NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

UNIVERSITY OF ALBERTA

A STUDY OF INTRALYSOSOMAL AMINO ACID CONCENTRATIONS IN NORMAL, AND MUTANT HUMAN FIBROBLASTS

BY

DR. ROSE GIRGIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PEDIATRICS

EDMONTON, ALBERTA

SPRING 1991

ISBN 0-315-66602-1

Canada

UNIVERSITY OF ALBERTA
RELEASE FORM

NAME OF AUTHOR: DR. ROSE GIRGIS

TITLE OF THESIS: A STUDY OF INTRALYSOSOMAL AMINO ACID CONCENTRATIONS IN NORMAL AND MUTANT HUMAN FIBROBLASTS

DEGREE: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: 1991

Rose Girgis

84 McLeod Place
Edmonton, Alberta.
Canada
T5A 3A8.

Date: April 23, 1991

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **A STUDY OF INTRALYSOSOMAL AMINO ACID CONCENTRATIONS IN NORMAL, AND MUTANT HUMAN FIBROBLASTS** submitted by **DR. ROSE GIRGIS** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**

.....

Dr. P. Ferreira (supervisor)

.....

Dr. M. J. Poznansky

.....

Dr. J. Lin

.....

Dr. D. Spady

Date: April 23, 1991

TO MY HUSBAND, SAFWAT
MY SON, DANIEL
AND MY PARENTS, RAMSES AND KAMELIA

...WITH LOVE AND GRATITUDE

Abstract

An attempt to develop a method to measure the intralysosomal amino acid concentrations of normal and mutant human fibroblasts, is described. Cystinotic and I-cell disease fibroblasts were selected to test this method since they have previously been shown to have intralysosomal accumulation of cystine due to defective cystine lysosomal transport.

Normal, cystinotic, and I-cell disease fibroblast lines were grown in tissue culture, and lysosomal fractions were isolated on a self generating Percoll gradient. Subcellular fractionation of fibroblasts yielded two lysosomal marker enzyme (β-hexosaminidase) peaks (p= 1.049-1.054, and p= 1.096-1.107). Lysosomal membrane integrity was conserved to the extent of 75-85% in most granular fractions as judged by assaying β-hexosaminidase in the presence and absence of Triton X-100. The activities of marker enzymes succinate dehydrogenase for mitochondria, and 5'nucleotidase for plasma membranes were found in the buoyant fractions near the top of the Percoll gradient. Therefore, the heavy lysosomal fractions were mainly used in this study.

A novel approach was tried to determine the lysosomal free amino acid pool using a sensitive High Pressure Liquid Chromatography (HPLC) system. The advantage of this technique is that it allows the study of a whole amino acid profile in one tracing. To attain the required separation and sensitivity, the instrument is equipped with multiple solvent capability, an ion exchange column, and post column derivatization with ortho-phthalaldehyde (OPA) and fluorescence detection of the amino acid derivatives.

Amino acid analysis was performed in cystinotic, I-cell disease, and normal fibroblast homogenates and in their lysosomal fractions. In agreement with previous reports, the cystine content was found to be increased approximately ten fold in cystinotic fibroblasts compared to normal lines. Cystine was present in cystinotic lysosomal fractions, but was not detected in normal ones. Whereas, in I-cell disease fibroblast homogenates, cystine content varied from non detectable to slightly higher than control cell lines, and their lysosomal fractions showed only traces of cystine. The increased cystine content in cystinotic and I-cell lines was unique and did not reflect a generalized or specific increase in other amino acids content indicating that there are no other abnormal amino acid carriers.

The recovery of amino acids from fibroblast homogenates were found fairly reproducible, however intra lysosomal amino acid pool was found to be very variable, probably due to physiological heterogeneity of lysosomes and multiple experimental variables such as cell culture age and passage number, methods of sample preparation and storage temperature.

Acknowledgements

I wish to thank Dr Paddy Ferreira (supervisor) for his guidance and supervision throughout the program. I also sincerely thank him for patiently reading and editing my thesis.

