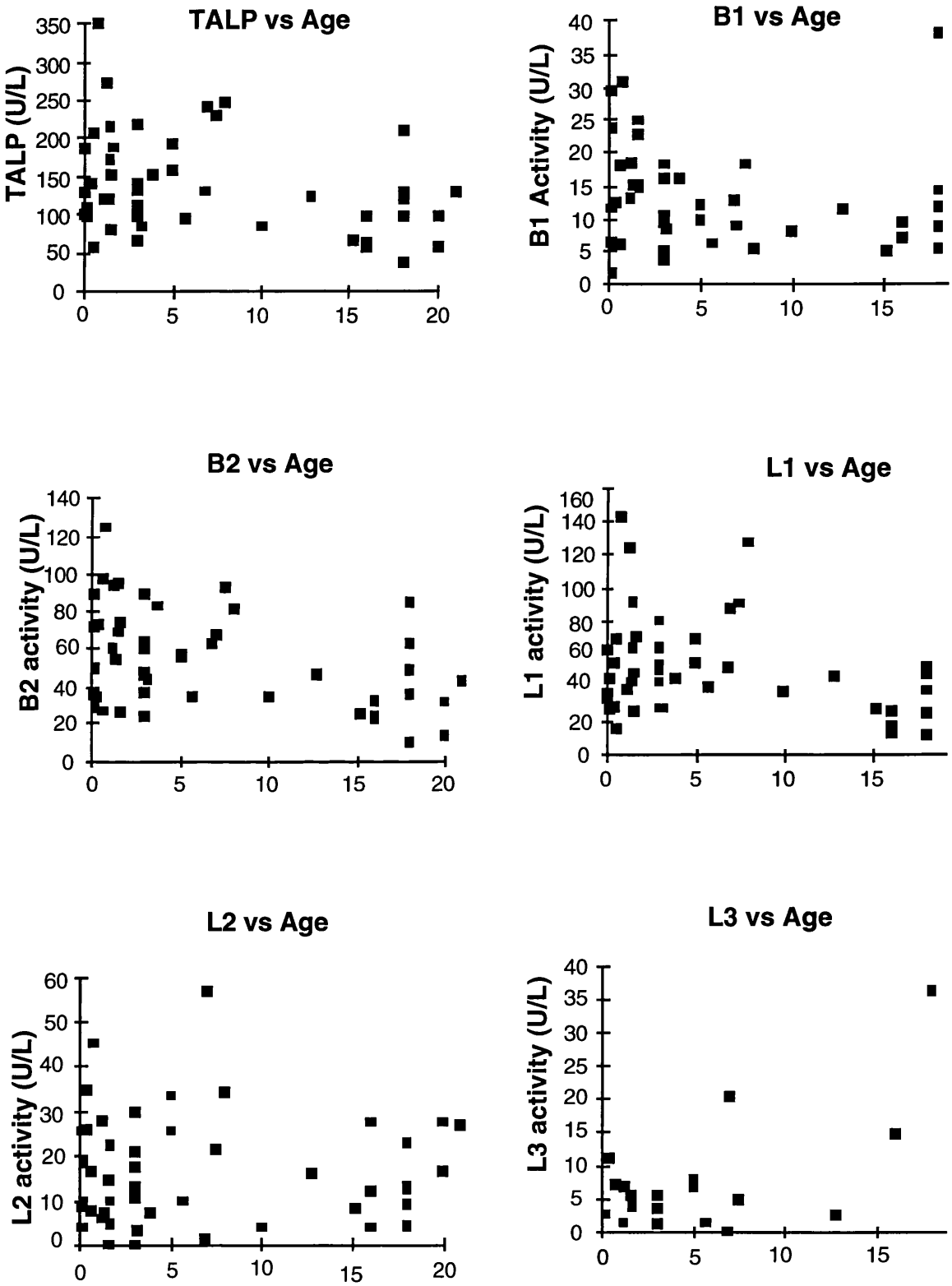


Fig.IV-14: *Correlations between age and individual isoenzyme activities*

Table IV-9: *Reference range for ALP isoenzymes determined by MCC in age groups up to 20 years*

0-6m

	TALP	B1	B2	L1	L2	L3
n	8	8	8	8	8	3
MEAN (U/L)	141.88	9.84	61.35	43.57	24.27	7.63
SD (U/L)	41.88	4.16	32.86	15.15	11.22	2.94
CV (%)	30	42	54	35	46	39
Range (U/L)	101-240	4.79-16.87	27.93-128.98	29.55-75.43	8.75-43.44	6.01-5.85

6-2Y

n	11	11	11	11	11	6
Mean (U/L)	175.27	17.55	72.41	66.75	14.72	4.77
SD (U/L)	85.29	6.61	30.13	39.34	13.09	2.20
CV (%)	49	38	42	59	89	46
Range (U/L)	56-350	5.93-30.70	26-17-124.71	15.81-141.96	4.80-45.36	1.20-7.07

3-10Y

n	17	17	17	17	17	8
Mean (U/L)	147.18	10.13	57.58	57.86	16.95	6.38
SD (U/L)	58.41	4.70	20.33	26.62	15.43	6.09
CV (%)	40	46	35	46	91	96
Range (U/L)	67-248	3.46-18.10	23.93-93.07	27.97-127.82	1.29-56.64	1.12-20.32

11-20Y

n	12	12	12	12	12	4
Mean (U/L)	89.25	9.94	32.78	29.06	14.8	6.72
SD (U/L)	32.5	9.21	14.95	14.49	8.48	5.91
CV (%)	36	93	46	50	57	88
Range (U/L)	36-128	2.94-11.74	10.42-62	10.95-47.7	3.91-27.67	2.28-14.9

Table V-10: *Correlation between total ALP and its isoenzymes (U/L) for the reference population*. For n=51, r> 0.2743 for p<0.05, r>0.3541 for p<0.01, r> 0.4433 for p<0.001.

	TALP	**B1**	**B2**	**L1**	**L2**
B1	0.5189				
B2	0.8877	0.6196			
L1	0.9363	0.3666	0.7575		
L2	0.5242	-0.1453	0.1688	0.5115	
L3	0.3107	0.0620	0.1786	0.0832	0.4352

respectively. L2 peak (29.74%) was on the top (for the reference population mean ± 2 SDs was 16.81 ± 17.62%) and L3 (24.45%) was increased (reference population: 8.61 ± 6.46%). No intestinal type peak could be detected (raw data in Appendix C4). As this clinical condition is not assumed to affect the liver, the interpretation of these findings was unclear. It could not be established if 'L2?' and 'L3?' peaks really originated from liver or if they were extra-fractions unresolved on MCC as only a very few number of sample (n=2) were available. These findings would be worth while following up as the aetiology of transient hyperphosphatasemia is still unknown.

The ALP pattern in neonates (cord blood samples) presented with substantial 'L1?' and 'L2 ?' peaks, a small B1 and a large B2 peak (see Fig.V-16). This result was in disagreement with the literature and the finding will be further addressed under Discussion.

5–3–3 **Pathological samples** – Interpretation of information given by MCC (data in Appendix C5)

A few samples from children with specific syndromes were analysed by MCC. The primary purpose was to see if patients with syndromes known to affect ALP but with a normal or mildly elevated total ALP activity would show some abnormalities in the distribution of their isoenzymes. It can be seen from Appendices C4 and C5 that all the results fall into the range for the reference population. This was also the case in patients with chronic renal failure (CRF) on total parenteral nutrition (TPN) where abnormal patterns would be expected [Tibi et al, 1991] as CRF is known to induce a raised bone fraction and TPN a raised liver fraction.

Too few samples were analysed to draw some conclusions from the results obtained but it would seem that alkaline phosphatase has a poor tissue specificity when its activity is within the normal range even in clinical conditions expected to affect the isoenzymes.

6 – Discussion

The aim of the work described in this chapter has been to apply the MCC method to the separation of ALP isoenzymes and isoforms. The outcome will be discussed in terms of methodology and clinical findings.

(i) Methodological aspects:

Two particular analytical features of this work differed from the procedures usually described in the literature. The first is the use of LiCl as the salt for the gradient. Typically

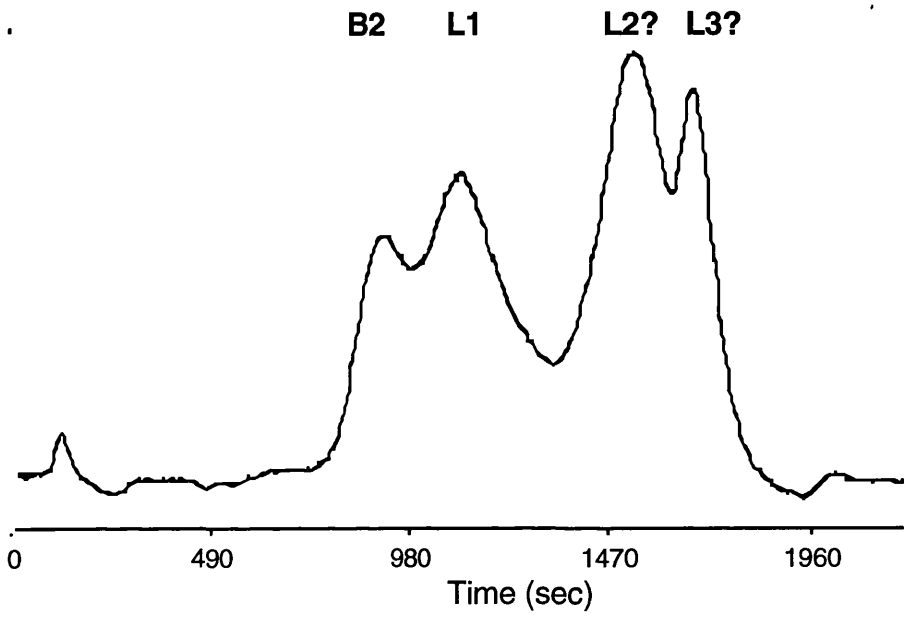


Fig.IV-15: *MCC analysis of a serum from a patient with transient hyperphosphatasemia.* B2, L1, L2 and L3 are as described in Fig.IV-3.

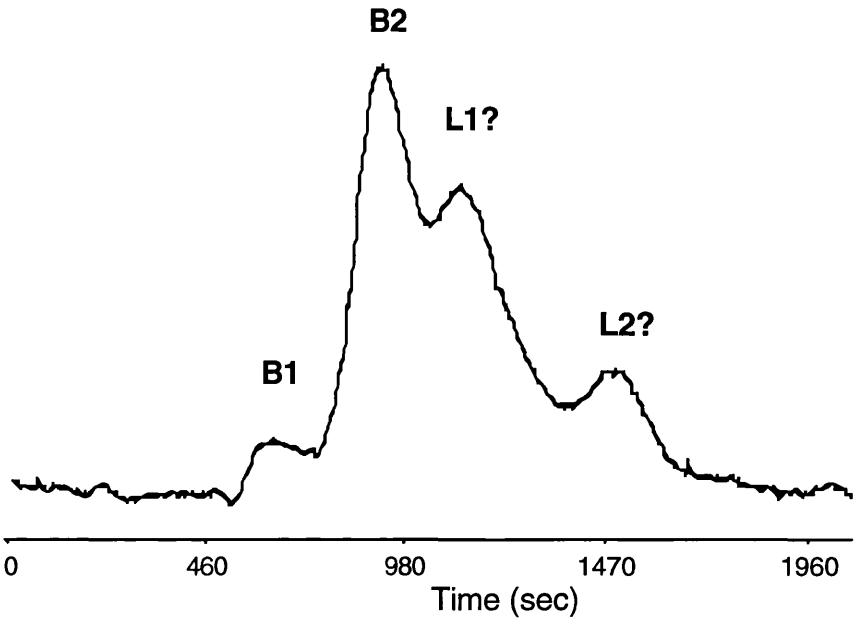


Fig.IV-16: *MCC analysis of a serum from a representative neonate.* B1, B2, L1, and L2 are as described in Fig.IV-3.

NaCl is used but Shoenau's group [Shoenau et al, 1986] noticed that LiCl gave a better resolution than other common salts on their Mono Q column. As the Polymer Laboratories packing material was chosen in this study partly because of its similarities to Mono Q, their findings could be adopted for this work. The second feature was the use of Triton X 100 in the eluting buffers. It is common in the literature to find a detergent in the substrate buffer mainly because it helps reduce band spreading (in this work it allowed a three minutes incubation period, which would normally be considered too long for non-segmented flow). However, electrophoretic methods contain Triton in the running buffer and the analytical gel as it is known to increase the resolution between the biliary and the liver isoenzymes. Based on this information it was decided to include Triton in the running buffers in order to maximise the resolution.

The analytical imprecision of the system used for separating ALP isoenzymes was found to be slightly greater than expected for a chromatographic analysis but in line with the data found for hexosaminidase isoenzymes analysis. Separating haemoglobin variants on a similar system Ersser et al obtained CVs ranging between 0.14% and 0.77% [Ersser et al, 1987]. The increase in imprecision using the present system was therefore thought to be due mainly to the post-column detection rather than the column chromatography. Slight variations in pressure or flow rate are amplified as they induce an increase of the incubation time. Filters placed on each side of the column became rapidly blocked (after a maximum of 30 samples) with fibrinogen and serum debris which resulted in raised pressures and also contributed to the decreasing precision.

Imprecision for ALP isoenzymes activities in clinical samples obtained during this study was high. This was thought to be connected mainly with the manual integration method used. An appropriately designed software providing an accurate and reproducible integration of the peaks would help improving the precision considerably.

One methodological point would need to be particularly addressed in further work, the identity of individual peaks. At present no reference method exist for the analysis of ALP isoenzymes and isoforms, and when Garrido et al [1992] compared three different methods designed specifically for the quantification of bone isoenzymes, they concluded that it is necessary to know what kind of information is required before choosing a method!

(ii) Clinical aspects:

There does not seem to have been any extensive chromatographic studies of ALP isoenzymes in paediatric samples. It was therefore necessary to establish age-dependent reference ranges before undertaking any investigation on clinical specimens.

The results obtained in neonatal samples were, as already mentioned, rather surprising with the finding of 'liver' isoenzymes already during this period of life. Although the samples used for healthy controls were cord blood, the possibility of contamination by maternal blood could be ruled out as the isoenzyme patterns was the same in clinical samples from children younger than 6 months. No chromatographic analysis of ALP on cord blood could be found in the literature, the only study found on neonates used electrophoresis [Crofton, 1987 and 1992]. Her work indicated that term neonates generally present with a single band on electrophoresis, which was identified as bone type by different methods [Crofton, 1987]. A partial desialylation showed a fraction called 'liver-like' but this was poorly defined and considered to be 'trailing' of bone isoenzyme. Her hypothesis for the absence of a liver isoform in infants was that there was either a delayed switch of the hepatic synthesis of the liver ALP, or an altered glycosylation of a liver-derived ALP.

The second hypothesis would allow an explanation for the findings obtained in this study. Taking in consideration that there are no independent criteria other than differences in glycosylation to determine the 'organ-source' of tissue-non-specific ALP isoenzymes, any slight change in charge could induce a 'false' result on electrophoresis and not affect the elution profile on MCC (or *vice versa*). If this was shown to be true, any clinical condition (or drug) likely to induce modifications of the protein glycosylation would have to be considered very carefully before interpretation of ALP isoenzymes analysis. This hypothesis could also explain the finding of liver type isoenzymes in conditions not related to liver disorder like transient hyperphosphatasemia.

The reference ranges in this study were established on the principle that neonates had some liver isoenzymes. When comparing the reference ranges obtained by electrophoresis [van Hoof et al, 1990] with those by MCC, the correlation with age seemed to be much less striking for MCC. However, the populations were smaller with an average age much lower than the one studied by electrophoresis. It would be interesting to carry out a comparison on a larger population with van Hoof's methods as it was noticed that 99% of the children in their study had a low activity of a fraction called 'bone variant' which disappeared in adulthood similarly to the fraction B1 obtained by MCC. Therefore, although the comparison between electrophoresis and MCC in this study showed very weak correlations, other methods may provide a better agreement.

Another interesting finding in this study was the absence of changes in the ALP isoenzyme pattern in very young paediatric patients with normal TALP but with conditions said to affect ALP. This finding is in agreement with previous observations using electrophoresis where the conclusion was drawn that ALP isoenzymes are of little diagnostic relevance in

children younger than 6 months [Crofton, 1992]. The explanation could again be age-related differences in the glycosylation of proteins.

7 – General conclusion

This study has shown that MCC has a potential for the analysis of alkaline phosphatase isoenzymes. Although additional work need to be done on the identification of peaks, some of the clinical results were sufficiently interesting to warrant a larger study in order to clarify some of these questions.

Chapter V: Separation of transferrin isoforms

A – Introduction

Transferrin is a monomeric glycoprotein with the property of reversibly binding iron. It was first isolated and characterised in 1947 by Laurell and Ingelman [Huebers and Finch, 1987].

1 – Biochemistry

1–1 Biosynthesis

The principal site for transferrin synthesis is the hepatocytes. However, two other sites have been shown to be important – the brain, where capillary endothelial cells and oligodendroglial cells synthesise transferrin, and the mammary gland during lactation [de Jong et al, 1990].

Studies of rat embryogenesis suggest that the ability to synthesise transferrin becomes restricted to certain cell types depending on the particular developmental pathway, but the mechanisms involved in the regulation of transcription and translation have not yet been fully elucidated [de Jong et al, 1990].

1–2 Structure of the molecule

Transferrin is a single polypeptide composed of 679 amino acids with two N-linked complex-type glycan chains, and a molecular weight of 79,570 dalton.

The protein is formed by two homologous globular domains which share 40% homology: the N–terminal domain, (Nt), comprises residues 1 to 336 whilst residues 337 to 679 make up the C terminal domain, (Ct). The carbohydrate moieties are located in the Ct domain on residues 413 and 611 (see Fig.V-1). Each domain also contains one metal binding site, binding a ferric ion with a Km of approximately 10^{22} M^{-1}. The Ct terminal site is called the acid stable site; its affinity for iron is 20 times greater than the Nt at pH 6.7. Under physiological conditions the effective affinity constant for the Nt and Ct sites are 1 and 6.10^{22} respectively. The binding of carbonate or bicarbonate at each site is concomitant with ferric ion binding [de Jong et al, 1990].

1–3 Glycosylation

The three main causes for molecular heterogeneity of transferrin are:

- The variations of the polypeptide chain – genetically-determined amino acid substitution or deletion (see Chapter I).
- The differences in iron content (see Section 3–1).
- The differences in the structure of N–linked glycan chains which will now be discussed.

The N–glycosylation is initiated in the rough endoplasmic reticulum (RER) by the synthesis of a lipid intermediate, dolichol–pyrophosphoryl–oligosaccharide with a stepwise addition of the sugar residues (see Fig.V-2). A membrane-associated oligosaccharyl transferase links the oligosaccharide to the transferrin polypeptide; this enzyme is highly specific and transfer will only be made to Asn residues in the sequence Asn–X–Ser/Thr (Cys); X may be any amino acid other than Pro or Asp. In transferrin, two possible sites are available and both are utilised. The last step, processing and elongation of the oligosaccharide chain to its final form, takes place in the Golgi apparatus by the action of mannosidases, glycosidases, glycosyl–phosphoryl–and sulfotransferases. The factors determining which type of glycan is added to a protein (hybrid, complex, high mannose or poly–N–lactosaminoglycan – see Chapter I) has not yet been identified [de Jong et al, 1990]. The transferrin molecule contains two complex-type glycan chains differing in their branching and terminal carbohydrate composition (see Fig.V-3).

Glycan synthesis does not take place via an accurate template mechanism as DNA and polypeptide synthesis. The micro-heterogeneity of transferrin is therefore considerable.

2 – Function of transferrin

2–1 Role in iron metabolism

The transport and delivery of iron to target cells and tissues is well understood and it is now known to be also essential for the growth and differentiation of a variety of cells, and to play a role in bacteriostasis [de Jong et al, 1990]. The adult human body contains between two and five grams of iron depending on body size and sex, equivalent to an average of 50 mg iron/kg. The iron content of haemoglobin represents 30 to 35 mg/kg (between two-thirds and four-fifths of the body iron), 15 mg/kg of surplus iron is stored as

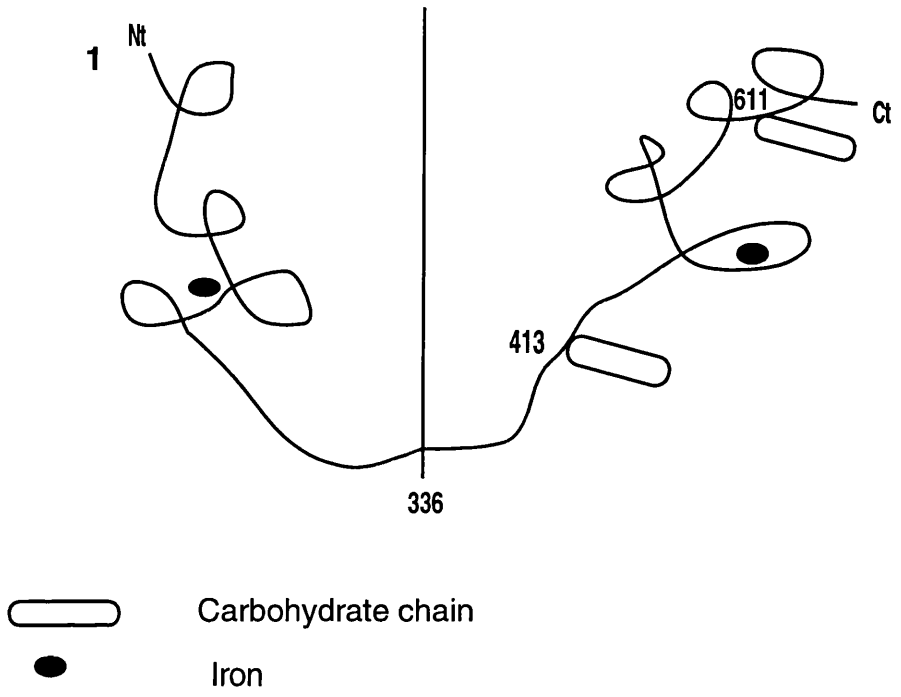
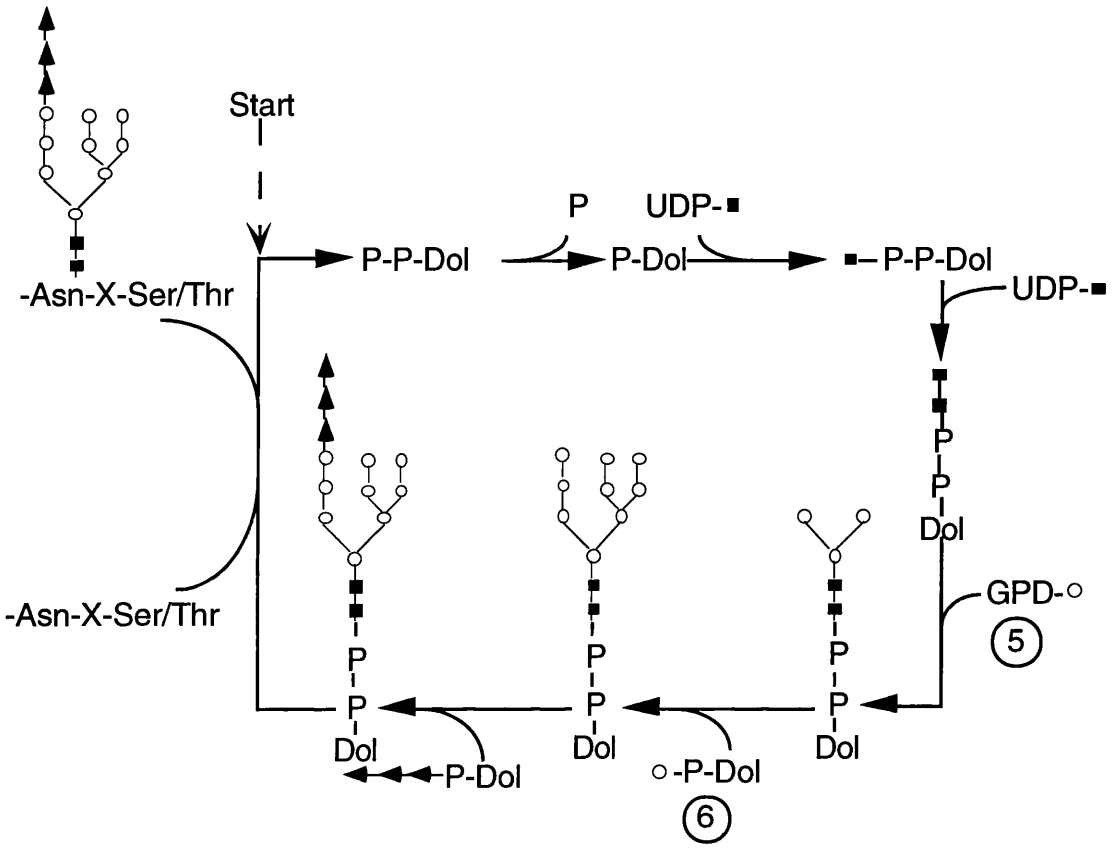


Fig.V-1: *Schematic structure of the transferrin molecule.* The numbers mark the amino acids position, Ct= carbon terminus, Nt= nitrogen teminus.



P-Dol : Dolichol phosphate

■ N-Acetylglucosamine

○ Mannose

▲ Glucose

Fig.V-2: *Stepwise addition of sugar residues.* Steps 5 and 6 are a 'summary' of a highly ordered process. 5= attachment of a β-mannosyl residue N-acetylglucosamine (GlcNac), followed by the attachment of mannosyl α 1–> 3 to the central mannose and mannosyl α-1–> 6, 6= elongation with mannosyl α-1–> 2 then assembly of a polymannose chain on the second branch. The whole process leads to the attachment of 9 mannose residues with GDP-Mannose as a donor.

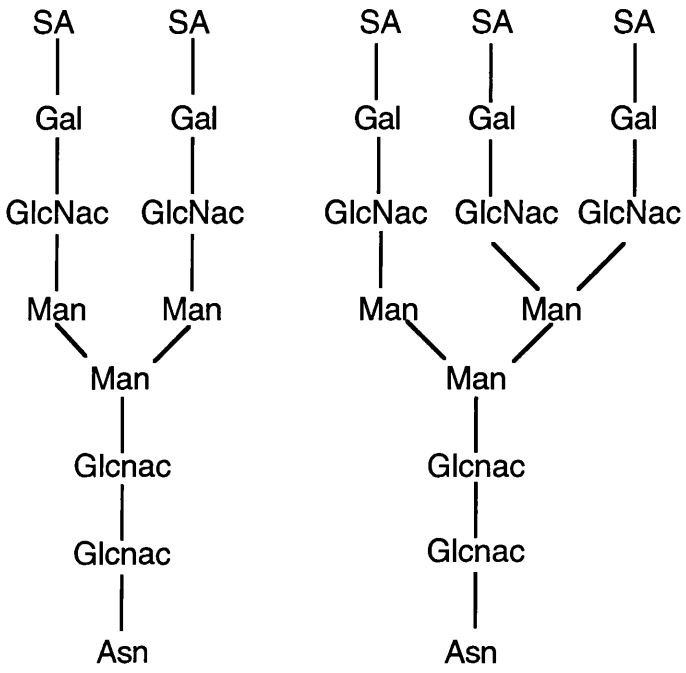


Fig.V-3: *Complex type oligosaccharide –bi and tri antennary– of the transferrin molecule.* SA= sialic acid, Gal= galactose, GlcNac= N-Acetyl-glucosamine, Man= mannose, Asn= asparagin.

ferritin and hemosiderin, mainly in reticulo endothelial cells and hepatocytes, and 4–8 mg/kg are spread throughout body tissues [Huebers and Finch, 1987].

In the normal adult human the physiological loss of iron amounts to 1 mg/day (due to occult bleeding from the intestine, iron in bile, urinary iron etc.) representing less than 1% of the plasma iron turnover. Iron in plasma is mainly bound to transferrin, the haemoglobin iron derived from intravascular hemolysis is less than 100 μg of iron per litre of plasma. Increased non-transferrin plasma iron is present in three situations [Huebers and Finch, 1987]:

- Iron poisoning
- Iron overload in thalassemia
- Tissue damage, iron–containing tissue ferritin can be released in significant amount.

Iron enters the body via the upper small intestine and is distributed to body tissues bound to transferrin.

The concentration of transferrin is regulated by a complex interplay of factors [Huebers and Finch, 1987]. It is known to increase in cases of iron deficiency resulting for example from infection, malignancy, or inflammation, in anaemia and pregnancy and to decrease in case of hemochromatosis (either primary, or acquired by repeated transfusions).

As has already been indicated the Km for the binding of iron to the two binding sites differs. Thus transferrin will exist in three different states under physiological conditions: apoferric (with no iron bound), monoferric and diferric. The total plasma concentration of transferrin is of the order of 4 μmol/l.

2–2 The receptors

Two kinds of tissue receptors bind the transferrin molecule– the asialoglycoprotein receptor and the 'transferrin' receptor.

(i)–The asialoglycoprotein receptor

The clearance of mammalian glycoproteins from the circulation following removal of sialic acid was first described in 1968. Proteins, such as transferrin, with an exposed galactose residue bind to asialoglycoprotein receptors (ASGP-R) with a high affinity (Kd= 10^{-9} to 10^{-8} M). Glycoproteins with the highest affinity ligand have at least three galactose moieties, which bind to specific sites on the receptor [Lodish, 1991]. For

transferrin, binding to the receptor is necessary, but may not be sufficient for endocytosis [Ashwell and Harford, 1982].

ASGP-R is the major protein on the sinusoidal or blood-facing surface of the hepatocyte plasma membrane and liver damage can result in a decrease of functional ASGP-R and therefore in an increased level of circulating asialo–glycoproteins [Drickamer, 1988]. ASGP-R is a stable hetero-oligomer constituted of two subunits, 58% identical in sequence, both of them being type II membrane proteins i.e. they span the membrane with Nt facing the cytoplasm. The biosynthesis, oligomerization, endocytosis and recycling of the two units are independent; the units can still interact to form a high affinity receptor for triantennary glycopeptides including transferrin [Lodish, 1991]. Once the ligand is transported to the lysosomes, the receptor is recycled to the plasma membrane.

It has been shown that the rate of synthesis of the receptors is lower than the rate of endocytosis and it was postulated that the receptors may reorient in the lysosomal membrane to avoid proteolysis and then recycled to the cell surface.

(ii)–The transferrin receptor

These receptors are essential for the supply of iron to body tissues as the total number of receptors determine the iron uptake. It has been shown that the number of receptors on erythroid bone marrow is proportional to the amount of iron required. As the red blood cells mature beyond the reticulocyte stage, their receptors, and thus their capacity to take up iron, are lost. In iron deficiency the number of both erythroids and non–erythroid receptors increases [Huebers et al, 1990].

The transferrin receptor is a large molecule, consisting of two identical subunits with a total MW of 76,000 Da, containing carbohydrate residues, and with a capacity to bind two molecules of transferrin. Each subunit is split into three parts, an extra-cellular fragment of 70,000 Da, an intra-cellular fragment of 5,000 Da and a fragment of 15,000 Da, which spans the cell membrane. The association constant is high although variable, usually 2.10^7 $M^{-1}min^{-1}$ to 2.10^8 $M^{-1}min^{-1}$ [Young and Bomford, 1984]. Neuraminidase does not destroy the function of the receptors, thus the carbohydrate moiety does not seem to be vital for transferrin binding [Young and Bomford, 1984].

Not all the plasma transferrin is saturated with iron but this does not interfere with the iron uptake as the monoferric form of transferrin is 1/7 as effective a donor as the diferric form.

Once the iron is released within cells it needs to be transferred to its site of utilisation, mainly for incorporation in haemoglobin, cytochromes, and some for storage in ferritin.

The receptor / transferrin complex is then recycled to the membrane. Since the transferrin half-life is 7–10 days, whilst that of iron is only one to two hours, the cycle may be repeated 300 times before degradation of transferrin occurs.

3 – Abnormal transferrin in human diseases

3–1 Transferrin and CSF leaks

There are no asialoglycoprotein receptors in the brain and therefore all the forms of transferrin can be detected in CSF. As asialotransferrin is only found in aqueous humour and perilymph in addition to CSF [Keir et al, 1992], the determination of asialotransferrin (or tau-protein) is used as a specific means of diagnosing liquorrhea, a generic term for the discharge of CSF from the nose or the ear. This may occur due to traumatic fracture, surgical procedures, encephalocoeles, infection and intra or extra-cranial tumours. The condition carries a high risk of inducing meningitis.

3–2 Alcoholism

Alcoholism is a world-wide problem, and a specific clinical marker for ethanol consumption has been sought for years. Ethanol is the most widely used centrally-acting compound known; it has immediate and direct biochemical and biophysical effects on cell membranes, which are similar to those of an anaesthetic, as well as metabolic consequences through its oxidation. It commonly produces simultaneous nutritional deficiencies and rapidly produces biological tolerance.

Present markers in plasma for detecting alcohol abuse include γ–glutamyltransferase (reported to be elevated in 54–88% of chronic alcohol abusers, which decreases slowly during abstinence), aspartate aminotransferase, glutamate dehydrogenase, and mean corpuscular volume (MCV) of red blood cells [Kwoh–Gain et al, 1990]. Increased concentrations of serum α–lipoprotein and iron in abusers have also been reported [Stibler et al, 1986]. These markers have the following disadvantages:

- Poor specificity, as disorders other than alcoholism may induce increased plasma levels of the marker.
- Insufficient sensitivity to detect abuse before organ damage has occurred.
- Lack of information about the kinetics of these markers relative to duration and amount of alcohol intake.

In 1976 Stibler and Kjellin reported an abnormal micro heterogeneity in transferrin in CSF and serum from patients with alcoholic cerebellar degeneration. They, and others, have subsequently shown that the heterogeneity is caused by transferrin molecules with a deficiency in their carbohydrate moiety. Carbohydrate deficient transferrin (CDT) has therefore been recommended as a specific marker for alcohol abuse as it is related to the accumulated effect of alcohol consumption. Extensive studies have shown that CDT appears in plasma after the intake of 50–80 g of ethanol/day for at least a week and disappears within 10 days of abstinence. The appearance of CDT is not affected by other disorders or concomitant liver disease [Stibler et al, 1986; Kwoh–Gain et al, 1990; Jeppsson et al, 1993].

The mechanism of the formation of CDT is not known. The only explanation offered to date [Stibler, 1991] suggests that ethanol interferes with several steps in glycoconjugate turnover. The CDT is shown to be unrelated to the total concentration of transferrin, and therefore, ethanol is unlikely to affect the synthesis of transferrin [Stibler, 1991]. Some studies on rat brain and liver demonstrate an inhibition of glycosyl transferase depending on blood alcohol concentration. In humans, impaired function of tubulin polymerisation necessary for the secretory process and, a reduced activity of three glycosyl transferases in serum have been reported.

Thus CDT appears to be a specific marker for alcohol-related diseases but could also be used to study the effect of alcohol on glycoprotein metabolism.

B – Development and assessment of a micro column chromato graphic method for transferrin

1– Aim

The purpose of the work described in this chapter was to investigate the potential of using MCC for separating isoforms of transferrin. The performance of MCC was compared with electrophoresis and IEF by analysing a variety of samples from subjects with clinical conditions known to change the isoform pattern of transferrin.

2– Materials

2–1 Chemicals

Bis-Tris ($C_8H_{19}NO_5$), Barbital CIV (barbitone,5,5-diethyl-barbituric acid) and Barbital CIV-sodium salt, 6,9-diamino-7-ethoxyacridine lactate (Rivanol), 3-amino-9-ethylcarbazole, 4-chloro-1-naphtol, *Vibrio cholerae* type II, *Clostridium perfringens* and human holo-transferrin (SIGMA, Poole, Dorset, UK)

IEF agarose, Pharmalytes 4-6.5 and Ampholine 4-6 (Pharmacia, Milton Keynes, UK), agarose ME (SEAKEM, Flowgen, Sittingbourne, UK), Nitro-cellulose (Sartorius, Epson, UK), Resolytes 4-8 (BDH Chemical, Poole, Dorset, UK) hydrogen peroxide (H_2O_2 30% Vol.) (Merk, Lutterworth, UK), Methanol AR Quality (Hayman Ltd, Essex), Desferral [Desferrioxamine Mesylate] (Ciba Pharmaceutical, Horsham, Sussex).

Anti-human transferrin IgG fraction goat (Incstar Ltd, Berkshire), rabbit immunoglobulins to human serum, peroxidase conjugated swine immunoglobulin to rabbit IgG and peroxidase conjugated rabbit anti-goat (Dako Ltd, Bucks, UK).

2–2 Packing materials

DEAE SEPHACEL, 'High Trap' cartridges and 'Resource Q' column (Pharmacia, Milton Keynes, UK); DEAE Affi-blue gel (Bio-Rad, Hertfordshire, UK).

2–3 Equipment

Pharmacia power packs Electrophoresis Constant Power Supply EPCS 3000/150 connected to Volt. Integrator VH-1 and Electrophoresis Power Supply EPS 500/400 were used.

Molecular Dynamics 'Personal Densitometer' with Image Quant software was used for scanning IEF and electrophoresis blots.

2–4 Samples

Serum and CSF samples received for routine analysis (for conditions assumed not to affect transferrin) were supplied by the Clinical Neurochemistry Laboratory, Institute of Neurology and Neurosurgery, London, and used to test the separation method.

Samples from patients with alcoholism related disorders were kindly provided by Dr R. Sherwood from King's College Hospital, London, and by the Department of Chemical Pathology at the Royal Free Hospital, London.

3 – Sample preparation

The first part of this study describes attempts to isolate transferrin from plasma before separating the isoforms of the protein as a post-column reaction could not be used.

3–1 Rivanol followed by ammonium sulphate

3–1–1 Methods

0.4 ml of distilled water was added to 1 ml of serum and 0.6 ml of 3% Rivanol solution was then added dropwise with vortexing. The mixture was centrifuged at 11,000 rpm for 10 min. The supernatant was then decanted and 0.85 ml 25% NaCl added, vortexed and the mixture centrifuged as previously. The clear supernatant contained IgG and transferrin. To remove IgG, one volume of the clear supernatant was mixed with one volume of saturated ammonium sulphate, the mixture allowed to stand for 10 minutes and centrifuged for 15 min as previously [Heide et Schwick, 1978]. Finally the samples were dialysed overnight against water.

3–1–2 Results

Purification of transferrin by Rivanol precipitation followed by ammonium sulphate precipitation and dialysis gave a yield of 20%. Fig.V-4 shows results following electrophoresis stained with Coomassie Blue of a serum from normal adult and from a patient with Carbohydrate Deficient Glycoproteins Syndrome (a condition known to affect transferrin isoforms, see Chapter VI) before and after purification. Only the transferrin bands stained after the purification which, appeared to be successful.

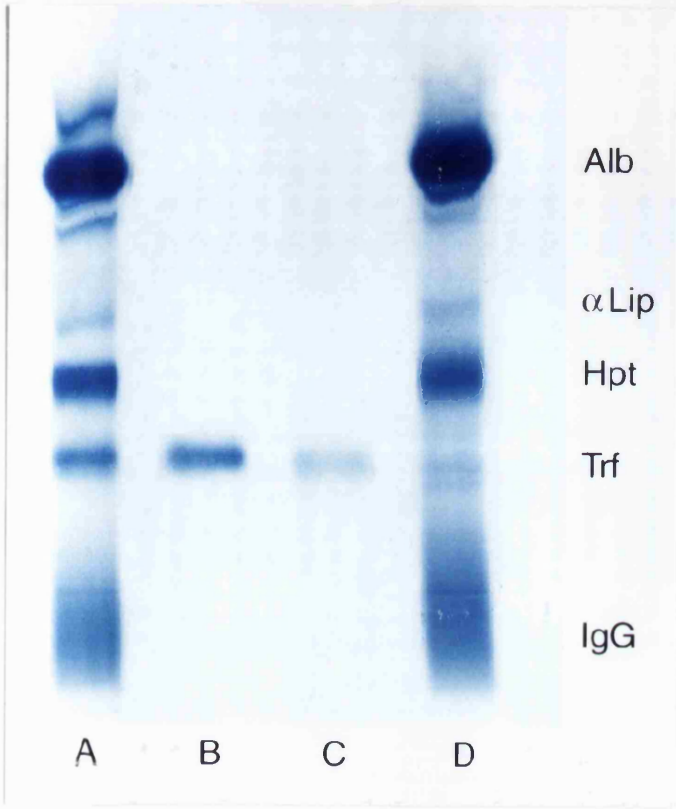


Fig.V-4: *Detection with Coomassie Blue stain of serum before and after treatment with Rivanol and ammonium sulphate.* Lane A= 1 µl of normal serum applied neat, Lane B= 5 µl of normal serum treated with Rivanol plus ammonium sulphate, Lane C= 5 µl of serum from a patient with CDGS treated with Rivanol plus ammonium sulphate, Lane D= 1 µl of serum from a patient with CDGS applied neat.

All the electrophoresis runs shown in this chapter are presented with the anode at the top of the figure.

However, when the same treated samples were immunostained or chromatographed (detection at 280 nm), the results were different. As can be seen from Fig.V-5 both immunostain and MCC showed albumin and immunoglobulins to be major contaminants. It was therefore decided that the level of purification obtained by this method was not sufficient to justify the time-consuming sample preparation.

3–2 DEAE-Affi blue gel

3–2–1 Method

DEAE-Affi blue gel combines the properties of ion exchange and pseudo-ligand-affinity. When used with 0.02mol/L phosphate buffer, pH= 7, it has been shown that all human serum proteins bind to the gel with the exception of transferrin and IgG [Werner et al, 1983].

3–2–2 Results

It was not possible to totally reproduce Werner's results [Werner et al, 1983]. A typical profile after chromatography (serum diluted 1/10) on DEAE Affi-blue gel and the corresponding fractions analysed on electrophoresis revealed with gold stain, is shown in Fig.V-6. The electrophoresis pattern indicated that immunoglobulinss are eluted in the first fractions (first peak), albumin is eluted with a high salt concentration only (1.4 mol/L), but transferrin is not resolved from the bulk of the proteins and is contained in one single peak together with haptoglobin and possibly α lipoproteins.

3–3 Albumin removal

The presence of albumin in serum could in theory cause at least two types of problems during the analysis of transferrin isoforms by the methods chosen in this study. Firstly, when IEF is used for the separation, albumin is known to create a 'shadow' which, sometimes covers the pI 5.7 isoforms (disialotransferrin) and thus impairing the quality of resolution [van Noort and van Eijk, 1990]. Secondly, serum contains much more albumin than transferrin (about 40 g/l and 4 g/l respectively) and, therefore, the albumin could saturate the column. In addition, it was shown in Fig.V-5 that a major fraction of albumin had a retention time similar to the main transferrin peak.

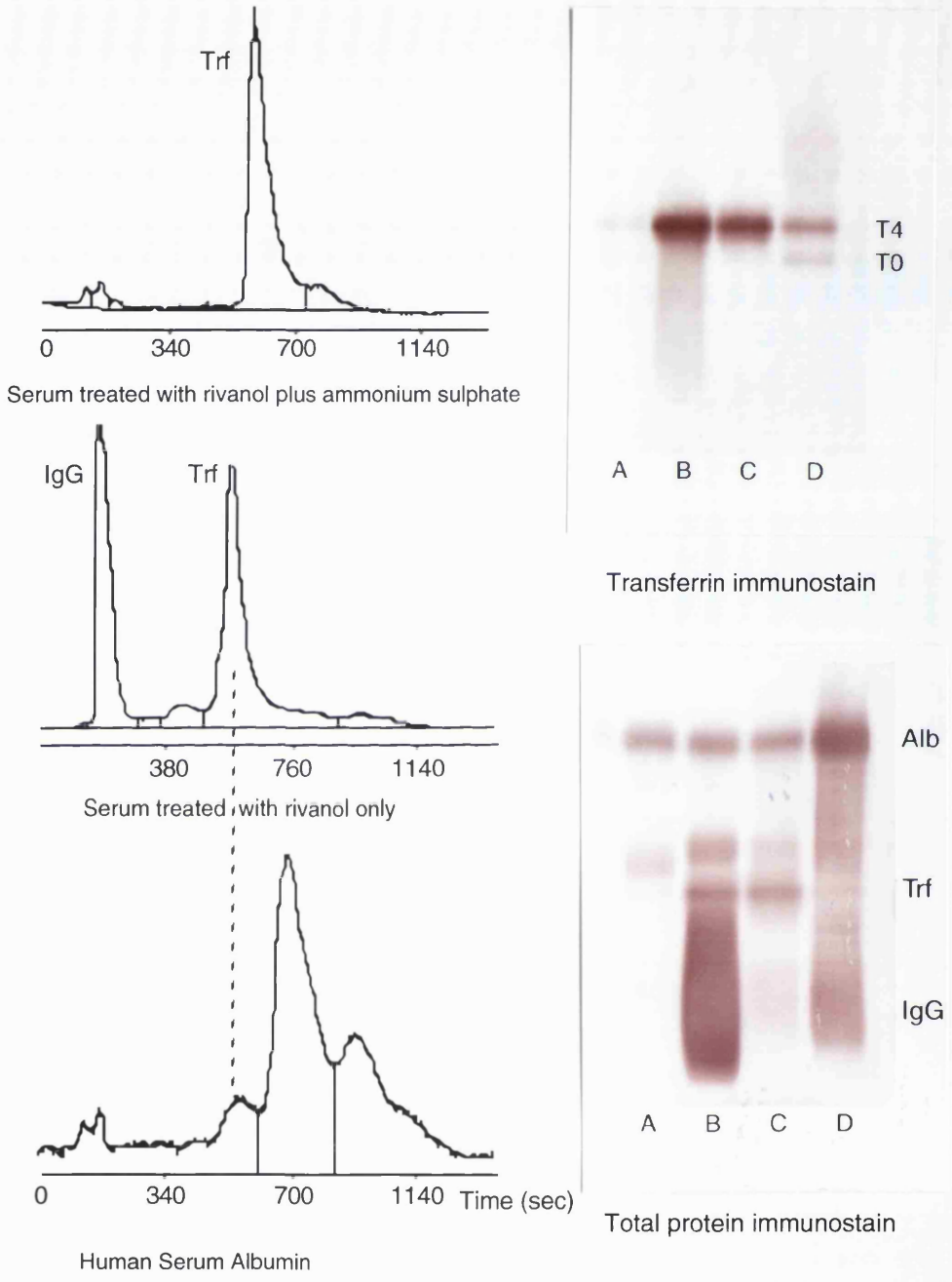
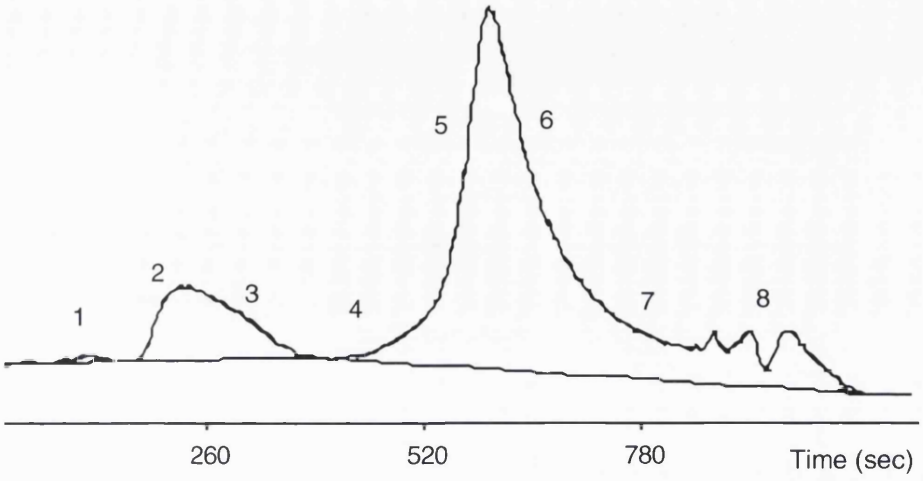
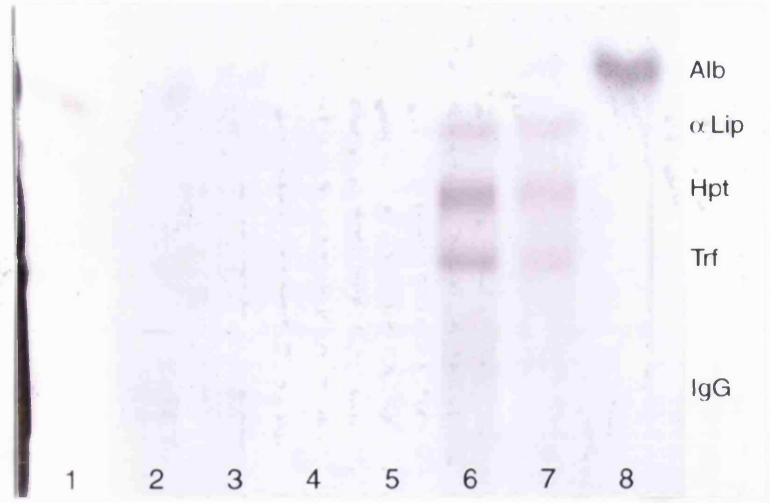


Fig.V-5: *Rivanol treated samples analysed by chromatography and electrophoresis.* Lane A= CSF, Lane B= Serum, both treated with Rivanol only, Lane C= Serum treated with Rivanol plus ammonium sulphate, Lane D= Control CSF. Alb= albumin, Trf= transferrin and IgG= Immunoglobulins G.



Chromatogram of serum proteins separated onto Affi-Blue gel column
(20 mm x 6.4 mm i.d)



Electrophoresis of the fractions collected followed by gold stain

Fig.V-6: *Level of purification of serum transferrin obtained with Affi-Blue gel.* Numbers represent the fractions collected and analysed on electrophoresis. Chromatographic detection= 280 nm. Alb= albumin, α-lip.= α–lipoproteins, Hpt= haptoglobin and Trf= transferrin. Electrophoretic detection= Gold stain, performed according to Keir and Thompson, 1988.

3–3–1 Methods

3–3–1–1 High trap

A High Trap column was used to remove the bulk of the albumin from the sample analysed. These cartridges are said to have a binding capacity of 20 mg of human albumin per ml of gel and to give an effective resolution with a flow rate up to 4 ml/min, and at a pH of 4-12 with any commonly used buffer.

The method used was as recommended by the manufacturer: Bis-Tris buffer pH 7.0, flow rate 1 ml/min, loading of 500 µl of serum diluted 1/10 with buffer (equivalent to 200 µg of albumin) and collecting 500 µl fractions.

Immunostaining of the fractions collected from the column was performed as described in Section 5-2-2 of this chapter with rabbit IgG anti human serum proteins as a first antibody.

3–3–1–2 Immunoprecipitation

Albumin was also removed from serum by immunoprecipitation.

Serum samples, diluted 1/200 with water, were mixed with goat-anti-human albumin in a one to one ratio and left mixing overnight at room temperature. After centrifugation for 10 min the supernatant was used for analysis.

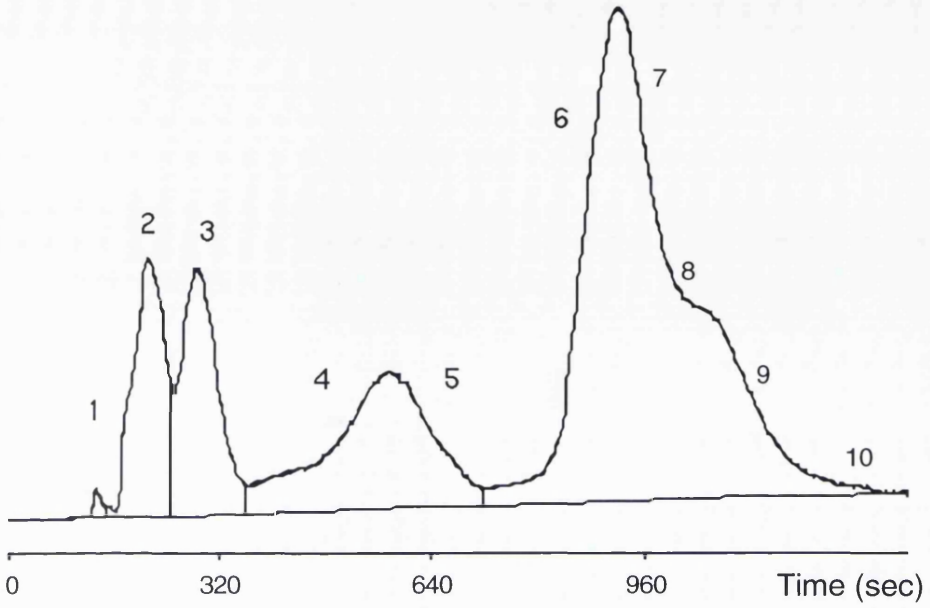
3–3–2 Results

Despite following the manufacturer instructions the High Trap cartridges were not sufficiently efficient in removing albumin, as it was present in every fraction collected (see Fig.V-7a).

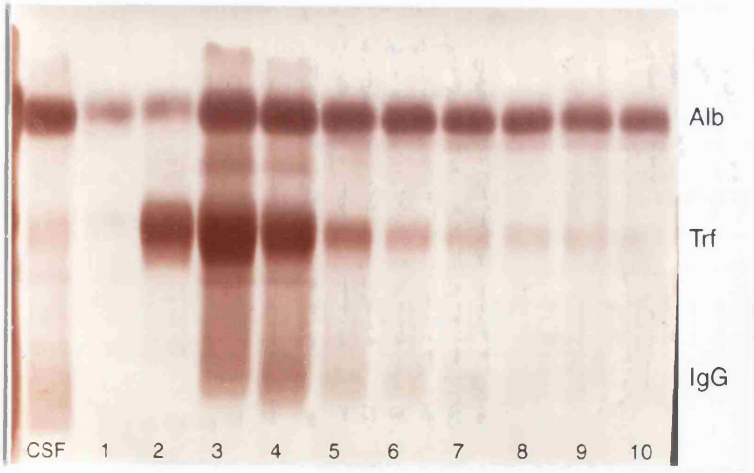
The only satisfactory method was immunoprecipitation (see Fig.V-7b) but it was not practical to use this method combined with MCC because of the amount of antibody required per sample.

3–4 Iron saturation and chelation

Although from the literature saturation of transferrin with iron seemed an important requirement for accurate analysis, it was not known whether it was important under the conditions used for IEF in this study. The purpose of iron saturation is to limit the number of peaks and bands by converting all the transferrin isoforms to their diferric form.



Profile of serum separated on High Trap cartridges



Electrophoresis of the fractions collected after chromatography followed by immunostain for human serum proteins

Fig.V-7a: *Albumin removal by High-Trap cartridges.* -numbers represent the fractions collected. Chromatographic detection= 280 nm. Pre-Alb= pre-albumin, Alb= albumin, Trf= transferrin. Antibody used for the electrophoresis= rabbit IgG anti human serum proteins.

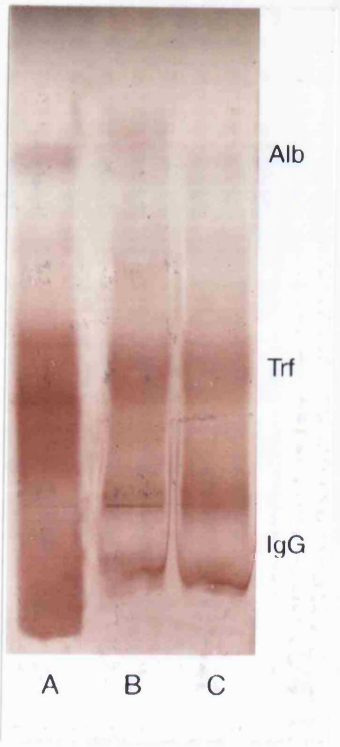


Fig.V-7b: *Albumin removal by immunoprecipitation.* Electrophoresis of the treated samples followed by immunostain for human serum proteins. A= Normal serum treated with Rivanol only, B= Normal serum treated with goat-anti-human albumin, C= Normal CSF treated with goat-anti-human albumin. Control level of albumin in normal serum using the same staining procedure can be seen in Fig.V-7a.

Ampholytes used for IEF are however known to be metal chelators and will compete with the complex Fe(III)-transferrin to form Fe-ampholytes. A long pre-focusing of the gel is necessary to neutralise this effect of ampholytes [van Noort and van Eijk, 1992]. Thus, it was felt necessary to investigate the effect of iron saturation on the resolution of transferrin isoforms by IEF.

Iron saturation has the added advantage of aiding detection following MCC as Fe-transferrin has an absorption maximum at 460 nm giving a 10 fold increase in sensitivity compared with detection at 280 nm [Jeppsson et al, 1993].

3–4–1 Methods

Iron chelation prior to analysis on IEF was performed with Desferrioxamine. Serum was diluted 1/10 in 0.1 mol/L acetate buffer pH 5.1 and 2 mg of Desferrioxamine added per 100 µl of serum (100 times excess). The procedure was carried out and compared at 4°C, room temperature and 37°C.

The samples were saturated with 10 µmol/L Fe(III) citrate in 0.5 mol/L $NaHCO_3$ at room temperature for one hour [de Jong and van Eijk, 1988], prior to analysis on chromatography in order to maximise the response at 460 nm [Jeppsson et al, 1993].

A recent recommendation to remove lipids and lipoproteins before analysis on chromatography was also incorporated, cleaning up the sample [Jeppsson et al, 1993]. 10 µl of Dextransulfate (100g/l) and 50 µl of $CaCl_2$ (1 mol/L) were added to 1 ml of serum. The supernatant was used for analysis after ultra centrifugation.

3–4–2 Results

Results of investigations on the iron status for IEF are shown Fig.V-21 and discussed in details in Section 6-2 of this chapter.

Based on Jeppsson's findings, iron saturation was incorporated in the sample preparation prior to MCC.

3–5 Conclusion

Sample purification using Rivanol and DEAE-Affi blue gel were not adequate for the purpose of this study. Because albumin has been shown to impair the resolution of transferrin isoforms using IEF [van Noort and van Eijk, 1990], sample cleaning was still thought to be an important factor for the quality of analysis. Albumin removal with High

Trap cartridges was tried unsuccessfully and immunoprecipitation, although satisfactory, was not a practical alternative. However, as will be shown later in this chapter, the presence of albumin did not induce any significant differences in the IEF pattern when immunofixation was used.

The conclusion was that a specific detection method would provide a better approach to the resolution and detection of transferrin than sample purification. Therefore it was decided to detect iron-saturated transferrin at 460 nm after MCC analysis and to use immunofixation for IEF and electrophoretic methods.

4 – Micro column chromatography

4–1 Method

The MCC method described in this chapter differs to some extent from the general conditions established in Chapter II because of the nature of the protein to be separated.

4–1–1 Running conditions

Fig.V-8 shows a diagram of the system used.

Buffer A was Bis-Tris 20 mmol/L, pH 6.2, and buffer B Bis-Tris 20 mmol/L plus NaCl 0.5 mol/L pH 6.2.

The column was Resource Q 1 ml (Pharmacia).

The equipment chosen was the Glycomat as no peak was expected to elute in the void volume.

The gradient was a modified version of the gradient used by Jeppsson [Jeppsson et al, 1993]. A flow rate of 1 ml/min was initially tried but the capacity of the syringes did not allow a complete analysis cycle as the GMAT does not have the facility of being able to refill the syringes during a run. A flow rate of 0.5 ml/min with a longer time analysis was therefore chosen for the analysis and the gradient was as follows:

Time	%buffer B	Flow rate(ml/min)
0	0	0.5
180	0	0.5
300	7	0.5
1500	25	0.5
1700	50	0.5

followed by a wash at 1 ml/min with 0.5 mol/L NaCl to regenerate the column.

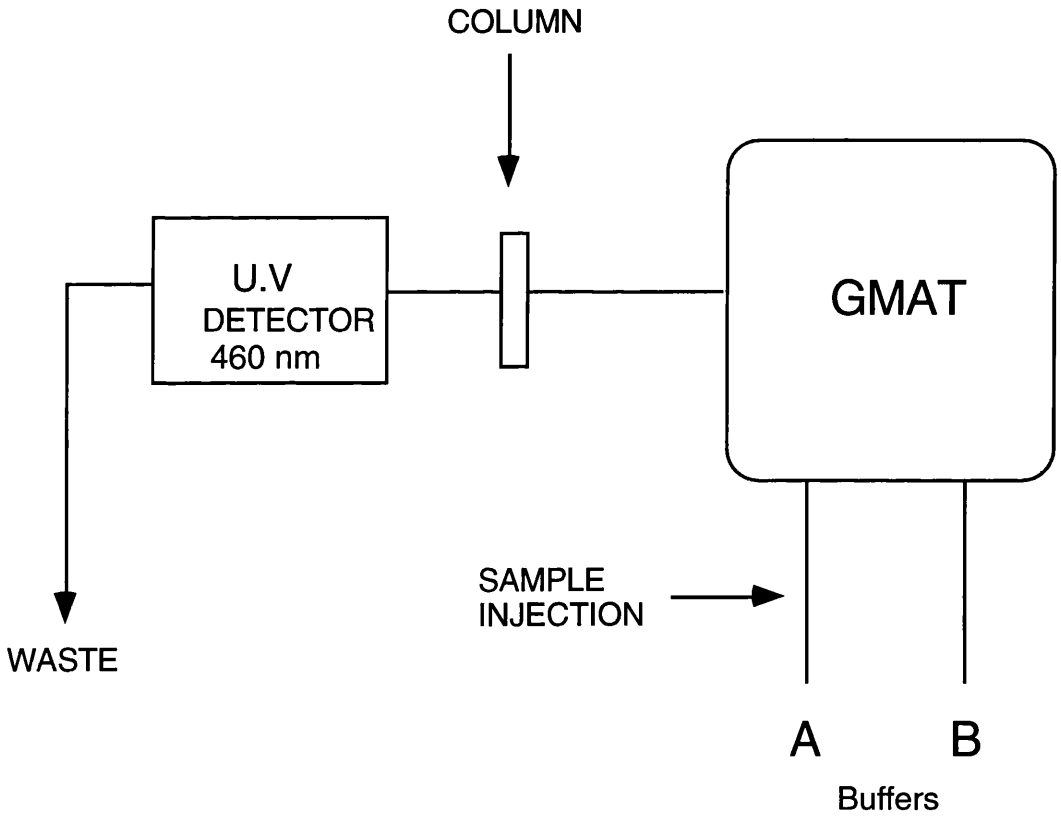


Fig.V-8: *Schematic representation of the system used for MCC of transferrin isoforms.*

4–1–2 Sample preparation

Jeppsson's method was chosen (see Section 3–4–1). The supernatant was diluted five times with the Bis-Tris 20 mmol/L, pH 6.2 buffer and a total of 170 µl was loaded onto the column.

Neuraminidase digestion was carried out according to the following procedure. Serum was diluted 1/100 in water and 5 µl of neuraminidase added for every 25 µl of diluted sample. The mixture was left to incubate for 2 or 24 hours at 37°C for a partial or a complete digestion respectively. The complete digestion was used to confirm the migration of asialotransferrin whereas a partial treatment allowed the presence of various isoforms.

4–1–3 Quantification of the peaks

As the DS 4000 software was not adequate for integrating the peaks in a reproducible manner, manual integration was adopted. Peaks were assumed to be gaussian in shape and their area was calculated according to the following formula:

Area = Height of the peak x width of the peak at half height.

The relative percentage of each peak was then calculated. As discussed in the previous chapter this method is likely to introduce bias and imprecision into the quantitation.