I gratefully acknowledge the valuable scientific advice of Dr Mark Poznansky and I wish to thank him and his staff for accommodating me with space to carry out my experiments in his laboratory.

I would like to thank Dr Jim Lin for his moral support throughout my training and for allowing me to carry out tissue culture in his laboratory.

I wish to thank Dr Don Spady for his follow up during my study.

I am very grateful to Dr Peter Olley for giving me the opportunity to pursue my research.

I wish to thank Dr David Schiff for his moral support and constant follow up throughout my graduate program.

I deeply appreciate the great efforts and skill of Mrs Luella Yakymyshyn, senior scientist, and the team of the Hereditary Diseases Investigation Program, department of Laboratory Medecine, in amino acid analysis.

Many thanks to Dr Richard Vriend for his skillful preparation of the electron micrographs and to Mr Tom Turner in photography for their development.

I also would like to thank Dr PD Thomas for his valuable experimental suggestions.

I would like to express my deep appreciation to Dr WC Taylor for his endless support, encouragement, and friendship.

I would like to thank the pediatric administration staff, especially, the division of medical genetics for welcoming me into the department and making me feel at home during this research period.

I am very grateful to Dr Thomas Overton, Department of Applied Sciences in Medicine, for kindly allowing me to use his word processor in typing this work.

I wish to thank the staff at the reference and circulation desks in John W. Scott Health Sciences Library for their efficient help.

This research was financed by the Central Research Fund, Research Services, University of Alberta.

Table of contents

List of Tables

List of figures

List of Micrographs

Chapter one: Literature review

1. Introduction: Subcellular organelles

In higher organisms, there is no "typical" cell, as eukaryotic cells vary according to their locations. However, there are major intracellular compartments or organelles that are common to all eukaryotic cells, and perform specific functions.

The limiting membrane of every cell, the plasma membrane, is a continuous bi-layered sheet of phospholipid molecules and other complex lipids, about 4-5 nm thick, in which various proteins are embedded. These proteins serve a variety of functions; some are pumps, channels, or carriers for transporting specific molecules into and out of the cell.

The nucleus contains the main genome and is actively involved in DNA synthesis and repair, and in RNA synthesis; while RNA processing and ribosome synthesis occur in the nucleolus.

The surrounding cytoplasm consists of the cytosol and the cytoplasmic organelles where the metabolism of carbohydrates, lipids, amino acids, and nucleotides, as well as protein synthesis takes place. The endoplasmic reticulum (ER) is formed of flattened sheets, sacs, and tubes of membrane extending throughout the cytoplasm. It consists of "rough" ER and "smooth" ER. The former is formed of flattened sheets studded on its outer surface with ribosomes engaged in proteins synthesis. The smooth ER is more tubular and lacks the attached ribosomes, and its major function is lipid metabolism. The Golgi apparatus is a specialized part of the smooth ER consisting of stacked, membrane-bounded, flattened sacs which receive carbohydrates, lipids and proteins from the ER, modify, sort, and export them to a variety of intracellular destinations.

Mitochondria contain two concentric membranes, an inner and an outer one, separated by an intermembrane space. The inner membrane is characterized by its plate like invaginations, the "cristae". These organelles provide energy for the cell: they generate most of the ATP utilized to drive biosynthetic reactions that require an input of free energy. They are also involved in oxidation of carbohydrates and lipids, in urea and heme synthesis, and in the control of cytosolic (Ca^{2+}).

Peroxisomes are membrane-bounded vesicles, containing oxidative enzymes which generate and destroy hydrogen peroxide. The cytoskeleton of the cell, which is formed by microfilaments and microtubules, plays a role in the cell morphology and in the motility of both the cell and the intracellular components.

Finally, lysosomes are membrane-bounded vesicles, containing digestive enzymes which sequester intracellular organelles and degrade macromolecules and particles taken up from outside the cell by endocytosis. In addition to the major membrane-bounded

organelles described above, cells contain many small vesicles that act as carriers between organelles, as well as other vesicles that communicate with the plasma membrane in the secretory and endocytic pathways.