4–2 Results

4–2–1 Analytical performance

4–2–1–1 Specificity

No suitable reference material could be found for the analysis of the transferrin isoforms. The commercial human transferrin (holo-Transferrin) was found to have a different mobility from that of normal serum. This was confirmed by Sherwood, 1993, (personal communication) and was therefore of little use. Thus the identification of each isoform was made by using a combination of two methods: (i) by comparing runs of different samples, and (ii) by collecting the fractions eluted off the column and analysing them by electrophoresis followed by immunostaining (for details see Section 5 of this chapter).

(i) Comparative runs: three isoforms can easily be identified, as shown in Fig.V-9a, by comparing a) normal serum, b) normal serum partially digested with *Clostridium Perfringens* and c) serum from a patient with alcohol-related disorder (ARD), where the disialotransferrin is known to be present (0.39% to 9.06%) [Kwoh-Gain et al, 1990]. With

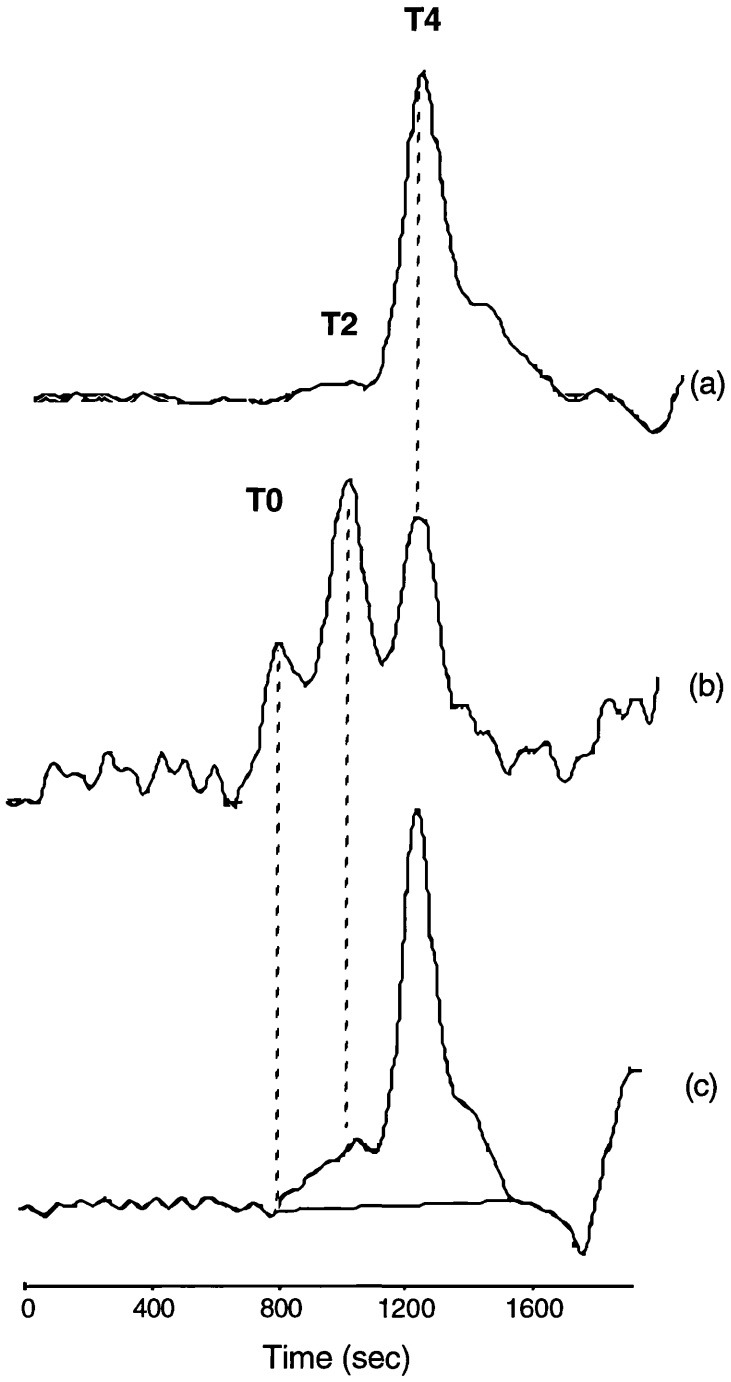


Fig.V-9a: *Identification of transferrin isoforms obtained by MCC using comparative runs.* a= Normal serum, b = Serum digested with *Clostridium Perfringens* for 2 hours at 37°C, c= Serum from a patient with alcoholism related disorder. T0= Asialo transferrin, T2= Disialotransferrin, T4= Tetrasialotransferrin.

a superimposition of those three chromatograms it could be deduced that the first peak is asialotransferrin (T0), the second peak disialotransferrin (T2), and the third peak tetrasialotransferrin (T4). Most of the chromatograms also showed a poorly resolved peak, eluting after T4, identified as being penta- and hexasialotransferrin (T5 and T6).

(ii) Collecting fractions from MCC: fractions were collected after chromatography of a serum from a patient with ARD and analysed by electrophoresis. The disialo- and tetrasialotransferrin have a difference in surface charges, which result in different electrophoretic mobilities. The migration of fraction 2 was more cathodal (less charged) than fractions 3, 4 and 5, but not as much as the T0 of the marker (see Fig.V-9b). It was therefore identified as disialotransferrin.

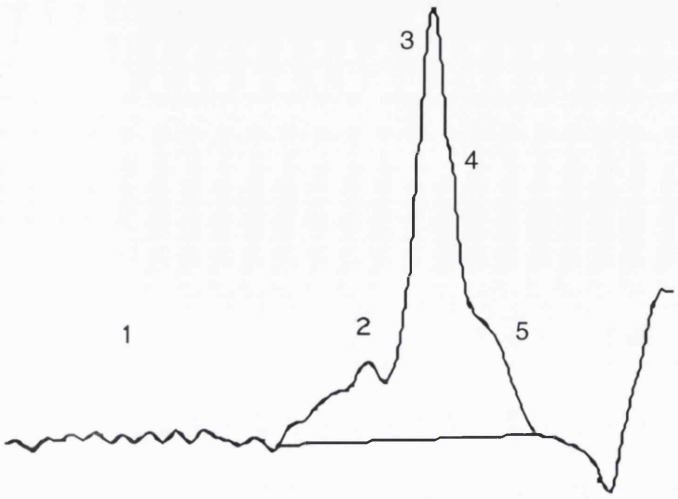
A peak was frequently noted to appear toward the end of the chromatogram. This peak was identified as protein-bound bilirubin which absorbs at 460 nm. The peak size was found to correspond to the concentration of bilirubin estimated in the routine laboratory (see Fig.V-10).

4–2–1–2 Retention times (RT)- (Raw data in Appendix D1)

The separation of the transferrin isoforms by MCC generally resulted in two distinct peaks (T2 and T4). This offered an opportunity to study the reproducibility of the retention times on Resource Q column in some detail. Only one column was used during this work.

(i) Within-batch variability: Two types of sera were analysed, from a normal adult and from a patient with alcohol related disorder. Table V-1 and Fig.V-11 show the results obtained with CVs less than 1%. Therefore the reproducibility of the retention times was considered to be good.

(ii) Between-batch variability: The same two samples were run in four consecutive batches over a period of 4 to 5 days. Table V-2 shows that between-batch CVs were larger (between 4.2% and 7.1%) than the within-batch CVs. However the CV for the relative retention times was less than 1% for the samples from both healthy subjects and patients with ARD, implying a good reproducibility of the gradient. As shown in Fig.V-12 the retention times increased with the batch number, therefore, the question of ageing of the column was addressed.



Chromatogram of serum sample from patient with alcoholism related disorder

Electrophoresis followed by transferrin immunostain

Fig.V-9b: *Identification of transferrin isoforms on MCC by analysing the collected fractions on electrophoresis.* Numbers represent the fractions collected from chromatogram. A= control CSF.

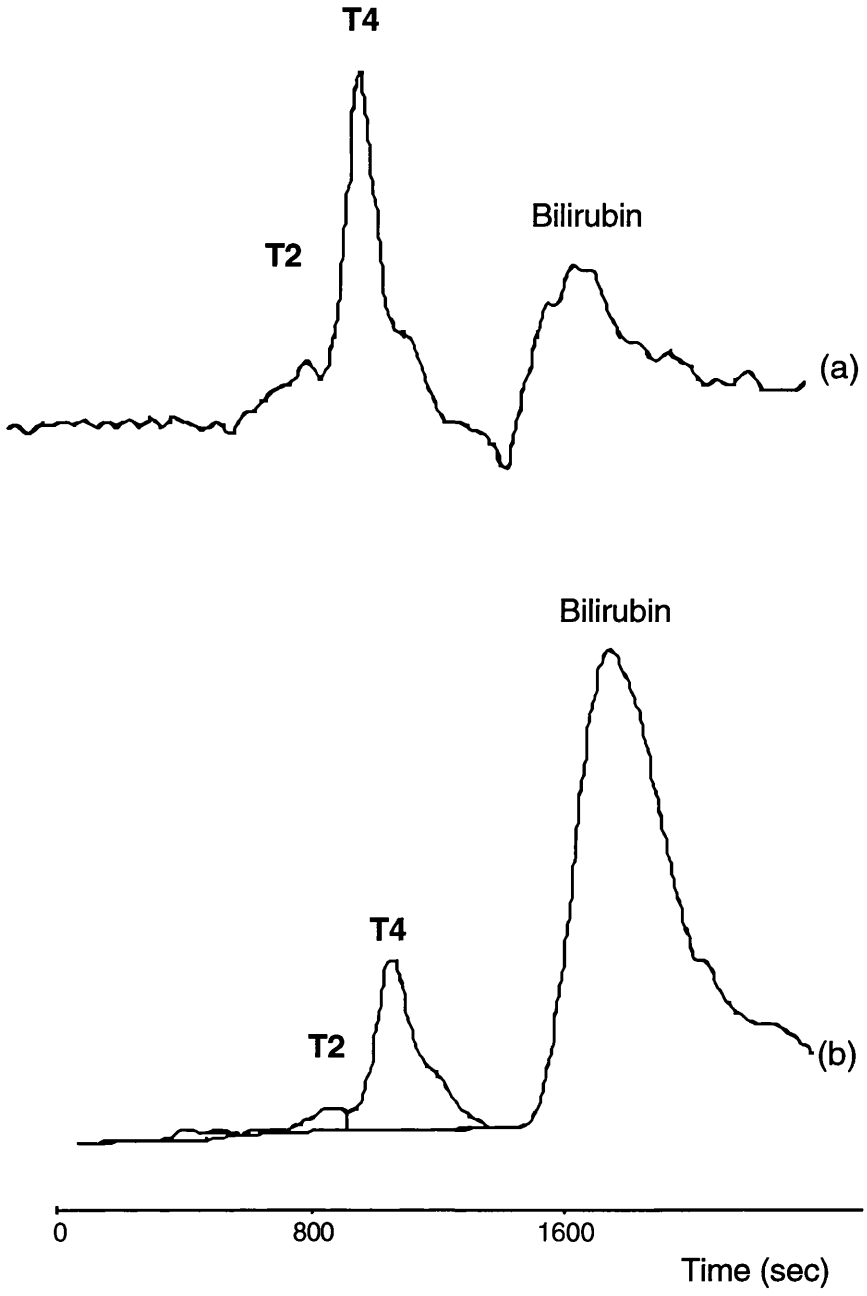


Fig.V-10: *Typical transferrin pattern obtained by MCC analysis in serum from patient with ARD showing protein-bound bilirubin.* a= Serum from a patient ARD, bilirubin = 7 µmol/L, b= Serum from another patient with similar condition, bilirubin= 63 µmol/L. T2 = Disialotransferrin, T4 = Tetrasialotransferrin.

Table V-1: *Statistical data for the within-batch variations of RTs for the isoforms of transferrin in two samples.* RT2= Rt for disialotransferrin, RT4= RT for tetrasialotransferrin.

	n	RT2	RT4	RT4/RT2
NORMAL	4			
Mean (sec)		1307	1346	1.03
SD (sec)		14.4	5.2	0.01
CV (%)		1.1	0.4	0.83
ARD	4			
Mean (sec)		1303	1338	1.03
SD (sec)		5.9	4.97	0.01
CV (%)		0.5	0.4	0.65

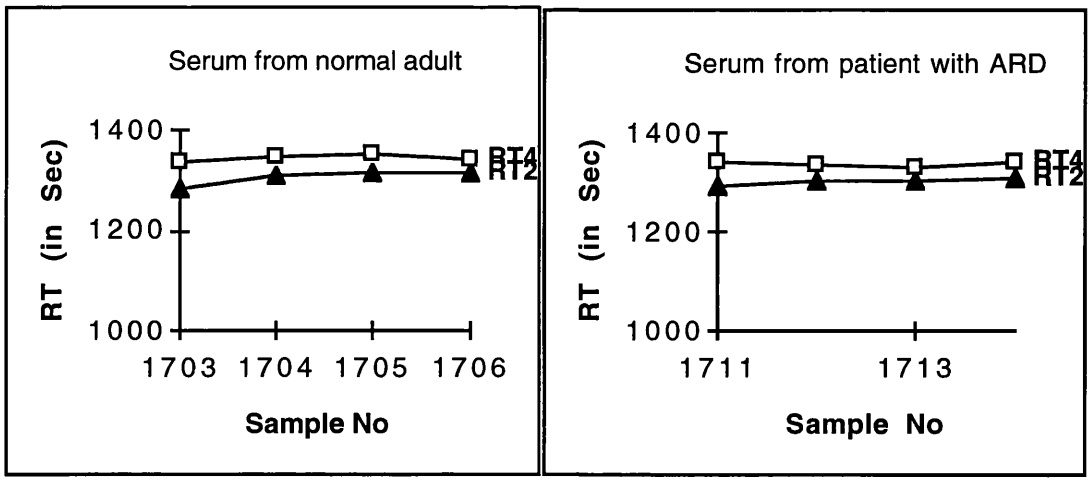


Fig.V-11: *Graphical representation of within-batch variations of RTs for the isoforms of transferrin in two samples.* RT2 and RT4 are as defined in Table V-1.

Table V-2: *Statistical data for between-batch variations of the RTs for the isoforms of transferrin in two samples.* (n= No of batch). RT2 and RT4 are as defined in Table V-1.

	n	RT2	RT4	RT4/RT2
NORMAL	4			
Mean (sec)		1305	1343	1.03
SD (sec)		92.8	84.3	0.01
CV (%)		7.1	6.3	0.91
ARD	4			
Mean (sec)		1315	1379	1.03
SD (sec)		65.8	57.6	0.00
CV (%)		5.0	4.2	0.31

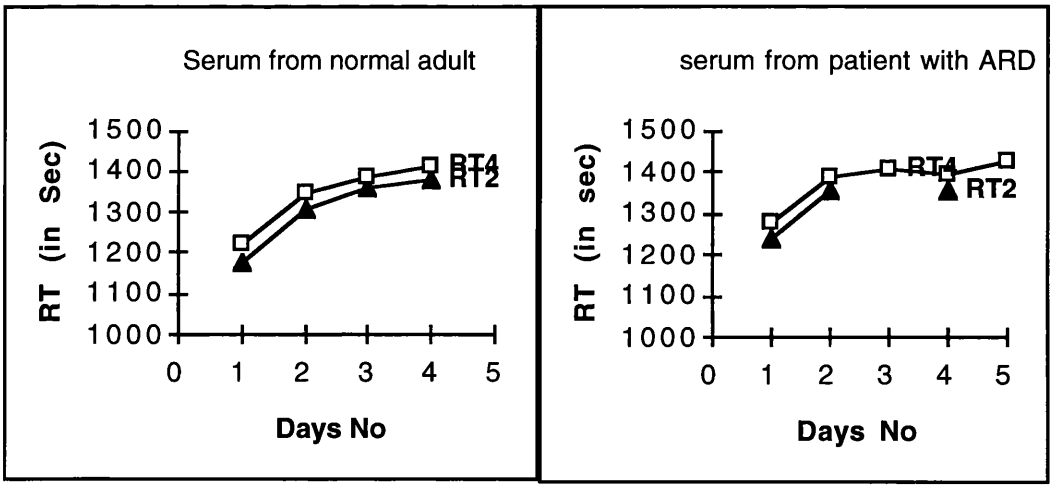


Fig.V-12: *Graphical representation of between-batch variations of RTs for the isoforms of transferrin in two samples.* RT2 and RT4 are as defined in Table V-1.

(iii) Long term variability: This was investigated in three ways.

Firstly, all the samples analysed (n=70) were taken as one group, analysed in 5 batches over a period of 6 days. The CVs for the RTs were similar to those obtained in the between-batch study (see Table V-3).

Secondly, the changes in the RTs were examined over a 24 hours period (25 samples). Fig.V-13 showed a slight increase in RTs whereas the CVs remained constant (see Table V-4). Three noticeable samples gave aberrant RTs (e.g. 1556-1586 sec for RT2-RT4 whereas the mean for that day was 1336-1368 sec). None of these sample represented a particular clinical condition and the phenomenon was thought to be due to mechanical problems.

Thirdly, the mean RTs for each working day were plotted against time. As shown in Fig. V-14, retention times for the transferrin isoforms increased with time. The back pressure was also noticed to increase, especially towards the end of the study, and the increasing retention times observed with time were thought to result from ageing of the column.

(iv) Conclusion: These results indicate that retention times for transferrin isoforms separated on a Mono Q column are reproducible within one batch of analysis but tend to increase with time. This was thought to be mainly due to the column as it was not noticed in the previous studies of hexosaminidase and ALP. Two factors can be considered. Firstly, although the lipids had been removed from the samples, some debris from the matrix could still precipitate onto the top of the column. If debris are not removed during the regeneration of the column, continuous build up could be responsible for increasing the back pressure. Other groups have introduced a pre-column to reduce this effect [Jeppsson 1994, personnel communication]. Secondly, mechanical factors concerning the column have to be envisaged. As a sample is eluted through a column with a salt gradient the packing material naturally packs down and then recovers during the regeneration procedure. Although regeneration was carried out according to the manufacturer's recommendations, the column may not, on occasions, have recovered between the analysis of two samples causing aberrant results. It may also have gradually packed down with time, increasing the back-pressure and therefore reducing the flow rate which would result in a longer elution time. This may have accounted for the increased RTs seen in 3 of the 25 samples plotted on Fig.V-13.

Table V-3: *Statistical data for variations of RTs for 70 samples analysed over 6 days.* RT2 and RT4 are as defined in Table V-1.

n = 70	RT2	RT4	RT4/RT2
Mean (sec)	1295	1334	1.03
SD (sec)	85	79	0.01
CV (%)	6.5	5.9	0.9

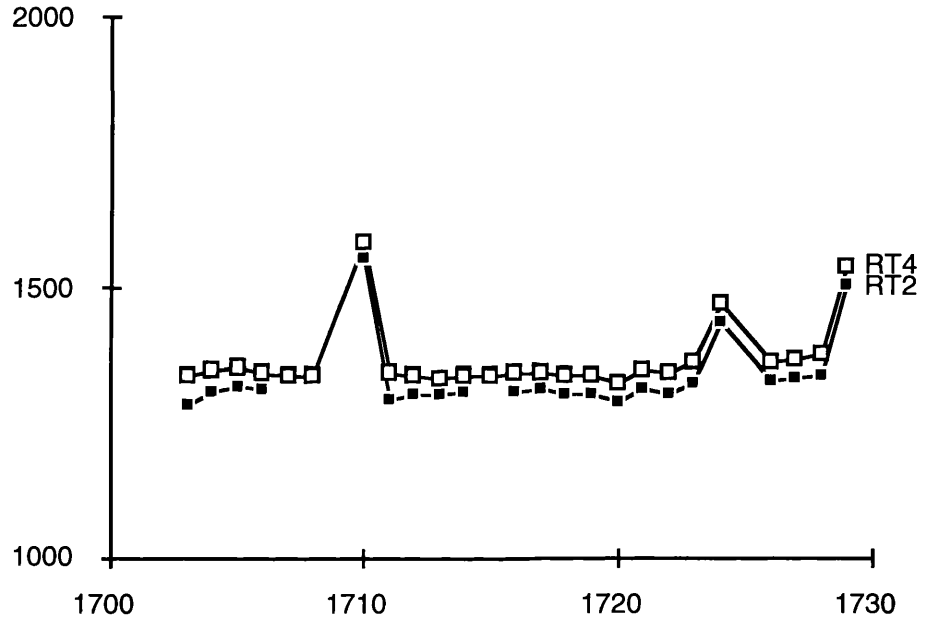


Fig.V-13: *Graphical representation of variations of RTs for 25 samples analysed over 24 hours.* The numbers on the x axis represent the samples in chronological order.

Table V-4: *Statistical data for variations of RTs for 25 samples analysed over a period of 24 hours.* RT2 and RT4 are as defined in Table V-1.

n = 25	RT2	RT4
Mean (sec)	1336	1368
SD (sec)	69.9	65.4
CV (%)	5.23	4.78

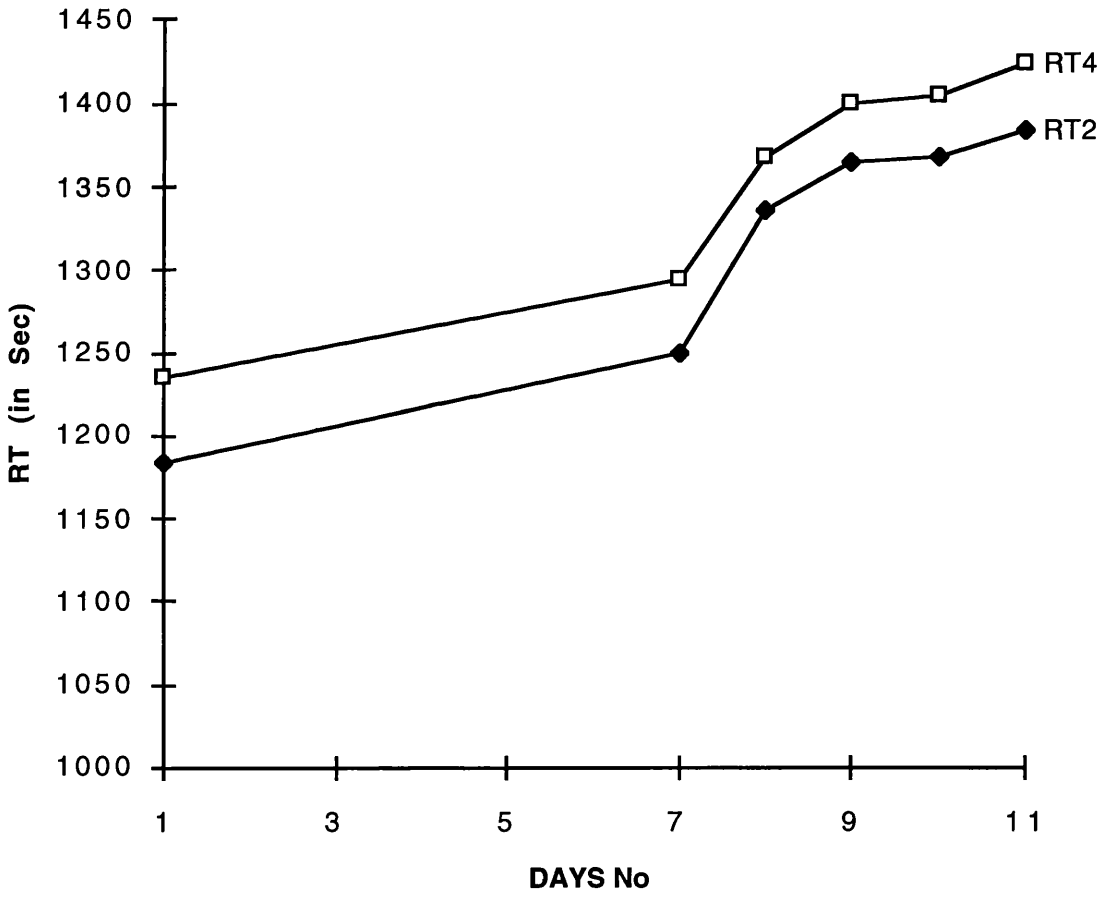


Fig.V-14: *Variations of RTs over six days using random samples.* Each pair of points represents the mean value for RT2 and RT4 on one working day. RT2 and RT4 are as defined in Table V-1.

4–2–1–3 Imprecision (Data in Appendix D2)

Imprecision of the quantitative measures of the transferrin isoforms was determined by analysing sera from adults with normal transferrin isoforms and sera from adults with ARD. Within-batch imprecision was measured by running 2 normals and 2 ARD samples on 3-5 occasions in a consecutive manner with re-equilibration of the column between each sample. The CVs were found substantial for T2 as could be expected (see below), and less than 1% for T4 in both normal and ARD sera (see Table V-5).

Between-batch imprecision was determined by running the same samples (one normal serum and 5 sera from ARD patients with different percentages of disialotransferrin) on four consecutive days. The samples were kept at 4°C between batches as transferrin is known to be stable at this temperature. The CVs were found to be of the same order of magnitude as those for within-batch imprecision (generally less than 15% for T2 and less than 2% for T4) with the exception of the normal serum (CV for T2 was 32%).

These results were not unexpected as overall imprecision is known to increase when measuring small peaks. In Fig.V-15 CV, within-batch and between-batch imprecision is plotted against the mean percentage of the isoforms. At a percentage less than 50% the CVs tend to increase. In addition, because T2 is such a minor peak (less than 2.5% of the total transferrin in normal serum), there were problems with reproducible integration. The large variations obtained for minor peaks would make it difficult to monitor changes in T2 during treatment of patient with ARD by MCC.

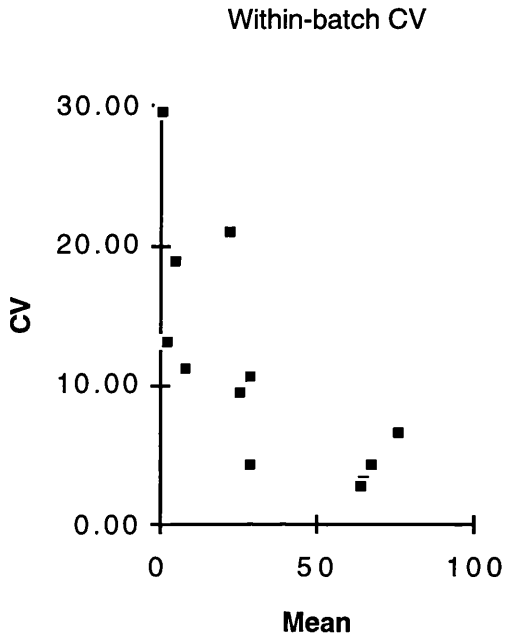
4–2–2 Typical patterns

Fig.V-9a showed chromatograms of sera from a normal adult and a patient with ARD. The disialotransferrin appears as a well defined peak in the ARD chromatogram allowing quantitation of the disialotransferrin concentration.

An interesting finding is the protein-bound bilirubin peak. This peak, previously not noticed, did not impair the transferrin isoforms analysis. Jeppsson et al did not refer to possible interferences by bilirubin, probably because their gradient stopped at 119 mmol/L NaCl, whereas in this study it went up to 250 mmol/L.

Table V-5 : *Within and between day imprecision for transferrin isoforms assayed by MCC.* Means are given in percentage of total transferrin.

Sample	Disialotransferrin	Tetra. + pentasialotransferrin
	Mean (CV%)	Mean (CV%)
Within day		
Normal serum		
n=3	1.43 (29.46)	98.80 (0.74)
n=4	2.48 (13.13)	97.40 (0.26)
ARD serum		
n=5	5.68 (18.73)	94.84 (0.88)
n=5	8.64 (11.20)	91.18 (1.34)
Between day		
Normal serum		
n=4	2.38 (31.99)	97.78 (0.74)
ARD serum		
n=6	5.73 (11.29)	94.74 (0.91)
n=4	5.68 (13.44)	94.40 (0.80)
n=5	13.52 (14.97)	86.16 (2.47)
n=4	9.45 (17.46)	90.58 (1.79)
n=5	6.98 (10.09)	93.08 (0.70)



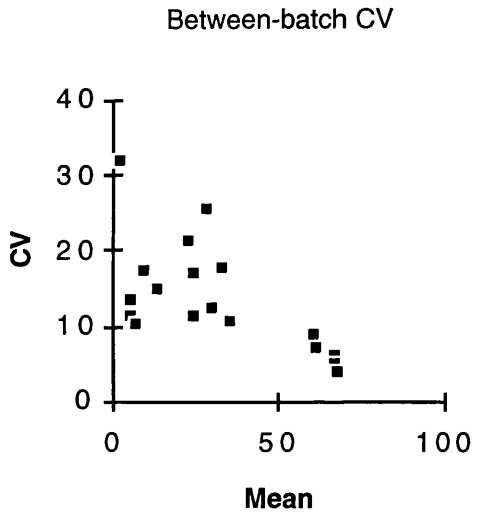


Fig.V-15: *Within and between-batches CVs for transferrin isoforms on MCC related to their concentration.* Mean= percentage of transferrin isoforms

5 – Electrophoresis

This method for analysis of transferrin isoforms was established to serve as one of the reference methods for MCC.

5–1 Method

1% ME agarose plates, 0.5 mm thick (as described in chapter IV).

Sera were diluted 1/100 (or 1/300 if incubated with antibody overnight). CSF was applied neat.

Typical running conditions were 400 V (about 65-75 mA) for one hour.

The following procedure was used to detect the transferrin isoforms. The surface of the gel was dried with a first membrane of nitro-cellulose and blotted for twenty minutes. The membrane was then blocked with 2% milk for twenty minutes and incubated in 0.2% milk with 1/1000 dilution of a goat IgG-anti-human transferrin overnight. A second antibody, peroxidase conjugated rabbit-anti goat IgG, was added with the same dilution factor of 1/1000 for two hours and finally a colour reagent was used to reveal the reaction.

Colour reagents for visualisation of the immunoreaction:

The colour reagent was prepared by mixing 20 ml of methanol with 20 mg of 3-amino-9-ethylcarbazole, which gave a red colour visualised by eye, followed by adding 100 ml of 0.1 mol/L acetate buffer and 100 µl of hydrogen peroxide.

If a scan was required, 4-chloro-1-naphtol was used as a purple-blue colour was more suitable for the red laser beam of the scanner used during this study.

5–2 Results

5–2–1 Coomassie Blue Stain

As can be seen from Fig.V-16 staining with Coomassie Blue after a prolonged run was sufficient to detect an unusual pattern of transferrin. A double band was clearly detected in the case of phenotypic variant (amino acid substitution of the protein core, for more details see Chapter I) and in the case of a patient with Carbohydrate Deficient Glycoprotein Syndrome (see Chapter VI for figures and details). Because of relatively low sensitivity of the detection method, no abnormalities were seen in the sera from patients with ARD and immunostain had to be used.

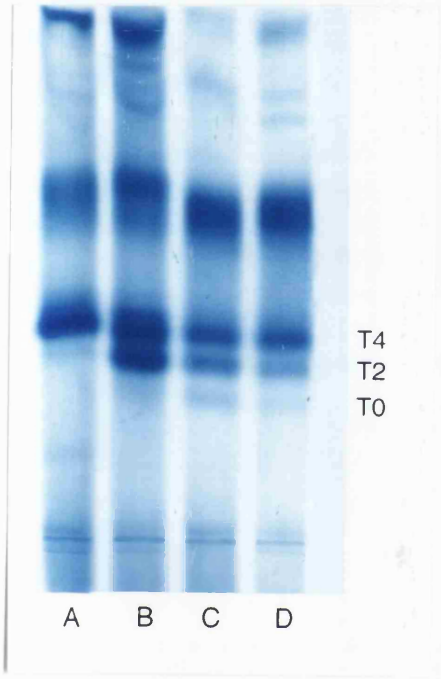


Fig.V-16: *Long run electrophoresis followed by Coomassie Blue stain.* Lane A= Normal serum, Lane B= Phenotypic variant, Lane C and D= sera from patients with a disorder affecting transferrin isoforms. T0= asialotransferrin, T2= disialotransferrin, T4= tetrasialotransferrin.

5–2–2 Immunostain

Typical patterns of serum and CSF transferrin after electrophoresis and immunostain are shown in Fig.V-17. A normal serum has only one detectable band corresponding to tetrasialotransferrin (Lane A) and a normal CSF has two bands, for asialotransferrin and tetrasialotransferrin (Lane C). In order to confirm the nature of each band samples were completely digested with a neuraminidase (*Vibrio Cholerae*, 24 hours) to convert all the isoform to asialotransferrin. Lanes B and D show serum and CSF after treatment, where only the most cathodal band is present, identified as ' asialo' form or T0. The anodal band is therefore likely to be the 'tetrasialo' form or T4.

For routine analysis a longer run (of two hours instead of one) was chosen in order to maximise the resolution between disialo-(T2) and tetrasialotransferrin. Fig.V-18 shows the electrophoretic pattern of a normal serum (Lane A), normal CSF (Lane B) and a serum from a patient with ARD (Lane D). Lane D shows a band running immediately before T4 which is characteristic of ARD and likely to be disialotransferrin (T2). In most cases, however, this band was not distinct enough to be resolved by the scanner introducing difficulties in quantifying the ratio T2/T4, which is used to monitor ARD. Lane C shows an example of a patient with a disorder known to modify the transferrin isoform pattern (CDGS) where T2 and T0 are clearly resolved (for more details see Chapter VI).

6 – Isoelectric focusing

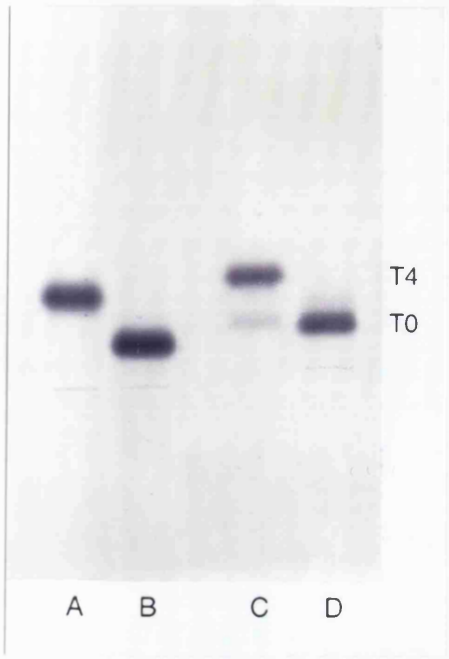
This method was developed as its high resolution would complement the information about abnormal patterns detected by electrophoresis and would allow quantitation of T2 in case of ARD.

6–1 Method

The gel was made as described for the analysis of hexosaminidase isoenzymes but the ampholytes were either Pharmalyte 4-6.5, Ampholine 4-6, Resolytes 4-8 or Servalytes 4-6.

The samples (2.5 µl of serum diluted 1/400 in water or neat CSF) were applied using an application foil.

During the course of establishing the isoelectric focusing method a series of different ampholytes run under different power conditions were studied in order to define the optimal conditions. Four gels were prepared with Ampholine 4-6, Pharmalyte 4-6.5, Resolyte 4-8, or Servalyte 4-6 respectively and all run at 5, 10, and 20 Watts. Each run was stopped at four stages: 100, 200, 400 and 800V/h to follow the protein migration.



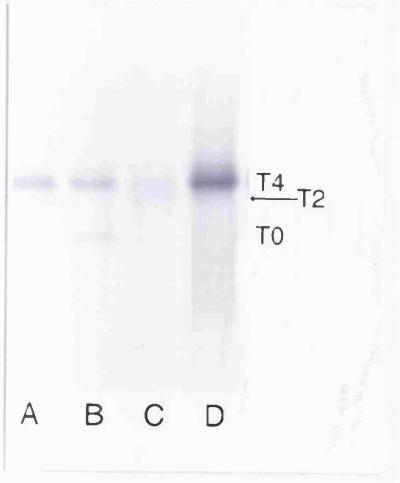
A = Normal serum

B = Serum digested overnight with *Vibrio Cholerae (VC)*

C = Normal CSF

D = CSF digested overnight with *VC*

Fig.V-17: *Transferrin electrophoresis of sample digested with neuraminidase.* Detection with immunofixation.



A = Normal serum

B = Normal CSF

C = Serum from patient with CDGS

D = serum from patient with alcoholism disorder

Fig.V-18: *Long run electrophoresis of transferrin followed by immunostain*

The optimal conditions for IEF were found to be: Resolyte 4-8 at a constant power of 20 Watt, with a maximum of 1250V and 150 mA for 1300 V/H (about 1 hour and 30 minutes). It was noticed that leaving the plate drying on the tank prior to application of the samples increased the conductivity and gave sharper bands.

The detection was by immunostain as described for electrophoresis in Section 5–1.

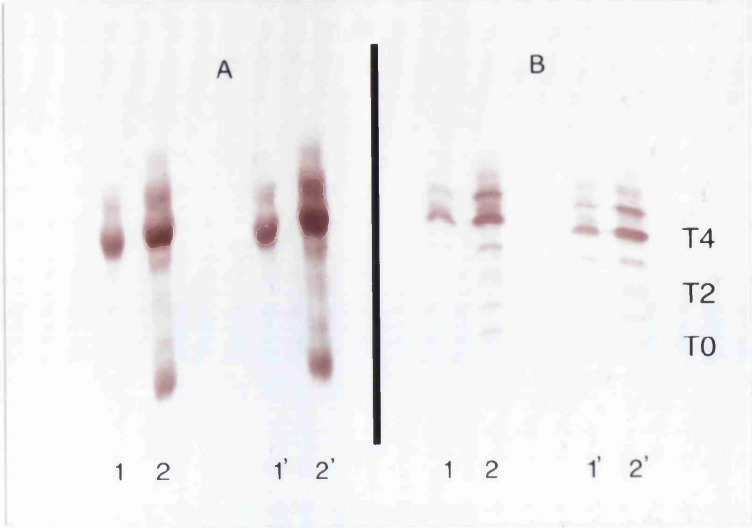
The densitometer used for the quantitation of the transferrin bands was constructed to work in transmission mode, i.e. it passed a laser beam through the medium under study. As transferrin immunoblots were on opaque nitro-cellulose membrane it severely limited the application of transmission densitometry. In order to overcome this problem, the blot had to be photographed on high contrast film and the negative produced used for scanning. This allowed for a better result, especially for the low level bands.

6–2 Results

6–2–1 Development of the method

Fig.V-19 shows the effect of iron chelation, iron saturation and removal of albumin (methods described in Sections 3–3 and 3–4) on the isoelectric focusing pattern. Fig.V-19a compares the results from normal serum and CSF obtained using Ampholine and Resolytes and clearly the latter gives a better resolution. Chelation did not induce any shift in migration of transferrin isoforms but the bands appeared sharper. The effect of iron saturation is shown in Fig.V-19b comparing normal and ARD sera and CSF treated at different temperatures. No significant differences were observed. The effect of albumin removal is shown in Fig.V-19c, and again there were no significant differences in mobility.

Fig.V-20a shows the patterns obtained after analysis of transferrin isoforms on a Pharmalyte 4-6 gel at 5, 10 and 20 Watts for 100, 200, 400 and 800 V/h. 20 Watts for 800V/h were the conditions which gave the best results, especially in the T4 region. Fig.V-20b demonstrates typical runs using the four types of ampholytes tested at 10 Watts, 800 V/h. Resolytes 4-8 (III) and Pharmalyte 4-6.5 (II) gave similar results for the resolutions of transferrin isoforms although the ratio T0/T4 for the CSF samples were found to differ in Resolytes 4-8 and Pharmalytes 4-6.5. This resulted in sharper resolution of the bands, especially with the Resolytes (see Fig.V-21a). Although differences in the T0/T4 ratios were still obtained when using the different ampholytes, Resolytes 4-8 were used during the rest of the study.

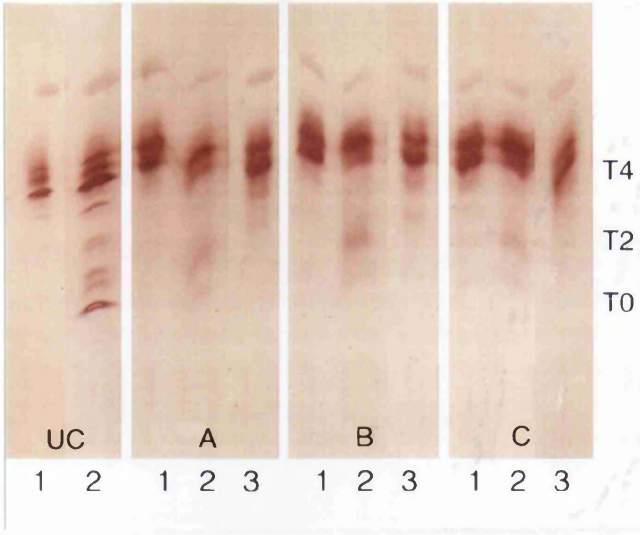


1-Normal serum
2-Normal CSF

1'-Normal serum saturated
2'-Normal CSF saturated

A = run on Ampholine 4-6
B = run on Resolytes 4-8

a-Iron saturation



1-Normal serum
2-Normal CSF
3-Serum from patient with ARD

UC = Untreated controls
A = Treated at 4°C
B = Treated at room temperature
C = Treated at 37°C

b-Iron chelation with Desferrioxamine

Fig.V-19 (a &b): *Effect of iron saturation, iron chelation and albumin removal on the resolution of transferrin isoforms by IEF.*

All the IEF patterns shown in this chapter are presented with anode at the top and cathode at the bottom of the figure.

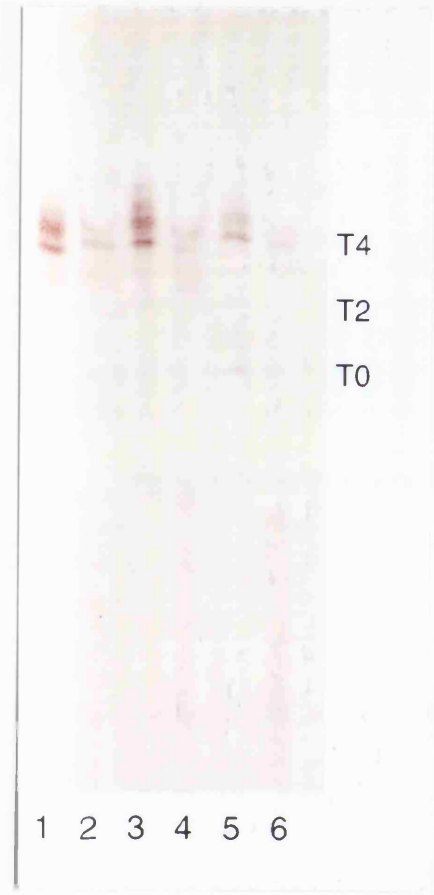


Fig.V-19(c): *Effect of iron saturation, iron chelation and albumin removal on the resolution of transferrin isoforms by IEF.* 1 & 5= Normal CSF, 2 & 6= Normal CSF treated with goat anti-human albumin, 3= Normal serum, 4= Normal serum treated with goat anti-human albumin. T4= tetrasialotransferrin, T2= disialo-transferrin, T0= asialotransferrin.

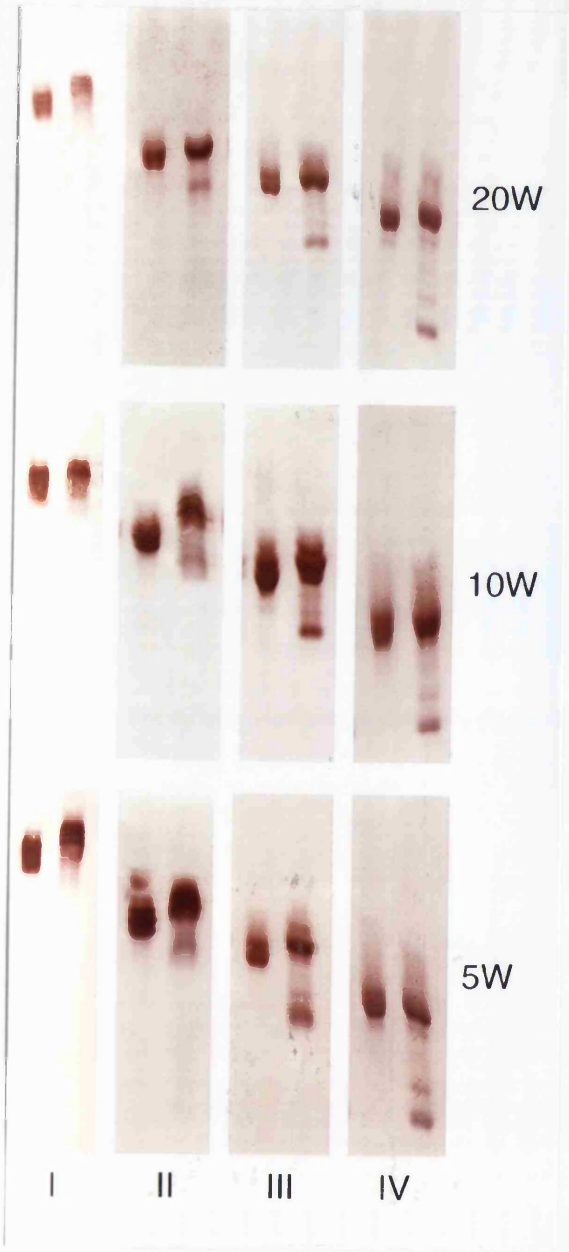


Fig.V-20: *Effect of varying the running conditions on the resolution of transferrin isoforms by IEF. a-Power.* I= 100 V/H, II= 200 V/H, III= 400 V/H and IV= 800 V/H. Each pair shows serum (left) and CSF (right).

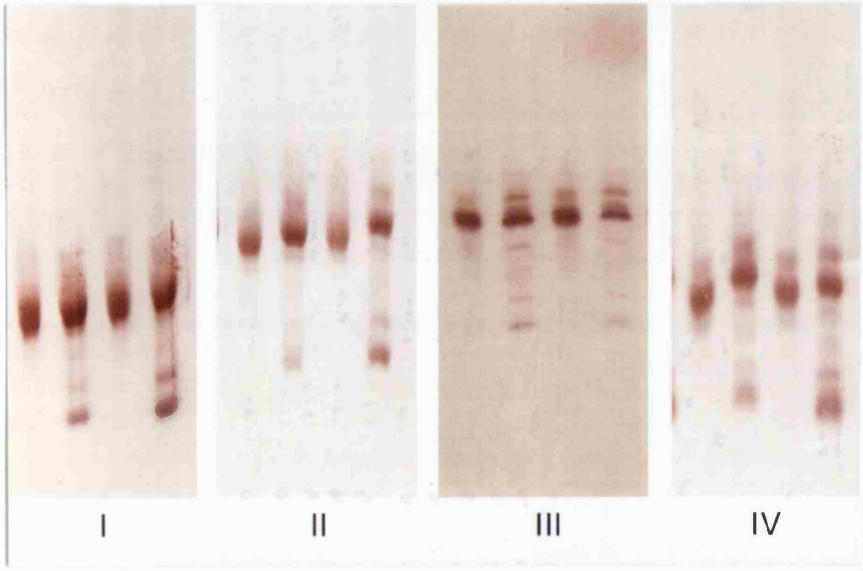


Fig.V-20: *Effect of varying the running conditions on the resolution of transferrin isoforms by IEF. b-Ampholytes.* I= Ampholine 4-6; II= Pharmalyte 4-6.5, III= Resolytes 4-8; IV= Servalytes 4-6, All run at 10 W for 800 V/H, each couple shows serum/CSF – serum/CSF.

6–2–2 Typical patterns

Fig.V-21a shows typical patterns obtained for (i) CSF with all the bands from asialotransferrin to hexasialotransferrin, later used as a marker for transferrin isoforms (Lane 1), (ii) normal serum with only tetrasialo- to hexasialotransferrin (Lane 2), and (iii) sera from patients with ARD showing the typical disialo- and trisialotransferrin (Lane 3 and 4). Lanes 5 and 6 show 2 examples of sera from patients with a syndrome affecting the distribution of transferrin isoforms (see chapter VI). Lanes 7 and 8 show a normal serum treated with (i) a neuraminidase known to digest the protein to T2 and (ii) with *Vibrio cholerae* to digest the protein to T0. As by electrophoresis this procedure helped confirming the nature of each band. It can be seen that with the high resolution of IEF multiple bands were obtained after a 'complete' digest with *Vibrio cholerae*, whereas electrophoresis showed a single sharp band for the sample. This finding was thought to result from the fact that optimal conditions were not used for the neuraminidase digestion (the sample was digested at pH 7.4, not at pH 5, which is the pH maximum for the action of neuraminidase). Fig.V-21b shows an example of phenotypic variant in Lanes 3 and 4, the doublet for every isoform appears clearly indicating a non-pathological pattern.

7 – Comparisons between methods (Data in Appendix D3)

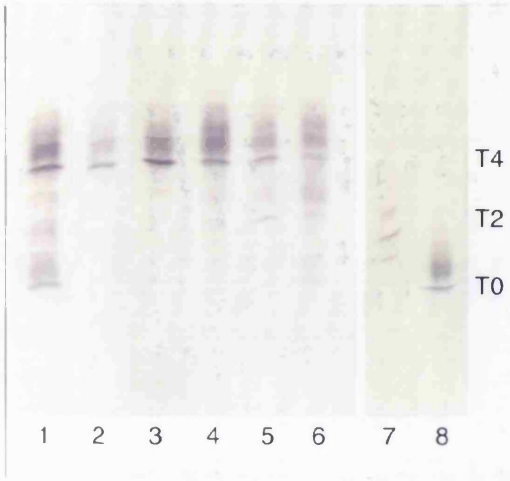
It was not possible to compare the electrophoresis method with MCC as was done for hexosaminidase and ALP as, (i) the transferrin concentration in CSF was too low to be detected by MCC, and (ii) the scanner could not resolve and quantitate the disialo-transferrin in sera from patients with ARD on electrophoresis (see pattern in Fig.V-18). Therefore, only the comparisons between IEF and MCC and IEF and electrophoresis could be carried out.

7–1 IEF and MCC

A comparison was carried out between these two methods with serum samples from patients with alcoholism related disorders.

The nitro-cellulose blots could not be scanned on the densitometer as the intensity of the bands of interest was too weak. A positive film of the blots was, therefore, used for the quantitation of these samples.

Fig.V-22 shows the results obtained when the percentage of disialotransferrin was compared using the two methods. A correlation of 0.85 was obtained but with a substantial scatter of the values.



Resolytes 4-8

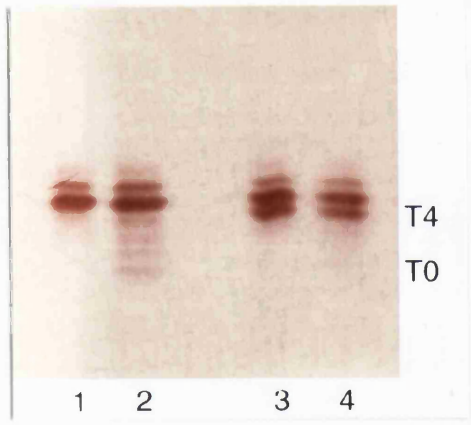
(a)

1-Normal CSF; 2-Normal serum

3 & 4-Serum from patient with ARD

5 & 6-Serum from patients with a disorder affecting transferrin isoforms

7 & 8-Normal serum digested with different neuraminidases



Pharmalytes 3-10

(b)

1 & 2-Normal serum/CSF

3 & 4-Phenotypic variant serum/CSF

Fig.V-21: *Typical patterns obtained by Isoelectric Focusing of transferrin isoforms*

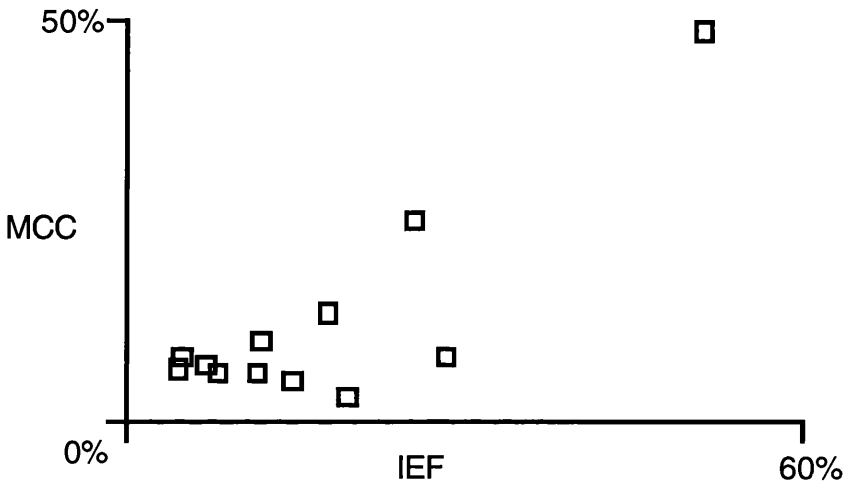


Fig.V-22: *Comparison of percentages of disialotransferrin using IEF and MCC in samples from patients with ARD.* The equation was as follow: MCC = (-4.09) + 0.95 x IEF, with r=0.85 for n=12.

7–2 IEF and electrophoresis

Electrophoresis of CSF generally gave a ratio of approximately 1 to 3 for the asialo to tetrasialo forms. It was however not clear how meaningful this ratio was because of an increasing stain intensity of the bands on IEF from the asialo to tetrasialo forms which varied depending on the type of ampholytes used. A comparison was made on 12 CSF run by both electrophoresis and IEF and scanned by densitometer. The qualitative information obtained was similar, with a correlation of 0.79 when comparing the percentages of asialotransferrin obtained following IEF and electrophoresis (Fig.V-23). During the scanning of the IEF plate a seventh band (following hexasialotransferrin) appeared to be quantitated. A cross reaction was assumed to be the reason as transferrin has only six isoforms and therefore the value was subtracted to calculate the correlation. More work would need to be done in order to identify the reason for this cross reaction.

8 – Discussion

8–1 Analytical aspect

8–1–1 Pre-analytical procedure

The methods outlined for hexosaminidase and ALP isoenzymes in this thesis were based on the development of specific post-column detection systems rather than sample purification. However, in the case of transferrin isoforms, this approach could not be considered in MCC, and therefore a sample purification and preparation methods were investigated.

Attempts to purify transferrin from the bulk of the proteins in serum were, however, a failure. DEAE Affi-blue gel did not provide any usable degree of purification, this was thought to be due to the type of column. Werner et al who used this technique successfully, had a much longer column, 15 cm x 1.5 cm, which obviously could provide a better resolution. Such a column could not be used in this study as, in order to keep the analysis at low pressure and automated, a column of 20 mm x 6.4 mm i.d was the maximum size for the equipment to handle the back pressure.

Rivanol purification initially looked promising, but presented three disadvantages for a routine analysis:(i) the high salt concentration necessitated dialysis steps which were time consuming, (ii) the recovery was only about 20%, and (iii) contamination could be detected with a sensitive detection system.

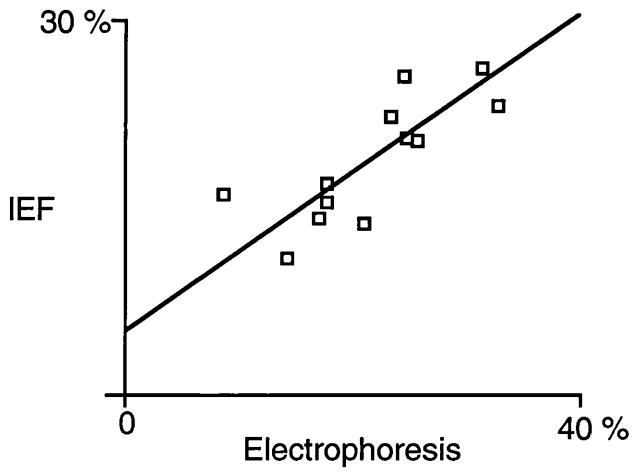


Fig.V-23: *Regression line obtained when comparing the percentages for asialotransferrin after analysis by IEF and by electrophoresis.* The equation was as follow: IEF = 6.62 + 0.6 x Electrophoresis, with r = 0.79 for n=12.

The second attempt of sample preparation, (removal of albumin, iron saturation or iron chelation) were not found to be helpful for IEF and electrophoresis despite indications in the literature [van Noort and van Eijk, 1990]. This was because of the much more specific detection system (immunoblotting) used in this study compared to the one generally employed (Coomassie Blue stain). For MCC, immunoprecipitation was the only method which provided an efficient removal of albumin but this procedure was not practical because of the large amount of antibody required. MCC was therefore carried out after removal of lipids from the sera and iron saturation following Jeppsson's recommendations.

8–1–2 Methodological aspect

(i) MCC:

The MCC method using Resource Mono Q column was shown to have an analytical imprecisions for the separation of transferrin isoforms comparable to that obtained by Ersser et al for the analysis of haemoglobin using a similar method [Ersser et al, 1987]. The system was reproducible and reliable despite the fragile packing material used which induced rapid ageing of the column. The imprecision of the calculated percentages for individual isoforms was found to be acceptable for the tetrasialotransferrin but increased up to 30% for the disialotransferrin. This was thought to be mainly due to the problems encountered during integration and manual quantitation of the peaks. As previously discussed, future efforts would have to be made to design suitable software for the automated integration of peaks obtained by MCC.

The literature and my own experience with transferrin analysis showed that Mono Q columns are made with a fairly 'fragile' packing needing long and complex cleaning procedures or preferably a pre-column to protect the analytical column. These specifications would not be 'ideal' for the purpose of this study (quick and easy automated analysis), therefore the resolution would have to be significantly different and provide a truly better clinical information for these disadvantages to be accepted.

(ii) IEF and Electrophoresis:

The differences seen in the ratio T0/T4 obtained by IEF and electrophoresis were thought to be due to the pH used for the running conditions. Electrophoresis was run at pH 8.6, whereas during IEF proteins are exposed to a range of pH. In addition there was probably also a difference in the iron content and bicarbonate binding resulting in slight differences

of net charge and therefore differences in the migration. As MCC was run at pH 6.2 it was considered to be more comparable to IEF than to electrophoresis.

In conclusion, the correlation coefficients were similar for the three methods, and they seemed to give the same information, although the scatter of values was considerable.

In future studies it would be of interest to compare the MCC method with other chromatographic methods such as those described by Stibler [see Table II-4c], or with some of the commercial kits which are now available for the monitoring of ARD.

8–2 Clinical aspect

For some conditions such as CSF leak or, as will be seen in the next chapter, carbohydrate deficient glycoprotein syndrome (CDGS), the purpose of transferrin analysis is principally to detect any abnormal isoform present in the fluid analysed. For patients with ARD, quantitation might be important as it may enable the monitoring of the disease. Electrophoresis and IEF have the advantage of being able to run more than 20 samples at one time but they give mainly qualitative information. However, they provide a quick and specific diagnosis of the patients with CDGS or CSF leak. A need for quantification has been expressed on many occasions, and it would be more easily obtained by chromatographic analysis, and therefore, in that situation MCC would be the method of choice.

8–3 General conclusion

This study showed that MCC was able to separate and quantitate transferrin isoforms, thus providing the potential of a rapid and simple method to monitor pathological conditions such as ARD.

Chapter VI: Carbohydrate Deficient Glycoprotein Syndrome (CDGS)

A – Introduction

Aim of this study

The purpose of this study was to apply the methods developed in the other sections to a newly recognised clinical condition namely CDGS. Extra-cellular hexosaminidase and transferrin have been shown to be abnormal in patients with this disorder and can be used as biochemical markers. During the course of this study several patients with CDGS were diagnosed which provided an opportunity to address the question of differences in charge of the carbohydrate moiety in the abnormal isoforms of transferrin and undertake some structural analysis to understand the molecular basis of the disorder.

1 – History

CDGS was first recognised in the early eighties [Jaeken et al, 1984]. In 1991, Jaeken reported that 29 patients (aged between 1 and 48 years) had been diagnosed and the number of diagnoses has continued to increase rapidly. In the United Kingdom, a number of neonates with olivopontocerebellar atrophy (OPCA) reported by Harding et al, 1988, were shown to have systemic and biochemical abnormalities similar to those of the Belgian patients with CDGS including an abnormal plasma transferrin pattern. Both groups of patients are now thought to suffer from different forms of the same disorder. The underlying genetic and enzymatic mechanisms have not yet been established but the current hypothesis is that there is a basic defect in the glycosylation pathway in the ER or the Golgi apparatus [Jaeken and Carchon, 1993].

2 – Clinical features

CDGS has been divided into four groups based on differences in the age of presentation (see Table VI-1). Some features, e.g. lipodystrophy, hepatopathy (increased transferase activity, hepatomegaly, abnormal histopathology) and retinitis pigmentosa are common to all forms of the disorder whereas others are associated with a particular age group. CDGS types II [Jaeken and Carchon, 1993] and III [Stibler et al, 1993] have now been described on the basis of biochemical differential diagnosis (see Section 3). Although the clinical

Table VI-1: *Clinical features of CDGS*

Group	specific clinical features	references
Infantile *multisystem*	•often stroke-like episodes •dysmorphic features •liver disease •neurological abnormalities •cardiac abnormalities	Harding et al, 1988 Horslen et al, 1991 Clayton et al, 1992 (a & b)
Childhood	•increased motor impairment, especially in lower limbs •increased mental impairment	Jaeken et al, 1984 Stibler & Jaeken, 1990
Teenage	•leg atrophy •able to communicate	Kristiansson et al, 1989
Adult *stable disability*	•hypogonadic – puberty normal in men, abnormal in women	Jaeken et al, 1991

features differ for the type II (no peripheral neuropathy, normal cerebellum), the general picture is similar for all types of CDGS.

3 – Biochemical aspects

In general, low plasma levels of albumin, thyroxin binding globulin (TBG), α1–antitrypsin, apolipoprotein B, cholesterol, transcorin, and increased plasma activities of transaminases, arylsulphatases, galactosidases, and N-acetyl glucosaminidase have been found. These changes are thought to be unspecific and related to secondary changes in intermediary metabolism and general organ damage. Type II CDGS patients have no proteinuria, no ALT, an increased serum AST, a normal serum albumin level and plasma arylsulphatase A activity. The unique biochemical marker shared by all patients, independent of age or severity of the syndrome, is the low serum level of total transferrin combined with abnormal ratios of the different isoforms. The typical pattern in CDGS type I presents 25% asialotransferrin, 25% disialo- and 50% tetrasialotransferrin, whereas CDGS type II is recognised by a marked increased disialotransferrin and a near absence of asialo-, tetrasialo-, and pentasialotransferrin. Type III shows equal concentrations of asialo-, monosialo- disialo- and trisialotransferrin with some normal isoforms.

The majority of human proteins are glycosylated, and recent investigations have revealed abnormalities of the carbohydrate moiety in many proteins in patients with CDGS.

4 – Prenatal diagnosis of CDGS

Stibler and Cederberg, 1993 have recently shown that transferrin in the paper blood spots collected from neonates can be analysed by isoelectric focusing and that glycosylation abnormalities in the transferrin can be detected in such samples. This raised the possibility of measuring transferrin or any other glycosylated protein in fetal blood and amniotic fluid for prenatal diagnosis of CDGS.

B – Results

1 – Study of hexosaminidase isoenzymes

1–1 Samples

Samples sent to the Institute of Neurology and Neurosurgery, London, for diagnostic purposes were studied. These included sera and fibroblasts from children with CDGS aged between neonate and 18 months, and sera from three older patients, aged 8, 9 and 10 years old.

Fetal blood and amniotic fluid were obtained at 19 weeks gestation from the Fetal Medicine Unit, University College Hospital, London.

All the methods referred to below have been described in the previous chapters.

1–2 Extracellular hexosaminidase activity

The total hexosaminidase activity measured in serum of patients with a severe form of CDGS was found to be greater than normal (between 4.6 and 9.3 μmol/hour/ml, compared with the reference range of 0.5 to 2.0 μ mol/hour/ml). The percentage of hexosaminidase A calculated both by heat denaturation and by chromatographic separation was lower than normal (between 26 and 37% compared to the reference range of 52 to 78% – see Table VI-2).

The three older children with a milder form of the syndrome had a serum activity within or just outside the normal range (1.4, 2.7 and 2.1 μmol/hour/ml) although the percentage of hexosaminidase A (37, 33 and 26% respectively) was still low. A study with N-acetylglucosamine-6-sulphate which has a higher affinity for the α-subunit of β-N-acetylglucosaminidase showed a normal level of hexosaminidase A activity despite the high total activity.

The analysis of serum hexosaminidase by MCC showed an abnormal mobility of all the isoforms (e.g. hexosaminidase A peak eluted at 1470 sec instead of 1214 sec). The variation in retention times is shown in Table VI-3 indicating a change in the net charge of the isoforms in patients with CDGS. Fig.VI-1 clearly shows abnormal peaks in the hexosaminidase B region and a very small hexosaminidase A peak in the CDGS sample when compared with a normal serum.

IEF of the serum hexosaminidase showed a complex pattern of bands with generally 'less negative' isoenzymes (see Fig.VI-2). Heterogeneity for the hexosaminidase A and multiple bands for hexosaminidase I and 'B like', (no hexosaminidase B could be detected) were common to all patients affected by CDGS.

Table VI-2: Total hexosaminidase activity and percentage of hexosaminidase A in serum from patients with CDGS.

Sample	Total activity (in µmol/hour/ml)	% Hex A
DC	8.6	25
JR	9.3	28
PH	2.1	26
TS	4.8	30
JS	4.7	39
TB	1.4	37
VB	2.7	33
CB (2 weeks)	6.7	23
CB (6 weeks)	3.0	37
Normal range	0.5 - 2	52 - 78

Table VI-3: *Retention times on MCC for hexosaminidase isoenzymes in normals and CDGS samples.* Retention times and SDs are given in seconds.

	Hex A (SD)	Hex I2 (SD)	Hex I1 (SD)	Hex B (SD)
Normal serum samples (n=18)	1214 (22.41)	975 (14.59)	661 (6.29)	496 (5.70)
CDGS serum samples (n=12)	1470 (15.85)	1090 (25.44)	644 (9.91)	404 (29.28)
Normal fibroblasts (n=4)	1219 (14.43)	none	none	771 (84.8)
CDGS fibroblasts (n=3)	1284 (128.52)	none	626 (10.97)	404 (0.58)

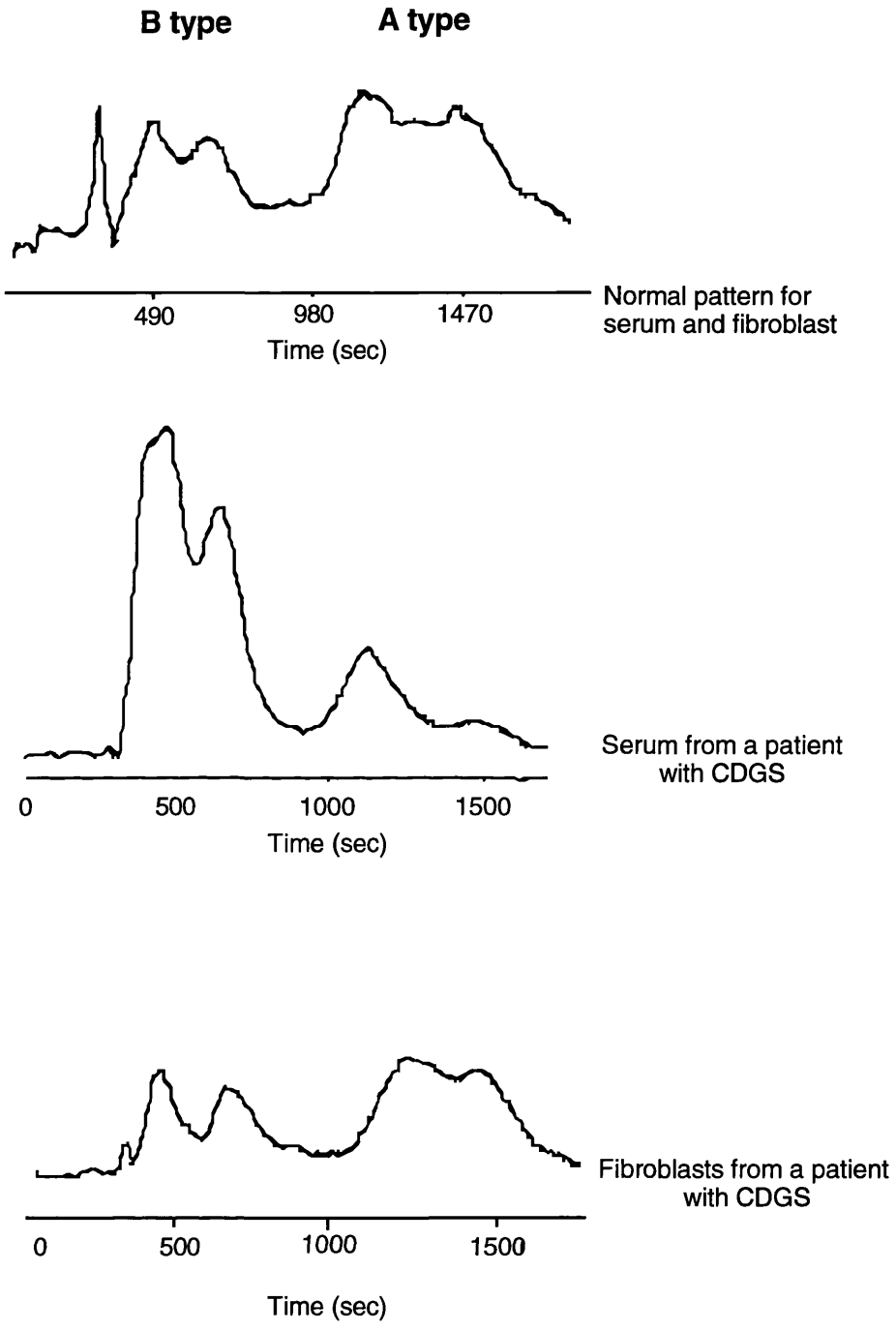


Fig.VI-1: *Chromatograms of hexosaminidase isoenzymes in serum and fibroblast from a normal control and a child affected by CDGS.* B= hexosaminidase B, A= hexosaminidase A, hexosaminidase activity is detected by fluorometry (for more details see Chapter III). Only one pattern is shown for control as the main difference between serum and fibroblast is the total activity. The sharp peak at the beginning of the chromatogram is created by the artefact described in previous chapters.

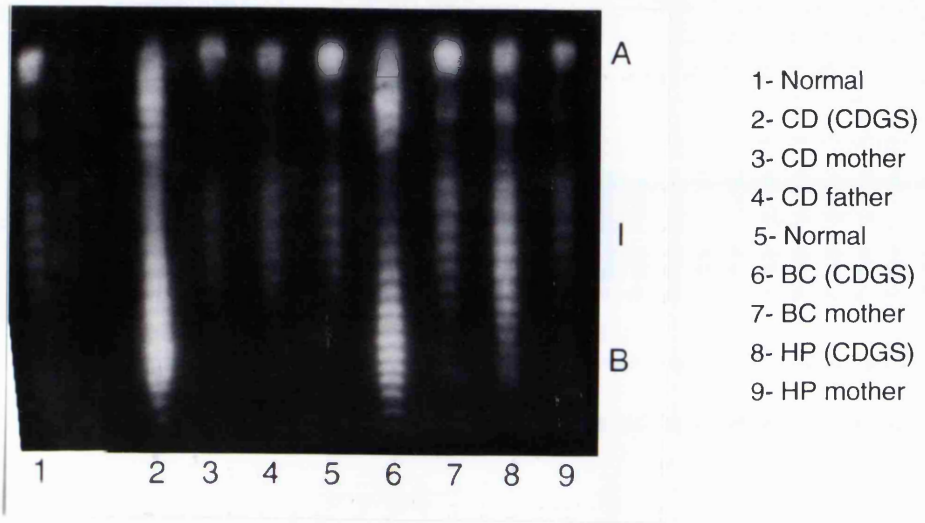


Fig.VI-2: Isoelectric focusing analysis of hexosaminidase isoenzymes of sera from patients with CDGS and from their parents. Analysis on Resolytes 4-8 with fluorometric detection as described in Chapter III. A= hexosaminidase A, I= hexosaminidase I, B= hexosaminidase B, the anode is at the top of the figure.

1–3 Intracellular hexosaminidase activity

The activity measured in cultured fibroblasts from CDGS patients was shown to be within the normal reference range (2.4 to 13.9 µmol/hour/ml for total activity with 43 to 62% for hexosaminidase A) and the pattern obtained by MCC analysis was comparable to that obtained in normal subject (see Fig VI-1). The retention times for hexosaminidase A was similar to that obtained in normal fibroblasts in contrast to serum, whereas a shorter retention time was found for hexosaminidase B in the CDGS patients (see Table VI-3).

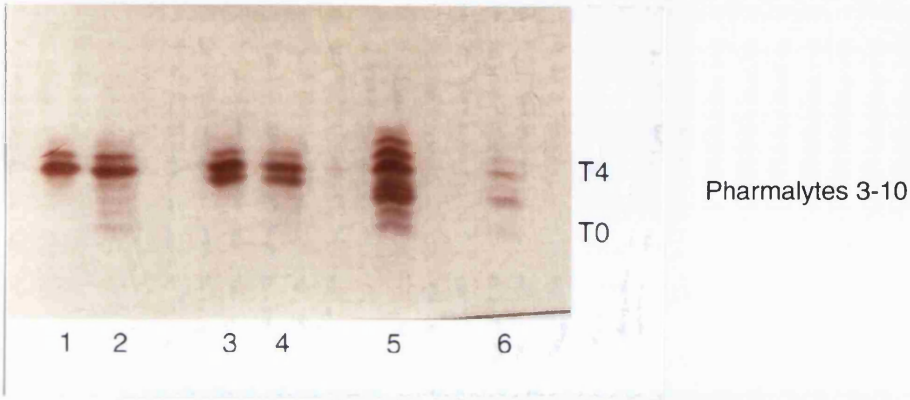
1–4 Discussion

An hypothesis can be made for the difference of behaviour between the circulating and intra-lysosomal forms of hexosaminidase. Lysosomal enzymes must be phosphorylated in order to be transported to the lysosomes and if the phosphorylation is defective the amount of the mature lysosomal enzyme decreases and the circulating extra cellular enzyme increases [Sonderfeld and Proia, 1989]. Phosphorylation and glycosylation of a protein are dependent on each other, so in patients affected by CDGS the abnormal glycosylation could lead to defective phosphorylation thus inducing an increased extra cellular concentration of hexosaminidase. The abnormal mobility of the different isoenzymes could be explained by the defective glycosylation impairing the net charge of the protein. Similar changes in lysosomal enzymes were also noted on isoelectric focusing by Jaeken et al, 1992, in untreated galactosaemic patients (called secondary CDGS). Subunit specific antibodies would be useful tools in the investigation of the patterns of hexosaminidase obtained after IEF and MCC analysis and hopefully provide further 'clues' to the underlying defect.

2 – Study of transferrin isoforms

2–1 As a diagnostic tool

In this work all the patients were diagnosed as CDGS type I with the typical IEF pattern of approximately 25% asialotransferrin, 25% disialotransferrin and 50% tetrasialotransferrin. Fig.VI-3 shows the pattern obtained in two sera: Lane 5 from a 9 years old with the mild form of CDGS and Lane 6 from a 3 months old with the acute form. Although the pI range used for the ampholytes was not optimal (from 3 to 9 rather than 4 to 8 – see Chapter V) all the isoforms of transferrin were clearly resolved and this pI range was also used for the routine diagnosis of CDGS.



1- Normal serum
2- Normal CSF
3- serum from a phenotypic variant
4- CSF from a phenotypic variant
5- Serum from patients with a mild form of CDGS
6- Serum from a patient with an acute form of CDGS

Fig.VI-3: *Isoelectric Focusing of transferrin isoforms in serum and CSF.* T0= asialotransferrin, T4= tetrasialotransferrin. Detection was by immunoassay as described in Chapter V. For details on phenotypic variants, see Section 3–2 of Chapter I. These samples were run in conjonction with sera from patients likely to be affected by CDGS in order to help confirming the pathological nature of the latter.

Fig.VI-4 shows the results obtained when sera from two patients with CDGS were analysed by MCC. Although the pattern is not as detailed as the one obtained by IEF, it is very different from that obtained in a normal serum and serum from an ARD patient (see chapter V). MCC would not however be the method of choice for screening for this syndrome as quantitation is not essential for diagnosis, but its specific abnormality (presence of T2) allows a diagnosis of this rare condition to be made.

Electrophoresis followed by immunostain as described in Chapter V is the method routinely used for the initial screen for CDGS. This gives a very specific pattern (see Fig.VI-5), which is instantly recognisable even on a simple routine Coomassie Blue stained gel. Confirmation of the type of CDGS can be made by IEF if necessary.

A similar transferrin pattern is also seen in untreated galactosaemic patients but the pattern reverts to normal after two weeks of a galactose-free diet. This observation has been confirmed by other workers [Jaeken et al, 1992] and galactosaemia is now classified as secondary CDGS as the hypoglycosylation is acquired (similar to alcoholism disorders).

2–2 Structural analysis: F.A.B.M.S

2–2–1 Method

Because of the relatively high concentration of transferrin in human serum (4g/l) and its well defined pattern of glycosylation, it was thought to be a good protein for structural analysis of the glycosylation changes in CDGS. Serum transferrin was purified from a normal adult, two children (aged 8 and 9 years old) affected with CDGS, and their parents. Two different methods were used.

(i) Successive precipitation with Rivanol followed by ammonium sulphate (for details see chapter V) which provided a mixture of all the isoforms.

(ii) Prolonged agarose electrophoresis: agarose electrophoresis was carried out as described in chapter V for four hours resulting in a maximal resolution of the transferrin isoforms. The individual isoforms were recovered by cutting out the bands, homogenising the gel and centrifuging at 12 000 rpm for 20 min. The supernatant contained the pure individual isoforms of transferrin (see Fig.VI-6).

These samples were used by Prof. A. Dell at Imperial college, London, for a study of the carbohydrate branches of transferrin [for details see Dell, 1987]. The protocol used is shown in Fig.VI-7.

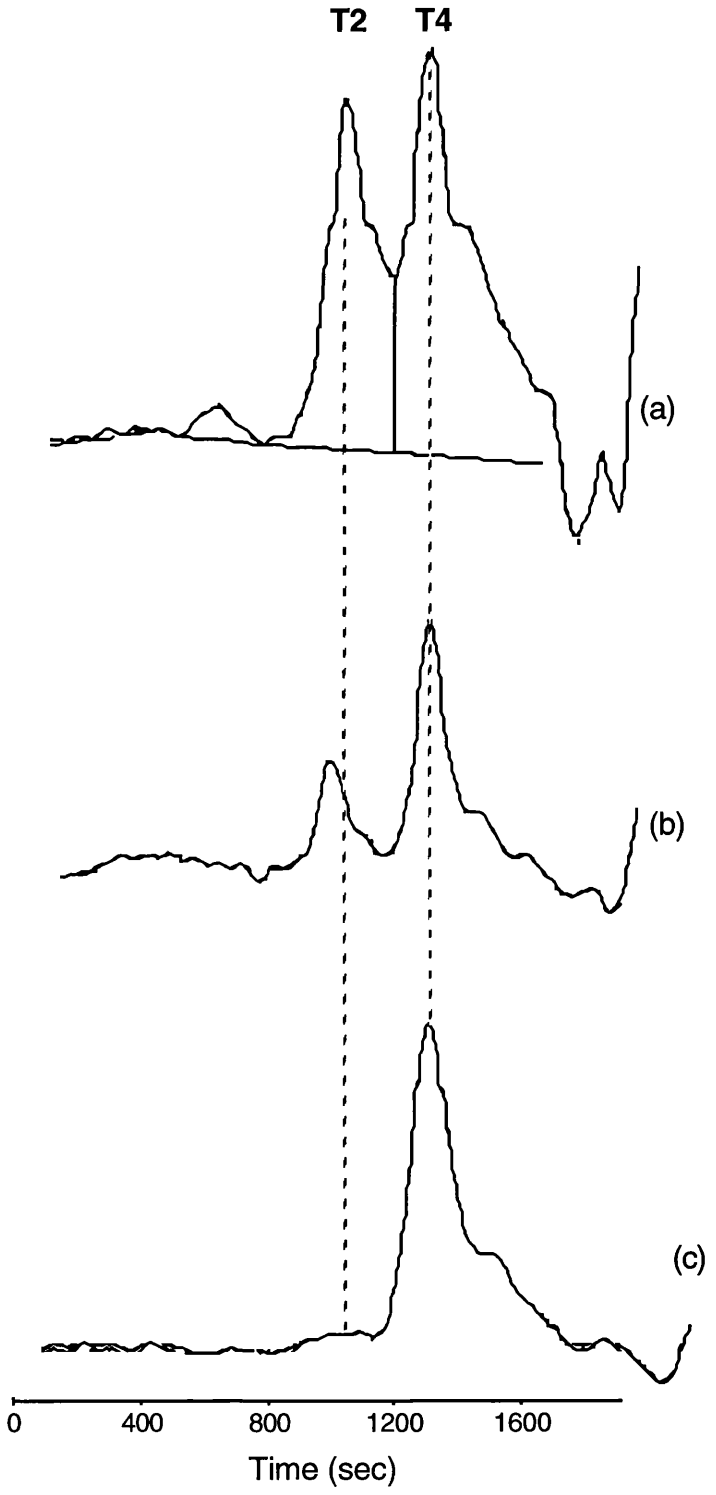
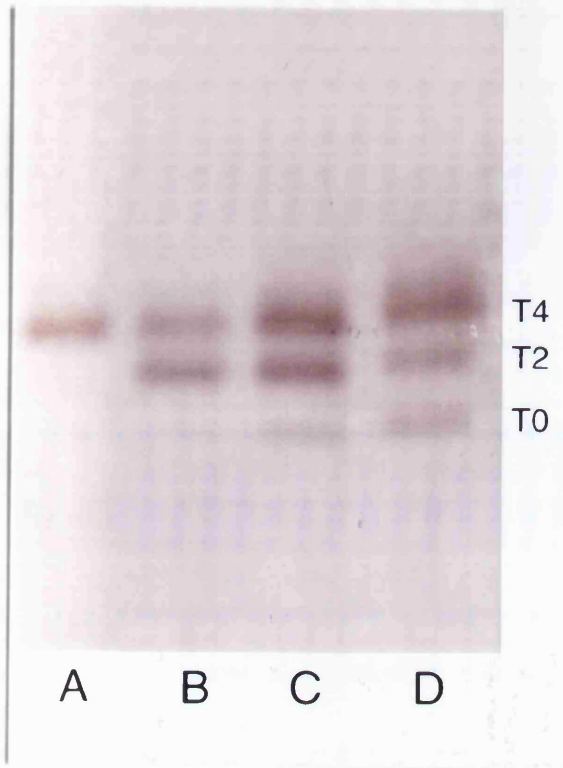


Fig.VI-4: *MCC analysis of serum transferrin from patients with CDGS.* a= TB, b= VB, 2 patients with mild form of CDGS, c= Normal serum. T2= disialotransferrin, T4= tetrasialo-transferrin. The samples were run as described in Chapter V with detection at 460 nm.



Lane A= normal

Lane B= phenotypic variant

Lane C= patient with CDGS

Lane D= patient with CDGS

Fig.VI-5: *Serum electrophoresis followed by immunostain for transferrin.* T2= disialotransferrin, T4= tetrasialotransferrin. Running conditions and detection were as described in Chapter V, the anode is at the top of the figure.

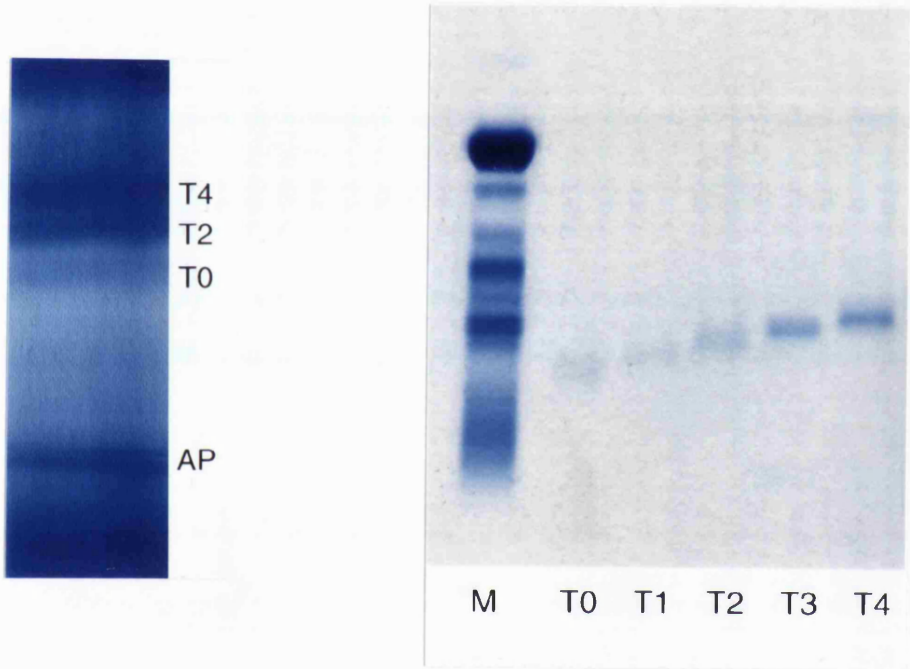


Fig.VI-6: *Extended agarose electrophoretic run for transferrin purification.* AP= application point, M= marker (normal serum), T0= asialotransferrin, T1= monosialotransferrin, T2= disialo-transferrin, T3= trisialotransferrin, T4= tetrasialotransferrin. Detection with Coomassie Blue.

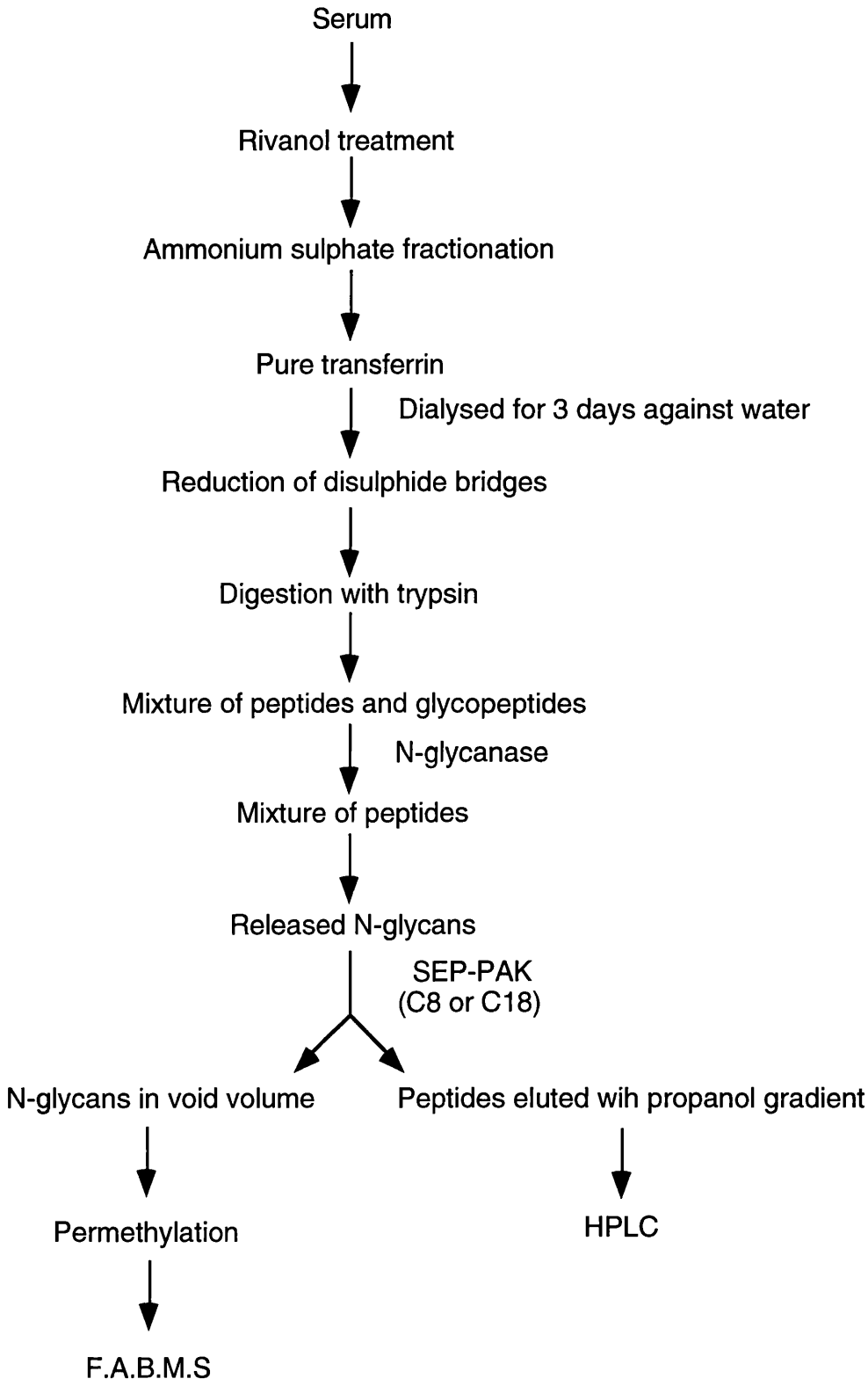


Fig.VI-7: *Sample preparation for F.A.B.M.S.*

2–2–2 Results and discussion

The initial hypothesis was that the transferrin in CDGS patients had a sialic acid-deficient carbohydrate chain. A mass spectrum of transferrin from CDGS patients would therefore be expected to show different mass fragments compared to normals. However, Fig.VI-8 shows the mass spectrum for transferrin observed from CDGS children. There were no differences when compared with a normal control. It was concluded that there was an underglycosylation of the sites (see Fig.VI-9), as this would not appear on F.A.B.M.S analysis. This hypothesis was confirmed by Wada et al [1992]. The question remains whether it is a random [Yamashita et al, 1993], or site-specific underglycosylation.

Further work would include a study of the peptides eluted after the tryptic digest and N-glycanase treatment (see protocol, Fig.VI-7). Two of the peptides contain the asparagine bearing the carbohydrate, which is modified to aspartic acid after the N-glycanase treatment. By studying the ratio of aspartic acid/asparagine in the peptides the underglycosylation could be determined and by looking at each peptide, (they are of different length and therefore, elute in different fractions after HPLC), the question of random/site-specific could be answered.

3 – Prenatal diagnosis of CDGS

During the course of this study, one of the families who already had had two babies affected by the accute form of CDGS (death by the age of 18 months) requested a prenatal diagnosis. As transferrin is 98% maternal in origin [Stibler and Kristiansson, 1991], other glycoprotein like α–foetoprotein, α1-antitrypsin or hexosaminidase could theoretically be used. Amniotic fluid and serum from the fetus at risks were obtained at 19 weeks gestation. The results were compared with normal fetal serum of similar gestational age.

Monitoring the serum hexosaminidase isoenzyme pattern in the fetus and early life of the infant indicated that the hexosaminidase pattern was normal in the fetus at 19 weeks gestation but the typical abnormalities of CDGS became apparent during the neonatal period (see Fig.VI-10). The peak corresponding to hexosaminidase A gradually disappeared whereas the peak corresponding to hexosaminidase B became much greater than in a normal infant. The hexosaminidase, transferrin and α1-antitrypsin patterns on IEF showed exactly the same progression of the abnormalities from birth (at 32 weeks gestation) to 4 weeks after birth when the pattern was the one of a typical CDGS case [Clayton et al, 1993], an example is shown of the hexosaminidase isoenzymes in Fig.VI-11. Prenatal diagnosis of CDGS by analysis of hexosaminidase or α1-antitrypsin in fetal serum cannot be offered.

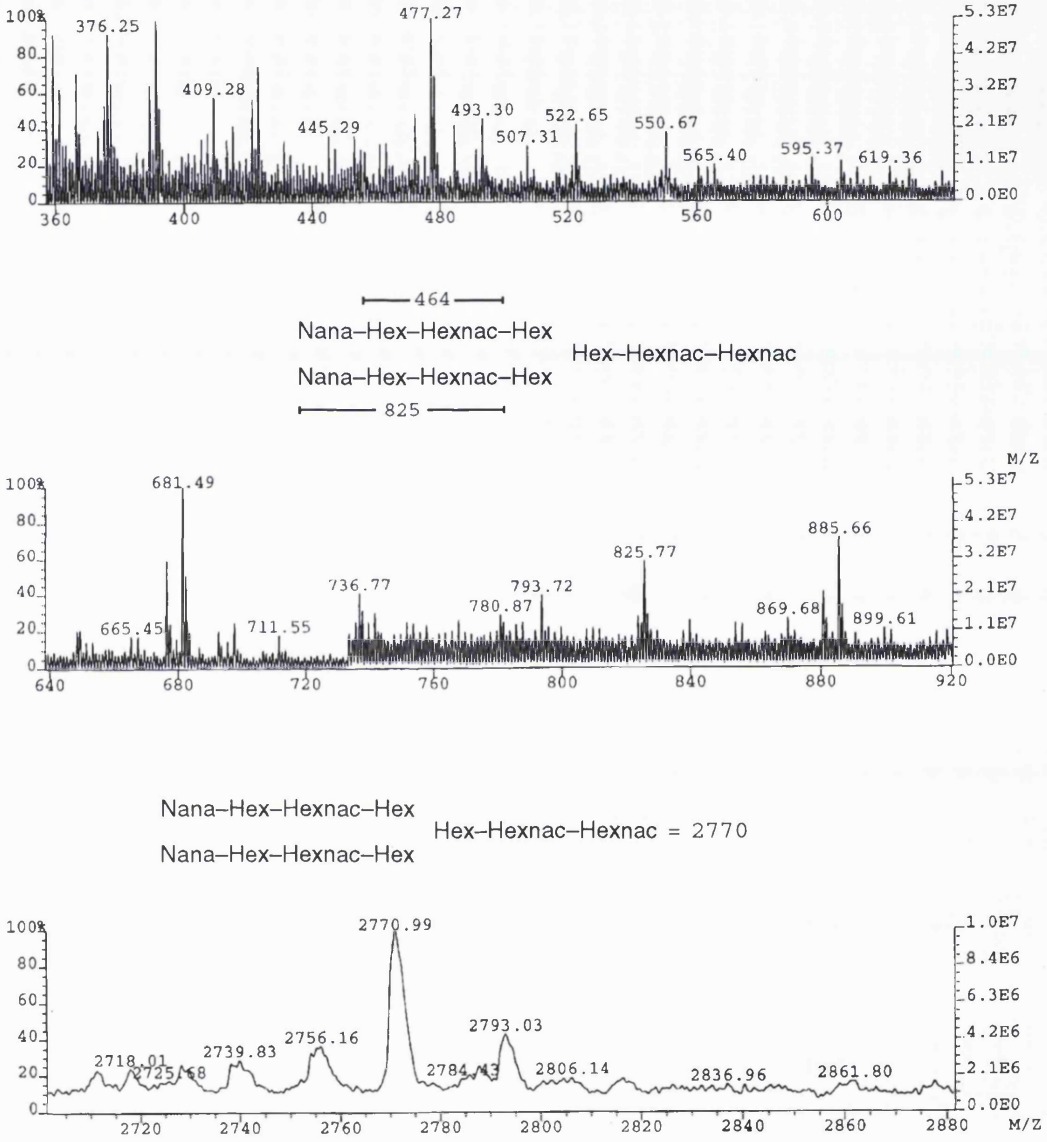


Fig.VI-8: *Mass spectra obtained for the transferrin carbohydrate analysis of CDGS children.* The numbers represent molecular mass of various fragments. Hex= hexose, Hexnac= N-acetyl-hexose, Nana= neuraminic acid.

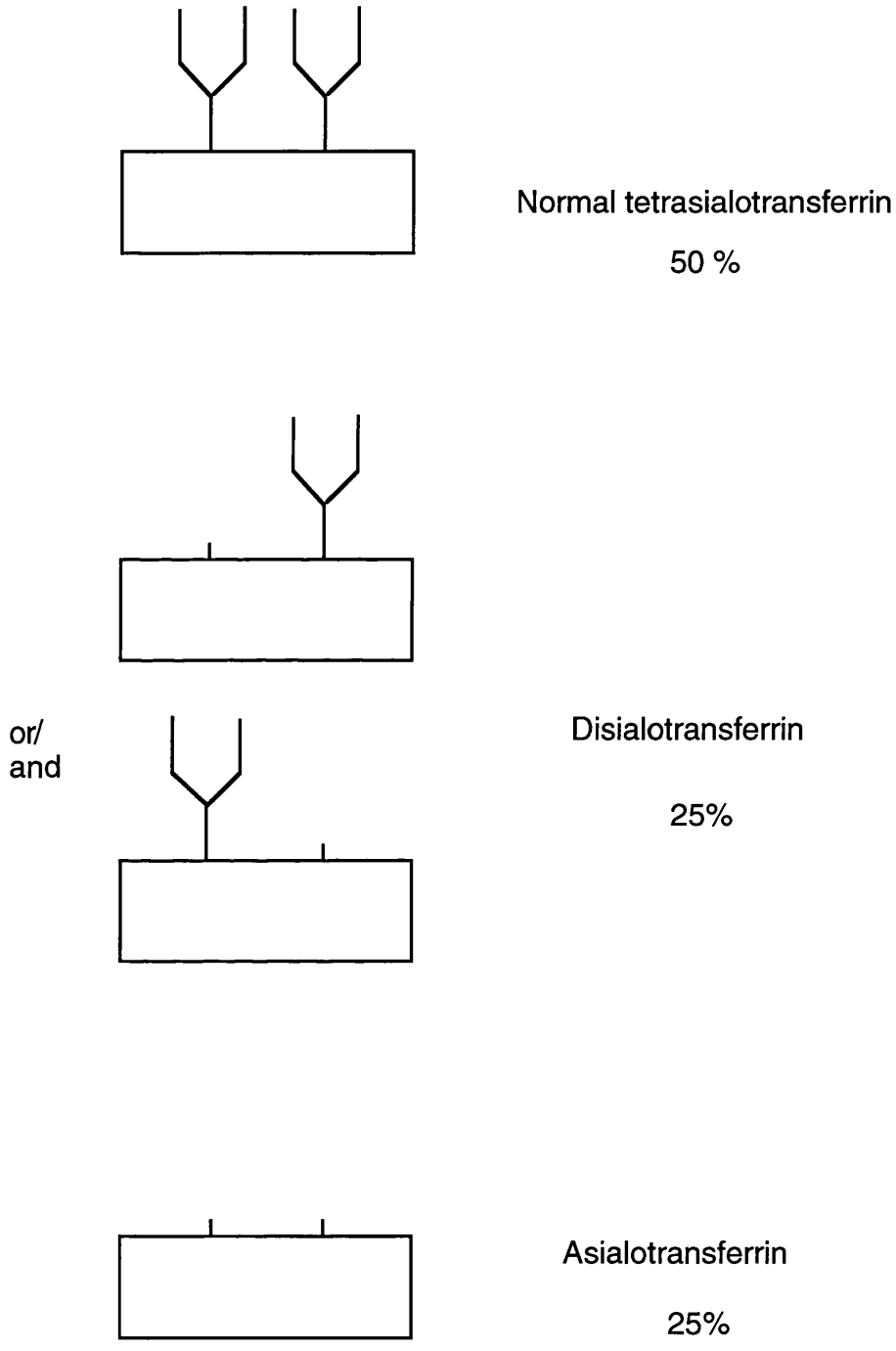


Fig.V-9: *Schematic representation of possible mechanism of underglycosylation of transferrin in CDGS.*

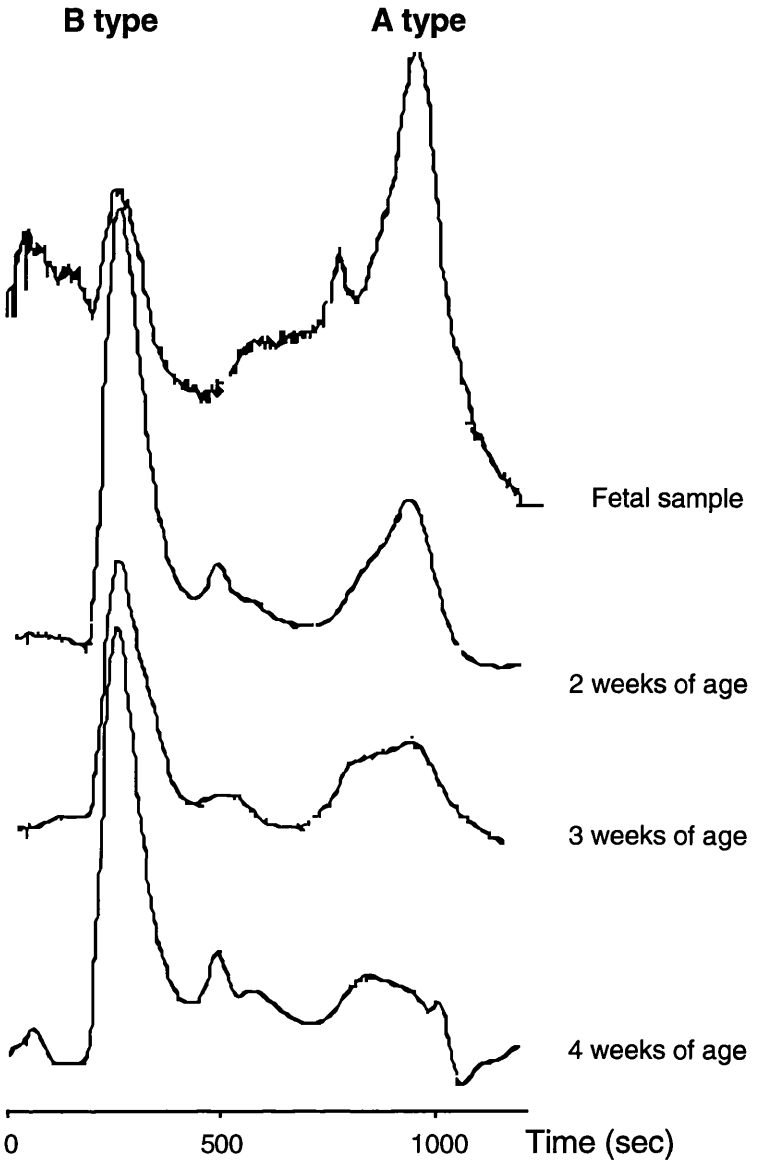
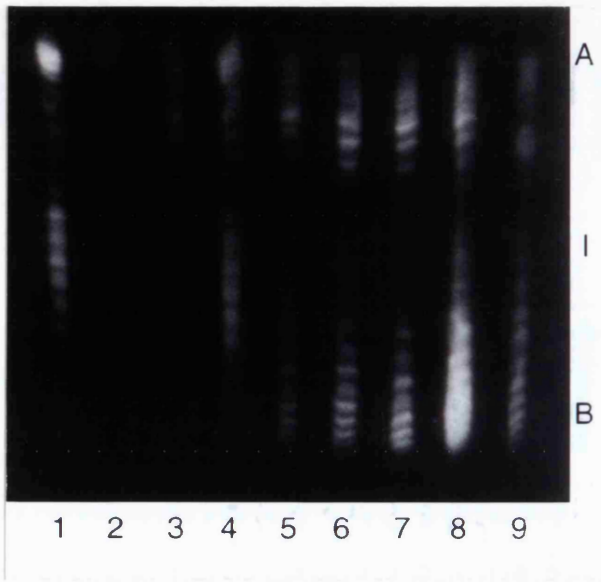


Fig.VI-10: *Hexosaminidase pattern by MCC in fetal plasma and the new born baby (up to 4 weeks of age) in a patient with CDGS* The fetal sample was taken at 19 weeks of gestation. Hexosaminidase isoenzymes were separated and detected as described in chapter III.



1-Neonatal control
2-Fetal control
3-Fetal from BC (19 weeks gestation)
4-BC 1 week of age
5-BC 2 weeks of age
6-BC 3 weeks of age
7-BC 4 weeks of age
8-Serum from CD (known CDGS)
9-Serum from BT (known mild CDGS)

Fig.VI-11: *Hexosaminidase isoenzymes analysis by IEF of sera from a fetus (19 weeks) and the new born baby (up to 4 weeks of age) in a patient with CDGS.* The isoelectric focusing was carried out in the conditions described in Chapter III. The anode is at the top and the cathode at the bottom of the figure.

4 – Conclusion

The high resolution of the different isoforms of transferrin and the simplicity of the interpretation of the pattern makes IEF the method of choice for the biochemical diagnosis of CDGS.

The results obtained during the prenatal and neonatal study led to the hypothesis of a 'switch' in the glycosylation pathways normally happening at birth (or at term) but defective in children with CDGS. The structural analysis with the F.A.B.M.S suggested that the glycosylation reaction was defective. This could arise in several ways: (i) the oligosaccharyl transferase could be defective, (ii) the supply of dolichol-pyrophosphoryl-oligosaccharide could be inadequate due to a defect in the assembly or supply of intermediates, and (iii) substrates or enzyme could be incorrectly localised. Further work on the cellular level would have to be carried out in order to answer these questions.

Chapter VII: General discussion

1 – Analytical aspects

1–1 Packing Materials

The large variations between packing materials of similar structure showed the importance of a careful choice for each application. In this study, the features of large pore size, rigid polymer matrix and a strong exchanger were found to be the most appropriate for separating isoenzymes and isoforms. The Mono Q packing gave a slightly better resolution on transferrin analysis, but PL SAX 1000 was found to be more robust. It would therefore be the first choice in future work.

1–2 Glycomat and G15

The two system, Glycomat and G15 showed some potential for the analysis of isoenzymes and isoforms. However a number of improvements would need to be made before considering their use on a routine basis.

The main drawback of the GMAT was the artefact created by the syringes system. If a delay of two minutes could be achieved before the loading of the sample onto the column, any fraction eluting in the void volume could be reliably analysed. The second problem was the fixed capacity of the syringes, the option of refilling the syringes during the course of analysis would allow the desired flexibility to design gradients without volume or time limitation. With these improvements, GMAT would be the equipment of choice in future work as its gradient reproducibility was shown much better than that of the G15. This was explained by the fact that GMAT has a much better resistance to back pressure and includes a mixing chamber for the gradient. The resistance to the back pressure in G15 was minimal which meant that the pump could not maintain a regular flow rate, which induced variations in the elution profile, especially when shallow gradients were used.

One limitation common to both systems was the number of channels available. Two buffers could only be delivered, and as a result it was impossible to use a wash solution different from the eluting buffers or to add extra reagents for post-column detection. They had to be delivered by an independent peristaltic pump which introduced background noise, as it was impossible to keep both pumps in phase.

1–3 Post-column detection system

Biological macromolecules are known to behave differently to small molecules in chromatographic systems as they need more time and space to bind and elute from the packing material whilst still retaining their tertiary conformation and activity. Therefore a slow flow rate (not greater than 0.55 ml/min) was chosen for the analysis of isoenzymes and isoforms of hexosaminidase, ALP and transferrin. As a consequence an important part of the developmental work was to find the right balance between the reaction time following elution from the column and band spreading. The proteins studied were large (>80 000 Da) and did not diffuse rapidly. Thus an open tubular reactor was chosen. Coiled teflon tubing are known to allow up to two minutes incubation (geometrical deformation, e.g. knitting or coiling, reduces the band broadening by @60% [Brinkman et al, 1989]). In this study up to three minutes incubation at 37°C could be carried out without impairment of the chromatographic resolution.

1–4 Reproducibility of MCC

The reproducibility of MCC chromatograms was in general acceptable in this study although not as good as that obtained by Ersser et al using a similar system. This was explained by a number of factors.

(i) Step gradients, used in this work, are known to have twice the imprecision compared to isocratic elution. Ideally, an internal standard for each isocratic part of the step gradient need to be included in order to obtain a good indication of the reproducibility.

(ii) The post-column detection system added noise to the whole system. The retention times are affected by the slight variations induced by the incubation step increasing the imprecision.

(iii) The integration system was not adapted to this kind of analysis. As the peaks obtained were generally broad (due to the post-column reaction) the room for variation during the electronic integration of the peak is greater than in a standard HPLC system and the software available for the study was not able to treat this type of data in a reliable manner.

1–5 Methods comparison

Relatively small numbers have been used for methods comparison during this work and a bigger study would need to be carried out in the future to confirm the preliminary results obtained for the performance of MCC. However, when comparing MCC with other analytical methods such as electrophoresis or isoelectric focusing, the correlations for

hexosaminidase and transferrin isoenzymes and isoforms showed a fairly good agreement between the methods. For alkaline phosphatase, the poor correlation between methods may be explained by a different method specificity; as recently shown [Moss, 1994] this point is of particular importance when a study is aimed at evaluating a new method for isoenzymes analysis.

2 – Clinical aspects

The MCC method showed the ability to differentiate between different isoenzymes and isoforms patterns, especially if a quantitation was important for the identification of a pathological condition. The identification of patients heterozygous for Tay-Sachs and Sandhoff disease was found to be quick, easy and reliable. The detection and monitoring of disialotransferrin in patients with ARD was shown to be possible although not totally reliable without mechanical improvements as suggested. The alkaline phosphatase isoforms of bone and liver could be resolved and quantitatively evaluated although with a high degree of imprecision. It was felt that MCC has the potential for a quick and easy measurement of the ALP isoenzymes of interest in paediatric but more work would have to be carried out to clarify the method specificity.

3 – General conclusion

Ellis et al were the first group to propose that the chromatography column used could be shortened without impairing the quality of analysis, providing a gain of time and sample. They chose hexosaminidase isoenzymes to develop their new method of low pressure micro column chromatography. In 1974 Ellis wrote:

"ion-exchange micro column chromatography coupled with automated assay... should be applicable to the resolution of the component forms of any enzyme for which a sensitive assay is available. The speed with which a run can be carried out makes it especially useful for labile enzymes." This study has vindicated this statement.

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ABSTRACT AND PUBLICATION RELATED TO THE THESIS

CARBOHYDRATE DEFICIENT GLYCOPROTEIN SYNDROME
The spectrum of clinical abnormalities in early infancy

Clayton, P.T., Di Tomaso, E., Prudden, M., Keir, G. and Winchester, B.
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We have encountered two further patients with the carbohydrate deficient glycoprotein (CDG) syndrome in its most severe form (neonatal olivopontocerebellar atrophy [NOPCA]). The diagnosis was made by electrophoresis of serum transferrin with immunofixation. The first was a boy born by Caesarean section at 37 weeks following premature rupture of membranes and failure to progress in labour. After 2 minutes he required intubation for hypoventilation. Further episodes of hypoventilation with desaturation occurred on the 1st and 3rd days of life. An unusual facies, large head (>97%), ill-formed ear cartilages, retracted nipples and prominent subcutaneous fat were noted. A chest X-ray showed increased vascular markings and hypoventilation only; ECG and echocardiogram were normal. By 3 weeks ventricular hypertrophy was apparent and by 9 weeks there was a severe hypertrophic obstructive cardiomyopathy. Other features included severe failure to thrive, hypotonia, cerebellar and cerebral atrophy, retinal dystrophy, macular hypoplasia and high myopia, long fingers, peau d'orange skin, flaring of iliac wings, acetabular hypoplasia and prolonged unconjugated hyperbilirubinaemia. The second patient, a girl, was born at 36 weeks following Caesarean section for antepartum haemorrhage. At birth she was noted to have oedema, a large head, prominent labia majora, episodes of twitching (not thought to be seizures) and roving eye movements (retinal dystrophy). She fed poorly and showed severe failure to thrive. At 6 months she showed hypotonia (cerebellar atrophy on CT and MRI scans), hepatomegaly, long fingers and toes, hypoplastic, inverted nipples and, on skeletal radiology, flared iliac wings, sloping acetabular roofs and inferior hooks on lumbar vertebrae. Our observations confirm that NOPCA is a multisystem disease which may present in many different ways.

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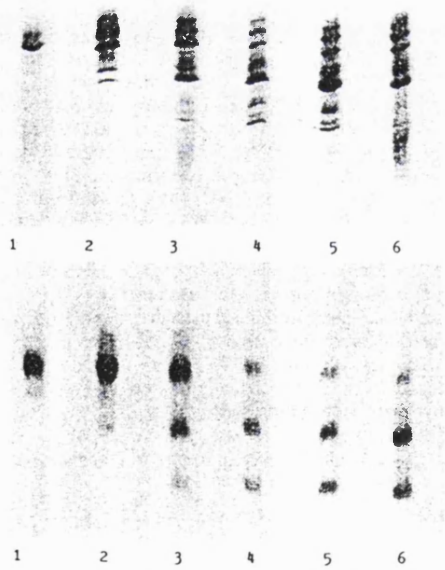
Carbohydrate-deficient glycoprotein syndrome: normal glycosylation in the fetus

SIR,—Some of the changes that occur in cells during fetal development, ageing, and malignant transformation are associated with changes in the oligosaccharide moieties of glycoproteins.[1] We present evidence that a recently recognised group of genetic diseases may help to define the role of age-specific enzymes in protein glycosylation.

Neonatal olivopontocerebellar atrophy[2,3] is the most severe form of primary carbohydrate-deficient glycoprotein (CDG) syndrome,[4,5] and is diagnosed by isoelectric focusing (IEF) of serum transferrin with immunofixation. Patients have reduced amounts of tetrasialotransferrin (TS_4) and increased amounts of disialotransferrin (TS_2) and asialotransferrin (TS_0). The TS_2 in two patients with CDG syndrome lacked one of the two N-linked sugar chains, suggesting a metabolic error in an early step in protein glycosylation.[6] Other serum proteins, including α1-antitrypsin and β-hexosaminidase, have abnormal IEF patterns due to altered glycosylation. β-hexosaminidase activity in plasma is increased; in contrast the plasma concentration of some glycoproteins (eg, thyroid-binding globulin) is reduced. Prenatal diagnosis of primary CDG syndromes should be possible based on the glycosylation pattern of fetal transferrin in early pregnancy.[7] We confirmed that, in blood samples from fetuses of 18–22 weeks' gestation, TS_4 was the major isoform, which indicates that it would be possible to detect a fetus with reduced TS_4 and increased TS_2 and TS_0. We offered to test the fetus of a couple at risk.[2]

Fetal blood was obtained at 19 weeks' gestation; the pattern was identical with that of normal fetuses, with TS_4 predominating. The total β-hexosaminidase activity was 1·0 μmol ml^{-1} h^{-1}, which was slightly higher than controls (0·59, 0·29, 0·55), but the percentage of heat labile activity and the IEF pattern were identical to those of controls. We believed the fetus to be unaffected. At 35 weeks a baby girl was delivered by emergency caesarean section because of decelerations in fetal heart rate. She required resuscitation, and had hypotonia, roving eye movements, restricted movements at the hips and knees, diarrhoea, failure to thrive, inverted nipples, long fingers and toes, fat pads above the buttocks, and lipoatrophy affecting the thighs—ie, the typical clinical picture of neonatal olivopontocerebellar atrophy. Blood samples were obtained at 10, 19, 30, and 32 days post-natally and analysed by IEF. The patterns for serum transferrin and α1-antitrypsin are shown in the figure. The carbohydrate-deficient isoforms of both proteins (and also of β-hexosaminidase) appeared in the 2nd to 3rd week of post-natal life (equivalent to 37 and 38 weeks' gestation).



Nitrocellulose immunoblots of serum α1-antitrypsin (upper) and transferrin (lower) after agarose IEF.

1 = 19 weeks' gestation (normal pattern). 2–5 = post-natal samples at 10, 19, 30, and 32 days, respectively; cathodal (carbohydrate-deficient) forms of both proteins are detectable at 10 days and become dominant by 30 days (cathode is at bottom). 6 = unrelated patient with neonatal olivopontocerebellar atrophy.

Our results indicate that abnormal glycosylation of serum proteins cannot be detected during pregnancy in fetuses affected by the CDG syndrome; prenatal diagnosis by IEF of transferrin from fetal blood is not possible. The likely explanation for the appearance of the abnormal glycosylation pattern of serum glycoproteins in an affected neonate is a failure to switch from the normal fetal pathway of glycosylation to a normal neonatal pathway. Other explanations include: inhibition of normal glycosylation when the neonate is exposed to some chemical from which the fetus is protected (as in galactosaemia[8]); or the affected fetus being unable to produce normal glycoproteins but maternal proteins cross the placenta and are responsible for the normal appearances on isoelectric focusing. While placental transfer could be true for transferrin, all the evidence suggests it is not true for α1-antitrypsin or β-hexosaminidase. The relation between the abnormalities of serum glycoproteins and the pathogenesis of the disease remains unclear, especially because our patient had clinical abnormalities (eg, dysmorphic features) before the development of the abnormalities of glycoproteins.

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H pylori in dental plaques

SIR,—Dr Banatvala and colleagues (Feb 6, p 380) report that 18 out of 21 Bangladeshi children in London studied had *Helicobacter pylori* in gastric biopsy specimens and also in dental plaque as detected by polymerase chain reaction (PCR). Moreover, 10 patients with negative gastric biopsy cultures were positive for *H pylori* by PCR of plaque.

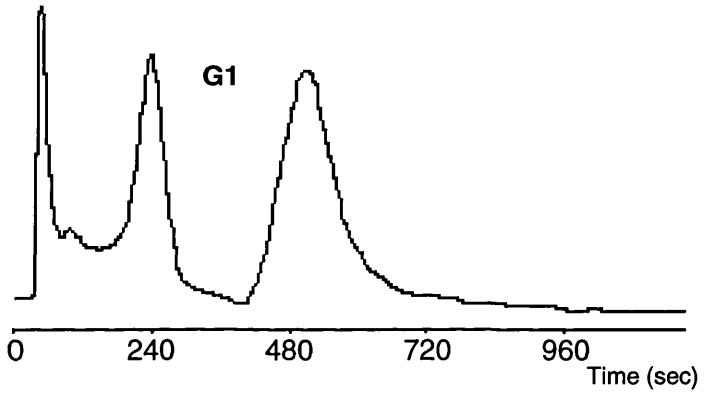
Our work with patients in southern Sweden aged between 22 and 74 years with various gastric symptoms involves comparing cultures, serology, and PCR of gastric biopsy and dental plaque samples. Of 6 biopsy-culture-positive patients, we found, like Banatvala and colleagues, a good correlation with PCR in 5 patients. Furthermore, we found *H pylori* in dental plaques of 13 out of 14 culture-negative patients.

As we use a rapid transport technique in an adequate medium,[1] it seems unlikely that inadequate biopsies or culture insensitivity, as suggested by Banatvala and colleagues, could explain our results. Our previous epidemiological studies in southern Sweden

Appendix A: Study of various gradients on two different packing materials

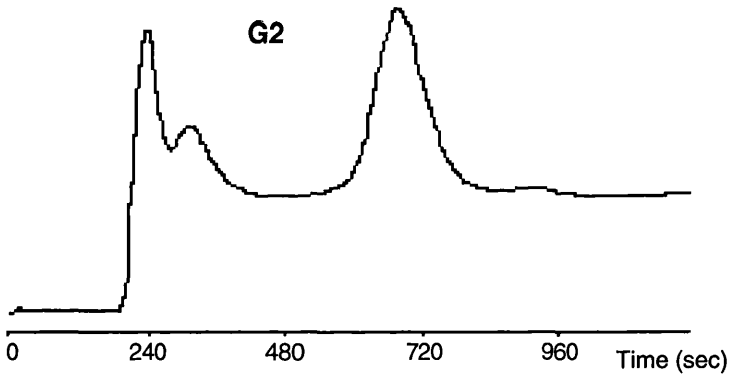
I-Fractogel

The enzyme analysed is ALP. Time is in secondes, flow rate in ml/min. The peak are not labelled as only the differences in resolution are of importance.



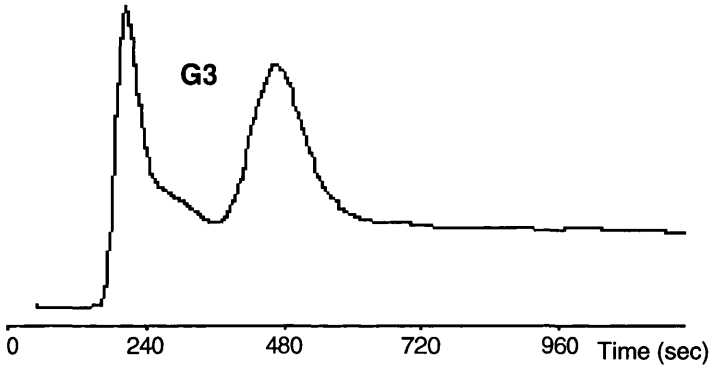
Time	%B	Flow rate
0	15	0.8
300	15	0.8
900	100	0.8
1200	100	0.8

B=500mmo/L LiCl



Time	%B	Flow rate
0	25	0.8
240	25	0.8
340	35	0.8
400	35	0.8
500	55	0.8
560	55	0.8
900	100	0.8
1200	100	0.8

B=500mmo/L LiCl



Time	%B	Flow rate
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300	25	0.8
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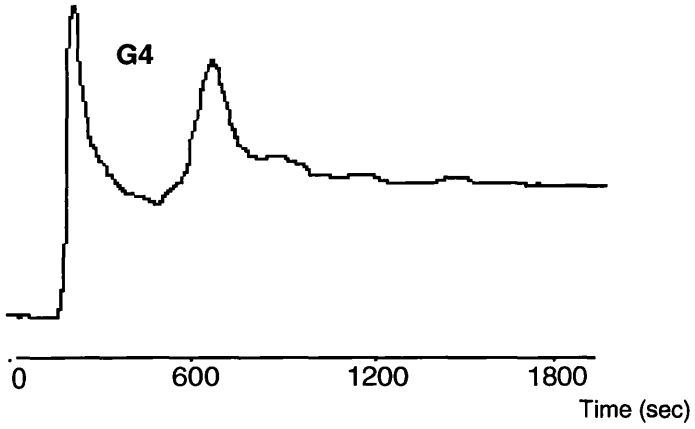
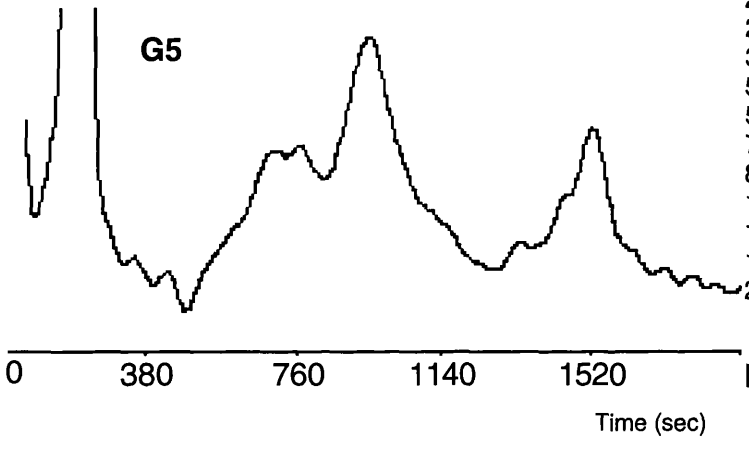
1200	60	0.8

B=500mmo/L LiCl



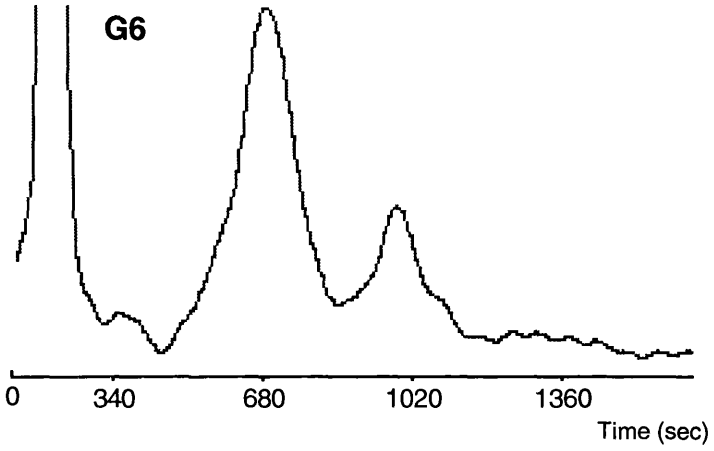
Time	%B	Flow rate
0	15	0.8
240	15	0.8
270	17	0.8
320	17	0.8
410	25.7	0.8
590	25.7	0.8
680	30	0.8
860	30	0.8
950	37.1	0.8
1130	37.1	0.8
1720	100	0.8
2020	100	0.8

B=350mmo/L LiCl



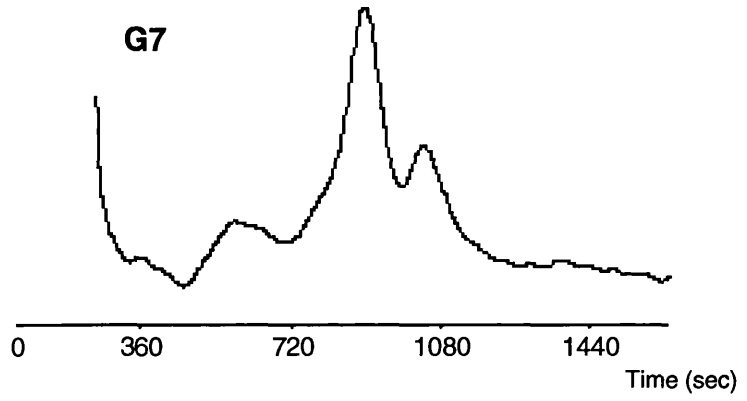
Time	%B	Flow rate
0	15	0.8
240	15	0.8
270	17	0.8
320	17	0.8
500	25.7	0.8
590	25.7	0.8
770	30	0.8
860	30	0.8
1040	37.1	0.8
1130	37.1	0.8
1720	100	0.8
2020	100	0.8

B=350mmo/L LiCl



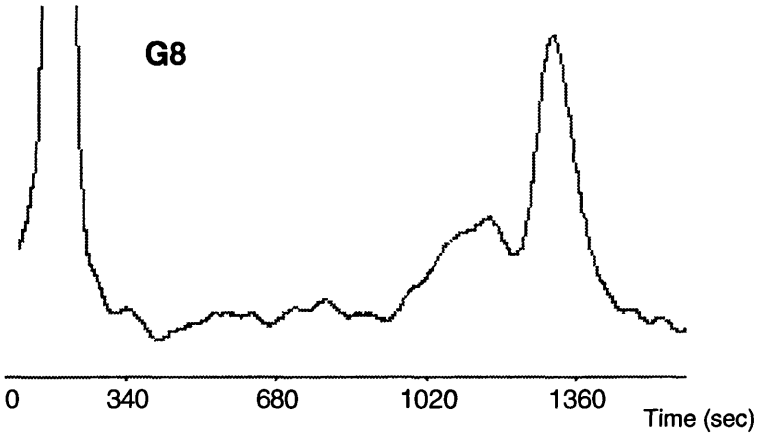
Time	%B	Flow rate
0	18	0.8
240	18	0.8
660	45	0.8
1080	95	0.8
1680	95	0.8

B=350mmo/L LiCl



Time	%B	Flow rate
0	18	0.8
240	18	0.8
660	45	0.8
1080	95	0.8
1680	95	0.8

B=250mmo/L LiCl

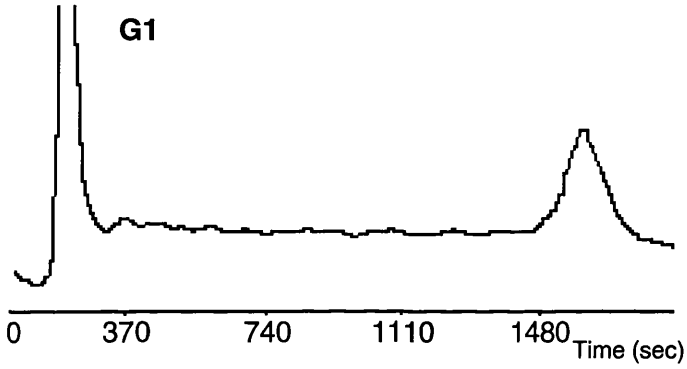


Time	%B	Flow rate
0	15	0.8
240	15	0.8
270	17	0.8
320	17	0.8
410	25.7	0.8
590	25.7	0.8
680	30	0.8
860	30	0.8
950	37.1	0.8
1130	37.1	0.8
1720	100	0.8
2020	100	0.8

B=250mmo/L LiCl

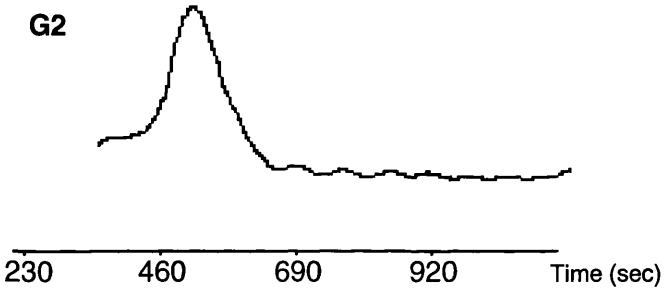
II-Zorbax

Enzyme= ALP, the time is given in secondes and the flow rates in ml/min



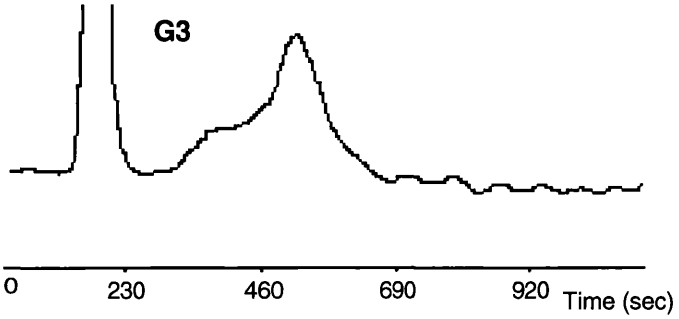
Time	%B	Flow rate
0	15	0.8
240	15	0.8
270	17	0.8
320	17	0.8
410	25.7	0.8
590	25.7	0.8
680	30	0.8
860	30	0.8
950	37.1	0.8
1130	37.1	0.8
1720	100	0.8
2020	100	0.8

B=350mmo/L LiCl



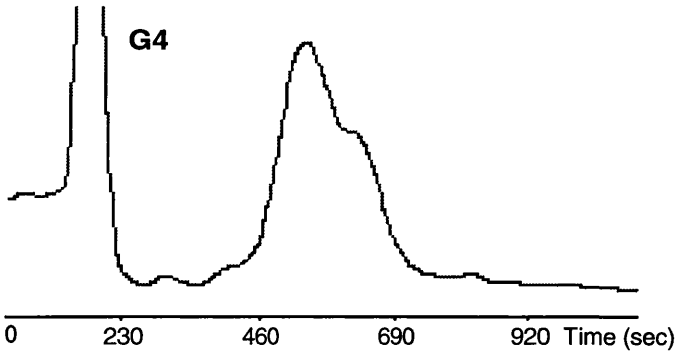
Time	%B	Flow rate
0	35	0.8
240	35	0.8
900	100	0.8

B= 500 mmol/L LiCl



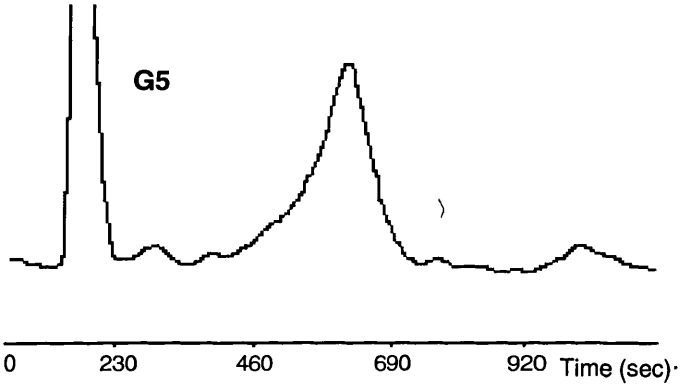
Time	%B	Flow rate
0	35	1
240	35	1
1200	100	1

B= 500 mmol/L LiCl



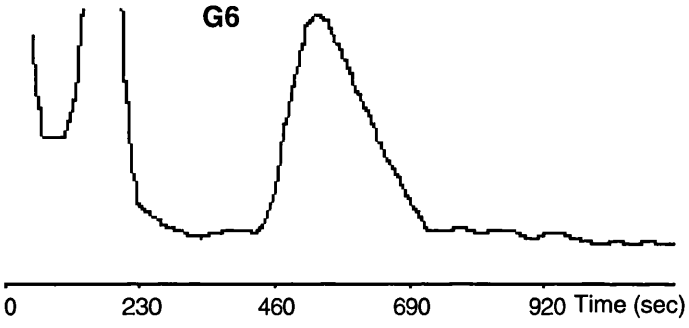
Time	%B	Flow rate
0	25	1
240	25	1
300	37	1
390	37	1
450	50	1
540	50	1
900	100	1
1200	100	1

B= 500 mmol/L LiCl



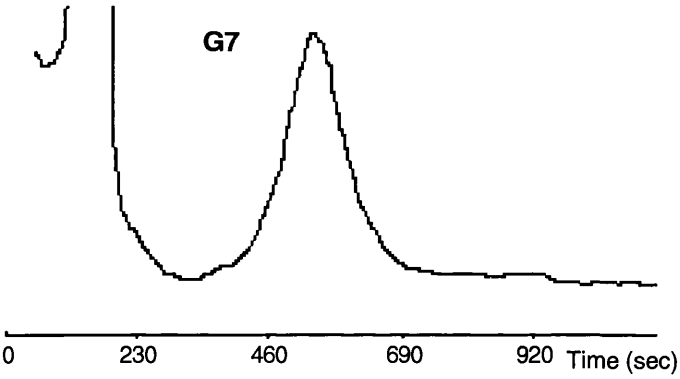
Time	%B	Flow rate
0	25	0.8
240	25	0.8
300	40	0.8
400	40	0.8
460	51.5	0.8
560	51.5	0.8
900	100	0.8
1200	100	0.8

B= 500 mmol/L LiCl



Time	%B	Flow rate
0	25	0.9
240	25	0.9
300	37.5	0.9
400	37.5	0.9
460	51.5	0.9
560	51.5	0.9
900	100	0.9
1200	100	0.9

B= 500 mmol/L LiCl



Time	%B	Flow rate
0	25	1
240	25	1
300	40	1
400	40	1
500	51.5	1
560	51.5	1
900	100	1
1200	100	1

B= 500 mmol/L LiCl

Appendix B1: Analytical imprecision for isoenzymes of hexosaminidase anlysed on MCC

I-Long term variations

1-Glycomat (GMAT) **2- G15**

retention times given in secondes, CV in percent.

ID	HEX.B	HEX.I1	HEX.I2	HEX.A	A/B	A/I1	A/I2	ID	Hex.B	Hex.A	HexA/HexB
1	490	657	968	1218	2.49	1.85	1.26	1	323	958	2.97
2	491	662	991	1217	2.48	1.84	1.23	2	277	940	3.39
3	498	662	966	1133	2.28	1.71	1.17	3	252	946	3.75
4	490	662	969	1218	2.49	1.84	1.26	4	266	945	3.55
5	491	660	965	1217	2.48	1.84	1.26	5	291	951	3.27
6	488	659	1002	1230	2.52	1.87	1.23	6	296	946	3.20
7	496	683	986	1216	2.45	1.78	1.23	7	279	940	3.37
8	496	662	967	1229	2.48	1.86	1.27	8	278	941	3.38
9	490	662	967	1207	2.46	1.82	1.25	9	278	960	3.45
10	496	660	968	1217	2.45	1.84	1.26	10	277	926	3.34
11	499	658	968	1207	2.42	1.83	1.25	11	308	946	3.07
12	498	663	968	1217	2.44	1.84	1.26	12	288	982	3.41
13	500	658	968	1240	2.48	1.88	1.28	13	273	971	3.56
14	499	661	966	1217	2.44	1.84	1.26	14	279	974	3.49
15	497	660	1012	1206	2.43	1.83	1.19	15	279	967	3.47
16	512	662	968	1230	2.40	1.86	1.27	16	278	968	3.48
17	499	651		1230				17	273	970	3.55
18	491			1206				18	280	971	3.47
								19	275	969	3.52
								20	275	955	3.47
								21	274	967	3.53
								22	300	954	3.18
								23	300	963	3.21
								24	283	971	3.43
								25	276	955	3.46
								26	281	963	3.43
								27		965	
Mean	**495.61**	**661.29**	**974.94**	**1214.17**	**2.45**	**1.83**	**1.25**		**282.27**	**957.65**	**3.40**
SD	**5.70**	**6.29**	**14.59**	**22.41**	**0.06**	**0.04**	**0.03**		**14.09**	**13.51**	**0.17**
CV	**1.15**	**0.95**	**1.50**	**1.85**	**2.25**	**2.16**	**2.32**		**4.99**	**1.41**	**4.96**

II- Variation within 1 column

	Hex A	Hex I2	Hex.I1	Hex B
11/2 (n=11)	1313	907	643	506
	1282	905	643	474
	1308	903	655	472
	1298	919	634	474
	1283	904		462
	1198	901	634	475
	1300		644	484
	1332	929		475
	1293	902	643	484
	1327	825	643	474
	1291	905	634	473
	1358	826	635	477
Mean	**1299**	**893**	**641**	**478**
SD	**38.70**	**34.55**	**6.70**	**10.60**
CV	**2.98**	**3.87**	**1.05**	**2.22**
12/2 (n=17)	1218	968	657	490
	1217	991	662	491
	1133	966	662	498
	1218	969	662	490
	1217	965	660	491
	1230	1002	659	488
	1216		683	496
	1229	986	662	496
	1207	967	662	490
	1217	967	660	496
	1207	968	658	499
	1217	968	663	498
	1217	968	658	499
	1206	968	661	497
	1230	966	660	512
	1230	1012	662	499
	1206	968	651	491
Mean	**1213**	**975**	**661**	**495**
SD	**22.12**	**14.59**	**6.29**	**5.77**
CV	**1.82**	**1.50**	**0.95**	**1.16**

	Hex A	Hex I2	HexI1	HexB
16/2 (n=11)	1177		579	371
	1176	707	499	371
		730	555	375
	1114	697	531	447
	1241	697	534	436
	1142	708	531	448
	1447	701		434
	1127	605		456
	1458	708		436
	1118	673	534	436
	1461	698	535	
	1251	695	555	429
Mean	**1246.1**	**692.4**	**537.25**	**421**
SD	**141.44**	**32.07**	**22.10**	**32.63**
CV	**11.35**	**4.63**	**4.11**	**7.75**

11/2 (n=11)	1299	893	641	478
12/2 (n=17)	1213	975	661	495
16/2 (n=11)	1246	692	537	421
	1252.67	**853.33**	**613.00**	**464.67**
	43.39	**145.61**	**66.57**	**38.76**
	3.46	**17.06**	**10.86**	**8.34**

III- Statistical data obtainrd for the test of variances

F-Test: Two-Sample for Variances

Batches		*Variable 1*	*Variable 2*	Batches	*Variable 1*	*Variable 2*
12 vs 16	Mean	1212.64706	1246.54545	11 vs 16	-1298.58333	1246.54545
	Variance	489.367647	20006.2727		1497.90152	20006.2727
	Observations	17	11		12	11
	df	16	10		11	10
	F	40.8818868			13.3562003	
	P(F<=f) one-tail	1.7491E-09			8.8503E-05	
	F Critical one-tail	2.02814476			2.24822827	

		Variable 1	*Variable 2*
11 vs 12	Mean	1298.58333	1212.64706
	Variance	1497.90152	489.367647
	Observations	12	17
	df	11	16
	F	3.060892	
	P(F<=f) one-tail	0.02093683	
	F Critical one-tail	2.45636755	

APPENDIX B2: Data for Hexosaminidase isoenzymes determined by MCC and used for calculation of imprecision

(Data = hexosaminidase A percentage)

	Within batch				Between batch
		Serum	Leucocytes		Serum
ID	Carrier	Normal	Carrier	Normal	Normal
1	50.5	62.2	35.2	74.6	62
2	39.7	62.8	36.4	65.6	63.3
3	50.81	58.67	35.57	68.48	61.2
4	42	63.9	36.8	64.54	60.0
5	50.8	61.8	30.5	69.4	61.6
6	50.6	60	32.98	75.5	61./9
7	45.55		29.28	75	
8	59.56		30.45	72.4	
9	55			77.2	
Mean	**49.39**	**61.56**	**33.79**	**71.64**	**61.66**
SD	**4.65**	**1.48**	**2.66**	**4.12**	**0.74**
CV	**9.41**	**2.41**	**7.37**	**5.75**	**1.2**

APPENDIX B3: Data for Hexosaminidase isoenzymes determined by MCC and electrophoresis and used for method comparison.

GMAT and electrophoresis (E)

(data = hexosaminidase A percentage)

Sample No	E.	GMAT	GMAT-E
Normal control	67	74	7
Carrier control	37	38	1
180906	77	70	-7
180907	69	72	3
180908	69	70	1
180909	67	68	1
180910	67	69	2
180911	73	71	-1
180912	76	78	2
180913	68	69	1
1180914	65	68	3
180915	75	68	-7
180816	66	69	3
180917	71	88	17
180918	76	74	-2
140567	57	57	0
141112	48	45	-3
140311	42	22	-20
146809	39	20	-19
140429	43	48	5
140604	54	58	4
140486	45	41	-4
140524	66	54	-12
140746	49	52	3
141164	51	42	-9
140618	38	54	16
146790	26	48	22
140717	57	48	-9
140575	47	31	-16
140417	32	38	6
141102	20	24	4
140524	66	58	-9

G15 and electrophoresis

No	E	G15	G15-E
	66	75	10
	42	50	8
1	67	74	7
2	66	72	6
3	36	41	5
4	64	70	6
5	43	40	-3
6	61	58	-3
7	33	35	3
8	62	68	6
9	61	62	1
10	71	62	-9
11	70	67	-3
12	56	70	14
13	82	83	1
14	75	59	-16
15	66	78	12
16	50	58	8
17	68	64	-4
18	42	55	14
19	66	56	-10
20	49	41	-8
21	51	70	19
22	64	87	23
23	46	51	5

Comparison between electrophoresis and G15 on QC samples

Sample No	E.	G15
1	66	75
2	64	74
3	48	41
4	65	70
5	53	40
6	43	35
7	64	68
8	63	62
9	66	67
10	50	70
11	84	83
12	77	59
13	62	78
14	43	58
15	66	64
16	36	55

Appendix C1: Data for the reproducibility of retention times (RT) for ALP isoenzymes analysis on MCC.

I-Within batch analysis. RTs, mean and SDs are given in seconds, CV in percent

1-Sample with high ALP activity: 582 U/L

sample ID	B1	B2	L1	L2	L3
17	669	926	1220	1537	1729
18	683	933	1251	1531	1712
19	670	927	1249	1515	1716
20	694	948	1217	1525	1729
21	679	932	1235	1519	1705
22	679	935	1207	1524	1720
23	667	927	1251	1515	1731
24	708	916	1204	1505	1718
25	668	928	1194	1503	1729
26	658	926	1201	1499	
28	669	916	1120	1497	
Mean	**676.73**	**928.55**	**1213.55**	**1515.45**	**1721**
SD	**14.19**	**8.86**	**37.42**	**13.26**	**9.11**
CV	**2.10**	**0.95**	**3.08**	**0.88**	**0.53**

2-Sample with a low activity (36 U/L)

ID	B2	L1	L2	L3
2		944	1143	1561
3	698	928	1148	1524
4		940	1146	1549
5	704	940	1149	1545
6		936	1133	1539
7	679	936	1192	1554
8	689	930	1222	1529
9	668	930	1144	1545
10		917	1146	1529
Mean	**687.60**	**933.44**	**1158.11**	**1541.67**
SD	**14.47**	**8.17**	**29.08**	**12.46**
CV	**2.10**	**0.88**	**2.51**	**0.81**

II- Between batch analysis RTs, mean and SDs are given in seconds, CV in percent

1-Normal serum from a neonate

ID	B1	B2	L1	L2	L3
1320	658	910	1120	1510	
1417	648	908	1108	1523	
1505	651	906	1132	1494	
1519	663	895	1118	1506	
1603	618	901	1113	1493	
1618	650	896	1117	1516	
1703	633	879	1111	1483	
1717	630	872	1116	1520	
Mean	**643.9**	**895.9**	**1116.9**	**1505.6**	
SD	**15.4**	**13.8**	**7.3**	**14.4**	
CV	**2.4**	**1.5**	**0.6**	**1.0**	

2-Serum from an adult with liver disorder

	B1	B2	L1	L2	L3
1321		996	1142	1465	1739
1410		999	1132	1491	1781
1418		981	1176	1471	1738
1506		981	1140	1444	1736
1520			1162	1479	1660
1604		951	1133	1456	1746
Mean		**981.6**	**1147.5**	**1467.7**	**1733.3**
SD		**19.0**	**17.7**	**16.7**	**39.7**
CV		**1.9**	**1.5**	**1.1**	**2.3**

III- Long term analysis RTs, mean and SDs are given in seconds, CV in percent

ID	B1	B2	L1	L2	L3	B2/B1	L1/B1	L2/B1	L3/B1
1318	638	921	1139	1494		1.44	1.79	2.34	
1319	658	924	1155	1506		1.40	1.76	2.29	
1320	658	910	1120	1510		1.38	1.70	2.29	
1321		996	1142	1465	1739				
1322	659	919	1109	1487		1.39	1.68	2.26	
1323	668	913	1119	1506	1727	1.37	1.68	2.25	2.59
1324	659	906	1152	1521		1.37	1.75	2.31	
1325	659	902	1126	1524		1.37	1.71	2.31	
1326	659	927	1123	1484		1.41	1.70	2.25	
1327	676	905	1123	1467		1.34	1.66	2.17	
1328	642	952	1152	1507		1.48	1.79	2.35	
1329	658	910	1110	1466	1787	1.38	1.69	2.23	2.72
1403	671	918	1126	1500		1.37	1.68	2.24	
1304	720	958	1171	1505		1.33	1.63	2.09	
1408	656	909	1106	1506	1758	1.39	1.69	2.30	2.68
1409	648	917	1088	1434	1781	1.42	1.68	2.21	2.75
1410		999	1132	1491	1781				
1411	639	915	1114	1476		1.43	1.74	2.31	
1412	676	908	1116	1484		1.34	1.65	2.20	
1416	692	921	1169	1506		1.33	1.69	2.18	
1417	648	908	1108	1523		1.40	1.71	2.35	
1418		981	1176	1471	1738				
1419	646	920	1131	1484		1.42	1.75	2.30	
1420	628	906	1115	1501		1.44	1.78	2.39	
1503	638	900	1120	1434		1.41	1.76	2.25	
1504	649	914	1131	1432		1.41	1.74	2.21	
1505	651	906	1132	1494		1.39	1.74	2.29	
1506		981	1140	1444	1736				
1507	629	892	1120	1464	1741	1.42	1.78	2.33	2.77
1508	637	885	1113	1452		1.39	1.75	2.28	
1509	629	894	1124	1454		1.42	1.79	2.31	
1510	638	901	1113	1391		1.41	1.74	2.18	
1511	629	902	1109	1466	1736	1.43	1.76	2.33	2.76
1512	648	901	1110	1494	1725	1.39	1.71	2.31	2.66
1513	641	932	1127	1445	1734	1.45	1.76	2.25	2.71
1517	644	898	1164	1498		1.39	1.81	2.33	
1518	685	898	1128	1505		1.31	1.65	2.20	
1519	663	895	1118	1506		1.35	1.69	2.27	
1520		930	1162	1479	1660				
1521	676	889	1011	1512	1749	1.32	1.50	2.24	2.59
1522	649	893	1068	1512	1687	1.38	1.65	2.33	2.60
1523	694	883	1055	1524		1.27	1.52	2.20	

1524	628	974	1090	1483		1.55	1.74	2.36	
1525	667	957	1078	1477		1.43	1.62	2.21	
1526	632	924	1053	1497		1.46	1.67	2.37	
1527	626	913	1053	1492		1.46	1.68	2.38	
1528	677	905	1041	1481	1798	1.34	1.54	2.19	2.66
1529	667	895	1116	1497	1790	1.34	1.67	2.24	2.68
1602	705	900	1114	1493	1781	1.28	1.58	2.12	2.53
1603	618	901	1113			1.46	1.80		
1604		951	1133	1456	1746				
1605	633	898	1101	1479	1781	1.42	1.74	2.34	2.81
1606	644	901	1114	1509	1714	1.40	1.73	2.34	2.66
1607		966	1135	1449					
1609	633	890	1100	1494		1.41	1.74	2.36	
1610	661	905	1084	1455		1.37	1.64	2.20	
1611	665	912	1122	1459	1764	1.37	1.69	2.19	2.65
1612	662	925	1125	1461	1759	1.40	1.70	2.21	2.66
1617	660	896	1117	1522		1.36	1.69	2.31	
1618	675	896	1117	1516	1683	1.33	1.65	2.25	2.49
1619	629	869	1105	1513	1692	1.38	1.76	2.41	2.69
1620		940	1186	1494	1660				
1621	739	872	1102	1518	1660	1.18	1.49	2.05	2.25
1622	659	901	1117	1449	1630	1.37	1.69	2.20	2.47
1623	671	880	1118	1521	1677	1.31	1.67	2.27	2.50
1624	642	890	1123	1464	1660	1.39	1.75	2.28	2.59
1625	641	897	1129	1442		1.40	1.76	2.25	
1626		913	1117	1510	1660				
1627	657	905	1118	1499	1660	1.38	1.70	2.28	2.53
1628	641	884	1102	1449		1.38	1.72	2.26	
1703	633	879	1111	1483		1.39	1.76	2.34	
1704	613	860	1109	1473	1740	1.40	1.81	2.40	2.84
1705		981	1140	1440	1726				
1707	613	885	1113	1462		1.44	1.82	2.38	
1708	626	893	1081	1443		1.43	1.73	2.31	
1709	605	850	1042	1470	1725	1.40	1.72	2.43	2.85
1710	613	878	1114	1453		1.43	1.82	2.37	
1711	614	850	1102	1469	1741	1.38	1.79	2.39	2.84
1712	612	936	1110	1425		1.53	1.81	2.33	
1713	613	908	1129	1442	1713	1.48	1.84	2.35	2.79
1714	631	877	1105	1464	1756	1.39	1.75	2.32	2.78
1717	630	872	1116	1520	1692	1.38	1.77	2.41	2.69
1718		927	1173	1497	1660				
1719	636	849	1111	1498	1685	1.33	1.75	2.36	2.65
1721	661	927	1102	1523	1660	1.40	1.67	2.30	2.51
1722	661	914	1105	1525	1674	1.38	1.67	2.31	2.53
1723	622	879	1089	1489	1682	1.41	1.75	2.39	2.70
1803	721	969	1133	1556	1778	1.34	1.57	2.16	2.47
1804	744	968	1203	1430		1.30	1.62	1.92	

1805			1071	1578	1781				
1818	714	891	1093	1480		1.25	1.53	2.07	
1819	613	881	1114	1502	1736	1.44	1.82	2.45	2.83
1820	680	911	1093	1435		1.34	1.61	2.11	
1821	631	900	1093	1440		1.43	1.73	2.28	
1822	714	954	1041	1432		1.34	1.46	2.01	
1823	694	921	1125	1397		1.33	1.62	2.01	
1824	661	903	1136	1418		1.37	1.72	2.15	
1825	653	931	1126	1367		1.43	1.72	2.09	
1826	702	948	1112	1495	1747	1.35	1.58	2.13	2.49
1816	652	857	1093	1419		1.31	1.68	2.18	
1817	631	904	1093	1443	1675	1.43	1.73	2.29	2.65
Mean	**653.7**	**911.0**	**1114.5**	**1478.7**	**1721.7**	**1.39**	**1.70**	**2.26**	**2.65**
SD	**29.3**	**31.0**	**30.7**	**35.2**	**45.3**	**0.06**	**0.08**	**0.10**	**0.13**
CV	**4.5**	**3.4**	**2.8**	**2.4**	**2.6**	**4.06**	**4.64**	**4.49**	**4.97**

Appendix C4: Data for ALP isoenzymes determined by MCC in a hospital population

I-Relative activities (%)

	Sample ID	Sex	Age	CLINICAL	I	B1	B2	L1	L2	L3
0 to 6 M	Hn	F	1M	transpos. great arteries		9.09	56.47	27.55	6.89	
	RM	M	1M	abdominal pains		6.2	35.24	33.08	25.5	
	W	F	4M	pre op Bailey W		4.09	24.32	39.3	24.48	7.82
	HC	F	5M	pre op 6A		8.73	51.53	21.11	18.64	
	9073			Cord blood		7.03	53.74	31.43	7.81	
	8889			Cord blood		4.09	23.87	29.9	37.13	5
	8867			Cord blood		6.73	37.43	32.92	18.34	4.59
	9997			Cord blood		5.65	42.56	28.4	23.4	
	8926			Cord blood		5.4	45.85	35.3	13.45	
	8937			Cord blood		9.05	51.51	30.16	9.28	
	MEAN					**6.57**	**40.37**	**30.76**	**19.83**	**5.80**
	SD					**1.99**	**13.71**	**5.59**	**10.56**	**1.76**
	RB	M	2M	pre op 5CE		15.89	48.47	33.61	2.04	
	GS	M	2M	anaemia-T		21.8	45.17	24.4	8.6	
	HN	F	2M	truncus repair		1.72	29.54	46.86	19.05	2.83
6M-2Y	DC	F	11M	hernia, pre op block shunt		7.44	33.38	38.85	17.79	3.26
	LC	M	12M	OOC encephal for repair		11.73	45.33	32.01	9.41	1.53
	AM	F*	14M	for shunt		10.96	50.5	32.82	4.75	1
	BC	M	15M	pre op 4CD		6.8	34.6	45.74	10.35	2.5
	GP	F	16M	VSD		12.36	45.03	36.76	5.85	
	WN	F	18M	open heart surg.		14.47	40.22	36.89	8.41	
	HC	M	18M	pre op 6CD		10.51	44.31	42.55		2.63
	CI	F	19M	VSD		9.91	48.98	32.2	6.5	2.41
	CK	M	19M	VSD	3.93	18.67	33.13	32.79	6.08	
	S	*	20M	pre op 3C		7.88	39.83	37.69	12.06	2.55
	PB	F	5M	preop 6B		13.2	46.37	33.04	4.6	2.8

	TR	M	7M	Nenopatic bladder		10.59	47.53	28.24	13.65	
	LF	M	7M	cerebral aneurysm	2.75	8.72	47.27	33.31	7.95	
	HI	M	9M	cardiac catheter		8.77	35.63	40.56	12.96	2.02
	Mean				**3.34**	**10.86**	**42.29**	**35.96**	**9.26**	**2.30**
	SD				**0.83**	**3.16**	**6.07**	**4.79**	**3.96**	**0.69**
	CV				**24.98**	**29.08**	**14.35**	**13.31**	**42.82**	**29.79**
3Y-10Y	RL	F	3	hydrocephalus		8.28	40.95	36.98	13.8	
	WJ	F	3	pre liver biop,pre op splen		3.99	37.08	41.08	13.48	4.37
	B	F	3	cardiac ITU		12.22	46.03	39.1		2.65
	DM	M	3	pre op 2C		5.16	35.71	41.75	15.72	1.67
	MC	M	3	pre op?		7.4	32.22	45.51	14.86	
	R	M	3	haemophagocitic syndrome		8.47	57.63	25.42		
	R2	M	3	idem		4.64	37.47	44.7	13.21	
	NE	F	3.2	patentductus arteriosus		9.95	52.13	33.66	3.91	
	FD	F*	3.8	pre op 7AB		10.65	54.56	30.16	4.63	
	CR	F	5	pre op T's & A's		7.68	35.63	35.53	16.37	4.8
	SS	M*	5	pre op?		5.03	29.41	35.69	17.43	3.52
	GA	F	5.5	eczema		5.91	36.29	46.77	8.06	2.69
	DC	M	5.7	coarctation		6.72	37.39	43.78	10.59	1.53
	HI	M*	6.8	cardiac catheter	1.33	9.72	48.62	40.32	1	
	GE	*	7	eczema		9.14	53.78	30.05	3.51	3.51
	AS	F	7	skin patient		3.6	27.71	36.4	23.5	8.43
	HU	F*	7.5	ASD		7.94	40.82	39.84	9.32	2.08
	TR	F	8	eczema		2.12	18.15	41.49	26.05	12.18
	McS	M	8	skin patient		2.17	32.55	51.54	13.74	
	RD	M	10	cardiac catheter		9.64	41.33	44.55	4.48	
	AK	F	*	sickle cell		3.61	26	38.91	23.54	7.93
	Mean					**6.86**	**39.12**	**39.20**	**12.48**	**4.61**
	SD					**2.89**	**10.21**	**6.21**	**7.20**	**3.27**
	CV					**42.06**	**26.09**	**15.84**	**57.67**	**70.84**

11Y-17Y	LE	M	13	Leg lengthen		9.19	37.4	38.46	13.1	1.85
	WA	F	15	pre op ?		7.7	38.19	41.56	12.55	
>17	4-890/1	M	16	school screen for Hex		7.06	22.62	26.73	28.24	15.36
	-684	F	20	idem		12.35	25.11	21.08	28.81	12.66
	-186	F	18	idem		32.25	40.66	20.73	3.61	
	649	F	20	idem		6.23	32.55	32.99	28.23	
	768	F	18	idem		11.98	36.08	38.88	13.06	
	-301	F	18	idem		14.94	28.94	30.41	25.44	
	-980	F	16	idem		12	42.13	24.29	21.58	
	-054	F	16	idem		14.98	51.28	27.53	6.21	
	-918/1	M	18	idem	**	6.83	41	25.02	11.12	17.43
	-657			idem		7.05	29.12	33.19	20.12	10.51
	097			idem		8.58	46.3	29.12	15.99	
	-233			idem		5.82	37.87	32.93	15.25	8.12
	-634			idem		7.11	23.34	43.49	26.05	
	-859			idem		5.06	18.68	43.96	31.87	1
	GREEN	F	18Y	mother of infant		6.55	47.33	36.41	9.71	
	Rawling	F	21Y	mother of infant	1.38	2.3	33.07	40.42	20.9	1.93
	Mean				**1.38**	**10.71**	**36.156**	**32.929**	**16.805**	**8.6075**
	SD					**6.556**	**8.9709**	**7.0824**	**8.8143**	**6.4569**
	CV					**61.18**	**24.812**	**21.508**	**52.45**	**75.015**
PATHOLO-GICAL SPECIMEN	2140		2M	?	3	17.17	28.04	29.27	20.18	
	2446		10.5Y	CRF		6.84	49.56	34.63	8.97	
	PP		2m	Intestinal obstruction		6.41	18.22	52.4	22.97	
	2443		11Y	CRF		8.69	44.55	34.49	10.79	1.48
	2405		12Y	CRF		5.99	44.49	35.66	11.03	2.84
	2265		3Y	Neuroblastoma			4.66	21.97	49.72	21.09
	259		<3M	Mn		5.92	34.6	35.13	18.3	6.05
	189		5M	post BMT		9.38	34.95	43.01	12.66	

184	2M	"	1.46	11.47	48.94	28.94	9	
187	2M	TPN Hernie	1.5	12.2	38.91	36.16	11.23	
296	3M	TPN		13.57	50.07	28.34	7.14	1
193	2.5M	AML		3.86	27.54	59.59	9.01	
228	5.5M	TPN	1	12.67	35.65	33.19	12.37	5.5
5242		Trans.hyperphos.		0.01	12.06	31.35	32.46	24.12
5214		"		1.13	14.45	32.65	27.02	24.77
2164	5Y	TPN		4.38	13.09	56	23.97	2.56
9594	2y10	ALL		5.98	40.45	37.05	13.36	3.17
2170	6M	ALL/TPN?		10.11	29.07	32.84	20.21	7.78
2377	2Y	ALL			24.82	49.06	26.26	8.85
2374	8Y	ALL		3.77	24.26	42.6	23.09	6.28
2173	8Y	AML		5.76	27.12	47.52	19.06	

II-Absolute activities for individual isoenzymes (in U/L), Means and SDs in U/L, CVs in %

0 to 6 M

ID	S	A	TALP	I	B1	B2	L1	L2	L3
Hn	F	1M	127		11.54	71.72	34.99	8.75	
RM	M	1M	101		6.26	35.59	33.41	25.76	
W	F	4M	141		5.77	34.29	55.41	34.52	11.03
HC	F	5M	140		12.22	72.14	29.55	26.10	
9073			240		16.87	128.98	75.43	18.74	
8889			117		4.79	27.93	34.98	43.44	5.85
8867			131		8.82	49.03	43.13	24.03	6.01
9997									
8926									
8937			138		12.49	71.08	41.62	12.81	
MEAN			**141.88**		**9.84**	**61.35**	**43.57**	**24.27**	**7.63**
SD			**41.88**		**4.16**	**32.86**	**15.15**	**11.22**	**2.94**
CV			**29.518**		**42.2**	**53.558**	**34.77**	**46.23**	**38.567**

6M-2Y

ID	Sex	A	TALP	I	B1	B2	L1	L2	L3
DC	F	11M	IS						
CL	M	12M	IS						
AM	F*	14M	120		13.15	60.60	39.38	5.70	1.20
BC	M	15M	272		18.50	94.11	124.41	28.15	6.80
GP	F	16M	120		14.83	54.04	44.11	7.02	
WN	F	18M	172		24.89	69.18	63.45	14.47	
HC	M	18M	215		22.60	95.27	91.48	0.00	5.65
CI	F	19M	151		14.96	73.96	48.62	9.82	3.64
CK	M	19M	79	3.105	14.75	26.17	25.90	4.80	
S	*	20M	187		14.74	74.48	70.48	22.55	4.77
PB	F	7M	IS						
TR	M	7M	56		5.93	26.62	15.81	7.64	
LF	M	7M	206	5.665	17.96	97.38	68.62	16.38	

	R	M	1M	185		29.40	89.67	62.18	3.77			HI	M	9M	350		30.70	124.71	141.96	45.36	7.07
	CS	M	2M	109		23.76	49.24	26.60	9.37			**Mean**			**175.27**	**4.38**	**17.55**	**72.41**	**66.75**	**14.72**	**4.77**
	HN	F	2M	97		1.67	28.65	45.45	18.48	2.75		**SD**			**85.29**	**1.81**	**6.61**	**30.13**	**39.34**	**13.09**	**2.20**
												CV			**48.66**		**37.69**	**41.62**	**58.93**	**88.94**	**46.11**
3Y-10Y	RL	F	3	218		18.05	89.27	80.62	30.08		**11-17Y**	lee	M	13	123		11.30	46.00	47.31	16.11	2.28
	WJt	F	3	129		5.15	47.83	52.99	17.39	5.64		william	F	15	65		5.01	24.82	27.01	8.16	
	B	F	3	131		16.01	60.30	51.22	0.00	3.47	**>17**	890/1	M	16	97		6.85	21.94	25.93	27.39	14.90
	DM	M	3	67		3.46	23.93	27.97	10.53	1.12		-684	F	20	57		7.04	14.31	12.02	16.42	7.22
	MC	M	3	140		10.36	45.11	63.71	20.80			-186	F	18	118		38.06	47.98	24.46	4.26	
	R	M	3	111		9.40	63.97	28.22	0.00			649	F	20	98		6.11	31.90	32.33	27.67	
	R2	M	3	99		4.59	37.10	44.25	13.08			768	F	18	98		11.74	35.36	38.10	12.80	
	N	F	3.2	84		8.36	43.79	28.27	3.28			-301	F	18	36		5.38	10.42	10.95	9.16	
	HD	F*	3.8	151		16.08	82.39	45.54	6.99			-980	F	16	57		6.84	24.01	13.85	12.30	
	CR	F	5	157		12.06	55.94	55.78	25.70	7.54		-054	F	16	63		9.44	32.31	17.34	3.91	
	SS	M*	5	192		9.66	56.47	68.52	33.47	6.76											
	DC	M	5.7	93		6.25	34.77	40.72	9.85	1.42		G	F	18	131		8.58	62.00	47.70	12.72	
	HI	M*	6.8	129	1.716	12.54	62.72	52.01	1.29	0.00		R	F	21	128	1.766	2.94	42.33	51.74	26.75	2.47
	AS	F	7	241		8.68	66.78	87.72	56.64	20.32		**Mean**			**89.25**	**1.766**	**9.94**	**32.782**	**29.06**	**14.8**	**6.7153**
	HU	F*	7.5	228		18.10	93.07	90.84	21.25	4.74		**SD**			**32.497**		**9.21**	**14.946**	**14.49**	**8.482**	**5.9149**
	McS	M	8	248		5.38	80.72	127.82	34.08			**CV**			**36.411**	**0**	**92.7**	**45.593**	**49.86**	**57.3**	**88.081**
	RD	M	10	84		8.10	34.72	37.42	3.76			-918/1	M	18	207		14.14	84.87	51.79	23.02	36.08
	Mean			**147.18**	**1.72**	**10.13**	**57.58**	**57.86**	**16.95**	**5.67**											
	SD			**58.41**		**4.70**	**20.33**	**26.62**	**15.43**	**6.08**											
	CV			**39.69**		**46.40**	**35.31**	**46.01**	**91.02**	**107.29**											

Appendix C5: Clinical information on the hospital population used for establishing a reference range for ALP isoenzymes

Sample I.D	Sex	Age	Clinical features	Laboratories investigations			Diagnosis
				Biochem	Microbiol	Histopath.	
HK	F	1m	No details	**high** Bil., potassium, phosphate **Low** Urea, calcium	None	None	Transposition of great arteries and extended arch repair
RB	M	2m	Birth weight = 1.12 kg PCO2 raised at 32 h of age	**High** Bil **Low** urea, potassium	Normal	none	prematurity, functional obstruction
GS	M	2m	No complete file		Sepsis		pulmonary atresia, VSD
HN	F	2m	6Lb 11oz, on Digoxin and diuretic since 1st day of life failure to thrive	Normal	Normal	none	Truncus arteriosus Type I, Repair with 9mm pulmonary homograft, gross motor delay.
PB	F	5m	No complete file				Congenital malformation, Bilateral open reduction of the hip by the medial approach
LF	M	7m	Child happy and alert, dilated rigth pupil with divergent squint	normal	normal	none	Intracavernous internal carotid aneurism
HI	M	9m	microcephaly, neuro-developmental delays and failure to thrive chiken pox 4 weeks before admission	**high** calcium	Staph Aureus	none	Perimembraneous ventricular septal defect
HH	F	12m	satisfactory O2 saturation on air, noisy breathing, bubbling in a naso-gastric tube	normal	Staph. Aureus	none	recurrent tracheo-oesophageal fistula, tracheo-malacia, gastro-oesophageal reflux, ASD
LC	M	12m	no past-medical history, alert and lively, neurological examination normal	normal	none	none	Parietal meningocele
WN	F	18m	chest infections, treated with amoxicillin	normal	none	none	secundum atrial septal defect
HC	M	18m	urinary tract infection, 3 weeks vomiting and fever, diarrhoea	normal	rotavirus, enterococcus	none	right pyelonephrosis, secondary to PUJ obstruction

CK	M	19m	failure to thrive, saturation @75% or less, on panel of drugs	**high** AST, **low** calcium, potassium, albumin	normal	none	multiple congenital anomalies (pulmonary atresia, VSD...)
CJ	F	19m	aortic saturation between 65-70%, chest clear, liver and spleen not palpable	normal	none	none	pulmonary atresia, VSD/right aortic arch. previous operations.
RL	F	3y	history of post-haemorrhagic communicating hydroicephalus, on examination alert and apyrexial	normal	Staph. Aureus	none	communicating hydrocephalus
WJ	F	3y	general delay, hearing loss, bilateral convergent strbismus, unable to take normal solids.	none	none	Liver: mild microglobular fatty changes	global neuro-developmental delay with severe speech and langage delay, mild pulmonary stenosi, Patent Ductus Arterosis...
DM	M	3y	History: right tonsillectomy and partial removal of right soft palate and uvula haemangioma	normal	commensals	none	Palatal haemangioma
NE	F	3.2y	frequent chest infections, asthma, on Becotide and Ventoline	normal	normal	none	Patent arterial duct for closure using catheter occlusion device.
CR	F	5y	numerous endoscopic procedure to drain the cyst prior to open surgery	none	none	none	Recuurent right ventricular cyst
DC	M	5.7y	dysmorphic with short neck and low set ears consistent with Noonan's syndrome	normal	normal	none	Coarctation of the aortarepaired with subclavian flap followed by recoarctation ballon dilatation.
LD	M	12.8y	very little informations, normal pre-op for ALP	none	none	none	Leg lenghtening lef Tib. and Fib., Olliers disease
WA	F	15.2y	No acute distress, unremarkable examination	normal	none	none	Pectus carinatum

Clinical information on the pathological specimen

296	M	3m	chronic diarrhoea, failure to thrive, on TPN, ALP normal on arrival, not repeated	**high** AST, Mn,	normal	none	Congenital enteropathy, cause unknown
187	M	2m	laparatomy at few days of age, several blood transfusions , disruption of wound on several occasions, on TPN	**high** Bil, AST,	wound sepsis	none	hepatosplenomegaly, no gallstones, no biliary, exact diagnosis unknown
189	M	5m	on TPN, post BMT	?	?	?	raised Mn
2170	M	6m		**high** ALT, **normal** AST	?	?	Acute Lymphoblastic Leukaemia
2173	M	8y					Acute Myeloblastic Leukaemia
193	M	2.5m	liver distented, spleen enlarged, on TPN, immunosupressed with acute RSV brochitis				AML pot ADE
2443	M	11y	under dialysis, waiting for renal transplant	**high** urea, creatinine, phosphate, **low** calcium, albumin			Chronic Renal Failure
2446	M	10.5y	awaiting for renal transplant, under series of drugs	**high** urea, creatinine, phosphate, AST			end stage renal failure secondary to juvenile nephronophtisis
2405	M	12y	bright, alert and looked well on examination	?			renal vascular disease, deteriorating renal function, problematic control of blood pressure

Appendix C2: Data for imprecision obtained after ALP isoenzymes analysis on MCC.

I-Within batch analysis – % of total activity

1-Sample with an elevated total ALP (582 U/L)

Sample ID	Bl	B2	L1	L2	L3	BONE	LIVER
017	1.32	7.06	22.66	51.18	17.78	8.38	91.62
018	1.35	8.62	26.95	46.48	16.6	9.97	90.03
019	1.28	7.49	28.99	47.78	14.45	8.77	91.22
020	1	6.62	24.92	52.05	15.44	7.62	92.41
021	2.02	6.67	26.69	47.32	17.29	8.69	91.3
022	2.43	7.93	29.12	47.32	13.21	10.36	89.65
023	1.49	6.83	30.3	47.92	13.47	8.32	91.69
024	1.63	7.81	30.38	48.41	11.77	9.44	90.56
025	1.61	8.05	31.55	48.28	10.52	9.66	90.35
026	1	7.02	41.13	42.11	9.36	8.02	92.6
MEAN	**1.51**	**7.41**	**29.27**	**47.89**	**13.99**	**8.92**	**91.14**
SD	**0.44**	**0.67**	**4.98**	**2.68**	**2.86**	**0.90**	**0.98**
CV	**29.28**	**9.10**	**17.00**	**5.60**	**20.45**	**10.04**	**1.08**

2-Sample with a low total ALP (36 U/L)

ID	Bl	B2	L1	L2	L3	BONE	LIVE
002		33.94	20.68	31.4	13.98	33.94	66.06
003	2.8	17.99	29.49	31.13	18.59	20.79	79.21
004		30.6	24.15	30.43	14.81	30.6	69.39
005	2.57	21.73	42.7	33		24.3	75.7
006		41.58	29.47	28.96		41.58	58.43
007	8.2	31.21	34.34	26.24		39.41	60.58
008	6.64	31.85	35.5	26.01		38.49	61.51
009	4.85	34.17	34.14	26.83		39.02	60.97
010		26.92	44.59	28.48		26.92	73.07
MEAN	**5.01**	**30.00**	**32.78**	**29.16**	**15.79**	**32.78**	**67.21**
SD	**2.43**	**7.02**	**7.84**	**2.49**	**2.46**	**7.49**	**7.49**
CV	**48.56**	**23.40**	**23.93**	**8.54**	**15.56**	**22.85**	**11.15**

II-Between batch analysis – % of total activity

1- Serum from a neonate

ID	B 1	B2	L1	L2	L3	BONE	LIVER
13-20	8.21	48.56	28.54	14.7		56.77	43.24
14-17	10.11	42	27.26	14.74		52.11	42
15-05	11.33	43.14	35.65	9.87		54.47	45.52
15-19	7.04	32.54	43.05	17.38		39.58	60.43
16-03	10.14	50.44	25.17	11.32	2.94	60.58	39.43
16-18	9.17	33.04	30.28	21.46	6.06	42.21	57.8
17-03	7.57	39.23	31.95	18.86		46.8	50.81
17-17	8.77	38.1	23.29	21.58	8.27	46.87	53.14
Mean	**9.04**	**40.88**	**30.65**	**16.24**	**5.76**	**49.92**	**49.05**
SD	**1.44**	**6.53**	**6.33**	**4.36**	**2.68**	**7.28**	**7.69**
CV	**15.94**	**15.97**	**20.66**	**26.88**	**46.52**	**14.59**	**15.69**

2- Serum from adult with a liver disorder

ID	B2	L1	L2	L3	BONE	LIVER
13-21	10.56	63.17	22.03	4.24	10.56	89.44
14-10	15.18	69.95	12.87	2.44	15.18	85.26
14-18	11.48	53.44	31.27	3.81	11.48	88.52
15-06	18.19	63.96	15.86	1.99	18.19	81.81
15-20	11.19	49.63	34.01	5.16	11.19	88.8
16-04	10.18	65.45	18.55	5.82	10.18	89.82
Mean	**12.80**	**60.93**	**22.43**	**3.91**	**12.80**	**87.28**
SD	**3.19**	**7.74**	**8.51**	**1.50**	**3.19**	**3.13**
CV	**24.94**	**12.71**	**37.93**	**38.24**	**24.94**	**3.59**

Appendix C3: Data obtained after analysis of ALP bone isoenzymes on MCC and electrophoresis.

Bone= percentage for the bone fraction obtained after scanning the electrophoresis plate

B1, B2, L1 =as defined previously, All = B1, B2 and L1 pooled together

ID	electrophoresis	B1	B2	L1	B1 +B2	All
1	34.50	2.66	38.65	43.62	41.31	84.93
2	92.52	9.04	40.88	30.65	49.92	80.57
3	75.74	5.40	45.85	35.30	51.25	86.55
4	80.31	5.91	36.29	46.77	42.20	88.97
5	59.00	9.14	53.78	30.05	62.92	92.97
6	94.10	3.61	26.00	38.91	29.61	68.52
7	71.85	3.26	27.71	36.40	30.97	67.37
8	80.67	5.82	37.87	32.93	43.69	76.62
9	56.85	7.05	29.12	33.19	36.17	69.36
10	60.86	8.58	46.30	29.12	54.88	84.00
11	54.11	17.60	46.93	29.89	64.53	94.42
12	60.91	19.15	37.61	36.68	56.76	93.44
13	68.16	12.00	42.13	24.29	54.13	78.42
14	54.85	3.99	29.20	45.49	33.19	78.68
15		14.98	51.28	27.53	66.26	93.79
16	75.50	6.23	32.55	32.99	38.78	71.77
17	62.32	6.83	41.00	25.02	47.83	72.85
18	73.20	4.66	21.97	49.72	26.63	76.35
19	70.10	6.32	34.81	21.56	41.13	62.69
20		5.99	44.49	35.66	50.48	86.14
21	91.18	11.39	43.45	34.16	54.84	89.00
22	70.50	5.76	27.12	47.52	32.88	80.40
23	81.59	8.49	44.55	34.49	53.04	87.53
24		10.11	29.07	32.84	39.18	72.02
25	24.20	1.13	14.45	32.65	15.58	48.23
26	10.30		12.80		12.80	12.80
27		6.41	18.22	52.40	24.63	77.03
28		4.66	9.69	25.51	14.35	39.86

Appendix D1: Analytical imprecision for isoforms of transferrin on MCC.

Mean and SD are given in secondes, CV in percent, Normal = serum from normal adult for transferrin isoforms, ARD = serum from a patient with Alcoholism Related Disorder, D= day.

I-Within Batch analysis

Normal	RT2	RT4	RT2/RT4
1703	1286	1339	1.04
1704	1309	1348	1.03
1705	1317	1351	1.03
1706	1316	1344	1.02
Mean	**1307**	**1346**	**1.03**
SD	**14.4**	**5.2**	**0.01**
CV	**1.1**	**0.4**	**0.83**
		excluded:	

ARD	RT2	RT4	RT2/RT4
1710	1556	1586	1.02
1711	1295	1343	1.04
1712	1305	1336	1.02
1713	1302	1332	1.02
1714	1309	1341	1.02
Mean	**1353**	**1388**	**1.03**
SD	**5.91**	**4.97**	**0.01**
CV	**0.4**	**0.4**	**0.65**
17010	1556	1586	30

II- Between batch analysis

Normal	RT2	RT4	RT2/RT4
D. 9 (n=3)	1174	1224	1.04
D.17(n=4)	1307	1346	1.03
D.18	1360	1389	1.02
D.19	1380	1414	1.02
Mean	**1305**	**1343**	**1.03**
SD	**92.8**	**84.3**	**0.01**
CV	**7.1**	**6.3**	**0.91**

ARD	RT2	RT4	RT2/RT4
D.16 (n=2)	1239	1279	1.03
D.17(n=4)	1353	1388	1.03
D.18		1407	
D.19(n=2)	1353	1393	1.03
D.20		1426	
Mean	**1315**	**1379**	**1.03**
SD	**65.82**	**57.6**	**0.00**
CV	**5.0**	**4.2**	**0.31**

III- Daily analysis

Sample	RT2	RT4	RT4/RT2
Day 1			
9005	1183	1228	1.038
9006	1167	1220	1.0454
9007	1172	1223	1.0435
9009	1169	1220	1.0436
9010	1166	1213	1.0403
9011	1170	1216	1.0393
9013	1186	1240	1.0455
9014	1162	1240	1.0671
9015	1182	1237	1.0465
9016	1187	1234	1.0396
9017	1187	1248	1.0514
9018	1194	1241	1.0394
9019	1191	1243	1.0437
9021	1209	1254	1.0372
9022	1203	1249	1.0382
9023	1197	1249	1.0434
MEAN	**1183**	**1235**	**1.04**
SD	**14.1**	**13.1**	**0.01**
CV	**1.19**	**1.06**	**0.70**

Sample	RT2	RT4	RT4/RT2
Day 7			
1603	1250	1289	1.03
1607	1241	1279	1.03
1608	1218	1278	1.05
1609	1236	1276	1.03
1610	1242	1281	1.03
1613	1253	1296	1.03
1614	1247	1298	1.04
1618	1270	1313	1.03
1619	1266	1310	1.03
1620	1274	1317	1.03
MEAN	**1250**	**1294**	**1.04**
SD	**17.04**	**15.51**	**0.01**
CV	**1.364**	**1.199**	**0.55**

Day 8	RT2	RT4	RT4/RT2
1703	1286	1339	1.04
1704	1309	1348	1.03
1705	1317	1351	1.03
1706	1316	1344	1.02
1707		1340	
1708		1338	
1710	1556	1586	1.02
1711	1295	1343	1.04
1712	1305	1336	1.02
1713	1302	1332	1.02
1714	1309	1339	1.02
1715		1338	
1716	1309	1341	1.02
1717	1313	1344	1.02

Day 9	RT2	RT4	RT4/RT2
1803	1360	1389	1.0213
1804	1358	1392	1.025
1805		1397	
1806		1407	
1807	1364	1401	1.0271
1808	1374	1410	1.0262
MEAN	**1364**	**1399**	**1.02**
SD	**7.118**	**8.26**	**0.00**
CV	**0.522**	**0.59**	**0.25**

1718	1302	1339	1.03
1719	1304	1337	1.03
1720	1288	1322	1.03
1721	1313	1346	1.03
1722	1305	1342	1.03
1723	1326	1362	1.03
1724	1437	1471	1.02
1726	1329	1365	1.03
1727	1332	1368	1.03
1728	1339	1379	1.03
1729	1504	1540	1.02
MEAN	**1336**	**1368**	**1.03**
SD	**69.9**	**65.4**	**0.00**
CV	**5.23**	**4.78**	**0.48**

Day 10			
1903	1380	1414	1.02
1904	1373	1401	1.02
1905	1354	1398	1.03
1906	1376	1414	1.03
1907	1373	1407	1.02
1908	1380	1414	1.02
1913	1366	1397	1.02
1914	1342	1400	1.04
1915	1330	1372	1.03
1916	1381	1414	1.02
1917	1378	1414	1.03
MEAN	**1367**	**1404**	**1.03**
SD	**17.24**	**12.82**	**0.01**
CV	**1.261**	**0.913**	**0.62**

Day 11	RT2	RT4	RT4/RT2
2003		1427	
2004	1375	1419	1.032
2005		1426	
2006	1395	1430	1.025
2007	1379	1412	1.024
MEAN	**1383**	**1423**	**1.03**
SD	**10.58**	**7.26**	**0.00**
CV	**0.765**	**0.51**	**0.42**

IV- Long term analysis

Sample number	RT2	RT4	RT4/RT2	Sample number	RT2	RT4	RT4/RT2
9005	1183	1228	1.04	1713	1302	1332	1.02
9006	1167	1220	1.05	1714	1309	1339	1.02
9007	1172	1223	1.04	1716	1309	1341	1.02
9009	1169	1220	1.04	1717	1313	1344	1.02
9010	1166	1213	1.04	1718	1302	1339	1.03
9011	1170	1216	1.04	1719	1304	1337	1.03
9013	1186	1240	1.05	1720	1288	1322	1.03
9014	1162	1240	1.07	1721	1313	1346	1.03
9015	1182	1237	1.05	1722	1305	1342	1.03
9016	1187	1234	1.04	1723	1326	1362	1.03
9017	1187	1248	1.05	1724	1437	1471	1.02
9018	1194	1241	1.04	1726	1329	1365	1.03
9019	1191	1243	1.04	1727	1332	1368	1.03
9021	1209	1254	1.04	1728	1339	1379	1.03
9022	1203	1249	1.04	1729	1504	1540	1.02
9023	1197	1249	1.04	1803	1360	1389	1.02
1603	1250	1289	1.03	1804	1358	1392	1.03
1607	1241	1279	1.03	1807	1364	1401	1.03
1608	1218	1278	1.05	1808	1374	1410	1.03
1609	1236	1276	1.03	1903	1380	1414	1.02
1610	1242	1281	1.03	1904	1373	1401	1.02
1613	1253	1296	1.03	1905	1354	1398	1.03
1614	1247	1298	1.04	1906	1376	1414	1.03
1618	1270	1313	1.03	1907	1373	1407	1.02
1619	1266	1310	1.03	1908	1380	1414	1.02
1620	1274	1317	1.03	1913	1366	1397	1.02
1703	1286	1339	1.04	1914	1342	1400	1.04
1704	1309	1348	1.03	1915	1330	1372	1.03
1705	1317	1351	1.03	1916	1381	1414	1.02
1706	1316	1344	1.02	1917	1378	1414	1.03
1710	1556	1586	1.02	2004	1375	1419	1.03
1711	1295	1343	1.04	2006	1395	1430	1.03
1712	1305	1336	1.02	2007	1379	1412	1.02

	RT2	RT4	RT4/RT2
Mean	**1294.788**	**1335.818**	**1.03**
SD	**84.999**	**78.868**	**0.01**
CV	**6.565**	**5.904**	**0.90**

APPENDIX D2: Imprecision for transferrin isoforms on MCC

I-Within-batch imprecision – % of total transferrin

	T2	T4	T5/6	T4 + T5/6	All
Normal serum					
Day 1	0.8	81.3	18.6	99.9	100.7
n=3	1.9	78.1	20	98.1	100
	1.6	68.4	30	98.4	100
Mean	**1.43**	**75.93**	**22.87**	**98.8**	**100.23**
SD	**0.42**	**5.02**	**4.76**	**0.73**	
CV	**29.46**	**6.61**	**20.8**	**0.74**	
Day 2	2	63.7	33.9	97.6	99.6
n=4	2.3	66.1	31.6	97.7	100
	2.8	68.6	28.5	97.1	99.9
	2.8	72.8	24.4	97.2	100
Mean	**2.48**	**67.8**	**29.6**	**97.4**	**99.88**
SD	**0.33**	**2.9**	**3.15**	**0.25**	
CV	**13.13**	**4.28**	**10.64**	**0.26**	
ARD					
161 894	6.1	64	29.9	93.9	100
n=5	6.9	67.8	27.4	95.2	102.1
	6.7	62.1	31.6	93.7	100.4
	4.3	66.9	28.9	95.8	100.1
	4.4	67.5	28.1	95.6	100
Mean	**5.68**	**65.66**	**29.18**	**94.84**	**100.52**
SD	**1.06**	**2.09**	**1.26**	**0.83**	
CV	**18.73**	**3.18**	**4.3**	**0.88**	
2643	8	65.1	26.9	92	100
n=5	9.6	66.9	23.5	90.4	100
	10.1	64.4	24.5	88.9	99
	7.1	61.1	31.9	93	100.1
	8.4	67	24.6	91.6	100
Mean	**8.64**	**64.9**	**26.28**	**91.18**	**99.82**
SD	**0.97**	**1.72**	**2.5**	**1.22**	
CV	**11.2**	**2.65**	**9.5**	**1.34**	

II-Between-batch imprecision – % of total transferrin

	T2	T4	T5/6	T4 + T5/6	All
Normal					
Day 1	1.43	75.93	22.87	98.8	100.23
Day2	2.48	67.8	29.6	97.4	99.88
Day 3	3.8	60.6	36.1	96.7	100.5
Day 4	1.8	66.9	31.3	98.2	100
Mean	**2.38**	**67.81**	**29.97**	**97.78**	**100.15**
SD	**0.76**	**4.06**	**3.73**	**0.72**	
CV	**31.99**	**5.99**	**12.46**	**0.74**	
ARD					
161894					
day 1	5.68	65.66	29.18	94.84	100.52
day 2	6.7	52.4	42.1	94.5	101.2
day 3	6.7	65	27.4	92.4	99.1
day 4	5.4	62.5	32.8	95.3	100.7
day 5	4.6	54	42.5	96.5	101.1
day 6	5.3	68.1	26.8	94.9	100.2
Mean	**5.73**	**61.28**	**33.46**	**94.74**	**100.47**
SD	**0.65**	**5.38**	**5.89**	**0.86**	
CV	**11.29**	**8.79**	**17.6**	**0.91**	
165445					
day 1	7.2	64.9	27.9	92.8	100
day 3	5.2	53.9	41	94.9	100.1
day 5	5	64.7	30.7	95.4	100.4
day 6	5.3	79.7	14.8	94.5	99.8
Mean	**5.68**	**65.8**	**28.6**	**94.4**	**100.08**
SD	**0.76**	**6.95**	**7.25**	**0.8**	
CV	**13.44**	**10.56**	**25.35**	**0.85**	
Excluded: day 2	11.5	67	21.4	88.4	99.9
Excluded: day 4	5.3			92.9	
161703					
day 1	12.1	60.5	27.4	87.9	100
day3	12.3	62.6	25.3	87.9	100.2
day4	11.1	71.6	16.4	88	99.1
day5	14.5	54	31.5	85.5	100
day6	17.6	59.2	22.3	81.5	99.1
Mean	**13.52**	**61.58**	**24.58**	**86.16**	**99.68**
SD	**2.02**	**4.42**	**4.18**	**2.13**	
CV	**14.97**	**7.17**	**17.02**	**2.47**	
Excluded: day 2	1.7	62.9	35.4	98.3	100

2044					
day 1	9.8	62.5	27.8	90.3	100.1
day 3	7.7	66.5	25.8	92.3	100
day 4	7.9	68	24.1	92.1	100
day 5	12.4	74.6	13	87.6	100
Mean	**9.45**	**67.9**	**22.68**	**90.58**	**100.03**
SD	**1.65**	**3.4**	**4.84**	**1.63**	
CV	**17.46**	**5.01**	**21.33**	**1.79**	
Excluded: day 2	3.3	62.4	35.5	97.9	101.2
2733					
day 1	7.7	66	26.4	92.4	100.1
day 2	7.8	67.4	24.8	92.2	100
day 3	6.7	65.4	27.9	93.3	100
day 4	5.5	67.1	27.4	94.5	100
day 5	7.2	75	18	93	100.2
Mean	**6.98**	**68.18**	**24.9**	**93.08**	**100.06**
SD	**0.7**	**2.73**	**2.8**	**0.66**	
CV	**10.09**	**4**	**11.24**	**0.7**	

III- Correlation between means and CVs for tetrasialotransferrin

Within-batch		**Between-batch**	
Mean	CV	Mean	CV
1.43	29.5	2.38	31.99
75.93	6.6	67.81	5.99
22.87	20.8	29.97	12.46
2.48	13.13	5.73	11.2
67.8	4.28	61.28	8.79
29.6	10.64	33.46	17.6
5.68	18.73	5.68	13.44
65.66	3.18	35.8	10.56
29.18	4.3	28.6	25.35
8.64	11.2	13.52	14.97
64.9	2.65	61.58	7.17
26.28	9.5	24.58	17.02
		9.45	17.46
		67.9	5.02
		22.68	21.33
		6.98	10.09
		68.18	4
		24.9	11.24

Appendix D3: Data obtained after analysis of disialotransferrin on MCC, electrophoresis and IEF

I- MCC and electrophoresis – CSF samples – % of total transferrin

Sample number	IEF	MCC
1	51.44	48.63
2	25.81	25.22
3	7.13	7.02
4	11.7	6.37
5	4.75	6.75
6	8.3	6
7	5.11	8.25
8	18	13.89
9	28.8	8.34
10	20	3.46
11	12.1	10.2
12	14.84	5.3

II-Electrophoresis and IEF – ARD samples – % of total transferrin

Sample number	Electrophoresis	IEF
1	8.92	17.12
2	17.89	16.55
3	23.58	22.85
4	14.4	12.23
5	26.11	21.13
6	25.11	21.21
7	33.04	23.79
8	31.67	26.55
9	24.85	25.86
10	17.24	15.3
11	18.01	17.96
12	21.31	14.83