Lysosomes stand out as a unique class against all other subcellular organelles by their polymorphism and by the variety of processes, both physiological and pathological, in which they are involved. The study of the lysosomal system has uncovered a great deal of details. For the purpose of my study, I shall emphasize those aspects of lysosomes which are relevant to my research; the intralysosomal amino acid concentrations of normal and mutant human fibroblasts and lysosomal transport defects.

2. Lysosomes

The discovery of lysosomes is attributed to de Duve et al (1955). de Duve, who was awarded the Nobel prize, in 1974, in recognition for his discovery of lysosomes and his subsequent work on these and other cellular structures, described the early development of the field in the first of the series of books on lysosomes edited by Dingle and associates (1969a,b; 1973; 1975).

2.1 Definition of lysosomes

Although varying considerably in ultrastructure, lysosomes can be defined as membrane limited sacs, containing a variety of hydrolytic enzymes. This lysosomal concept rests essentially on two biochemical criteria: a) the association of acid hydrolases within special cytoplasmic particles and, b) the structure linked latency of the enzymes concerned (de Duve, 1983). The role of lysosomes in physiological and pathological conditions is thought to involve the digestion of both exogenous and endogenous substrates. Therefore, the name "lysosomes" was appropriately suggested by de Duve: Lysosomal enzymes break up their substrates by means of water, i.e. hydro (lyse) them, and are contained within particles ("somes" form the Greek soma).

2.2 The Formation and fate of lysosomes

Lysosomal enzymes are produced in the rough endoplasmic reticulum, then transfered to a specific region of the smooth endoplasmic reticulum located in the inner surface of the Golgi apparatus. These proteins are sorted by different receptors as they leave the Golgi apparatus in separate transport vesicles to meet in the endolysosome which already contains endocytosed material for digestion *(Fig 1.1)*. The substrates which are available for digestion originate by two main mechanisms: Heterophagy and Autophagy (Lazarus et al, 1975).

Heterophagy is a process whereby the cell engulfs extracellular material. This occurs either by phagocytosis or by endocytosis. Phagocytosis describes the uptake of large particles such as bacteria, protozoa, cellular debris, etc, and it is an activity carried out by cells of the reticuloendothelial system such as macrophages and polymorphonuclear leucocytes. In general, this process involves the formation of cytoplasmic extentions or pseudopodia around such particles producing a *phagosome* which later acquires lysosomal enzymes to become a *phagolysosome* (Sabatini and Adesnik, 1989)

Endocytosis refers to a variety of processes involving the internalization of extracellular substances such as hormones, growth factors, and nutrient-carrier proteins e.g. cholesterol incorporated in low density lipoprotein (LDL) particles (Goldstein et al, 1985), a process known as "receptor mediated endocytosis", and to the uptake of extracellular fluid and solutes what is known as "fluid phase endocytosis" or "pinocytosis". Kinetically receptor-mediated-endocytosis shows typical Michaelis-Menten kinetics as the system can become saturated. In contrast, pinocytosis is characterized by linear dependency on the concentration of the ingested solute. Endocytosis occurs when a portion of the cell plasma membrane surrounds extracellular fluid or solid particles forming a membrane bounded cytoplasmic compartment. In receptor mediated endocytosis the extracellular particles interact with specific cell surface receptors which mediate their interiorization. The ligand-receptor complexes become concentrated in small plasma membrane invaginations known as "coated pits". These pits form coated vesicles soon after pinching off into the cytoplasm (Pearse,1980). The membranes of these coated vesicles contain several proteins, of which the best characterized is "clathrin". The vesicles lose their coats as they fuse with membrane bounded tubulovesicular organelles known as endosomes. The current view is that a subclass of endosomes near the Golgi apparatus undergoes conversion into lysosomes by losing endosomal membrane components and by acquiring the necessary complement of lysosomal hydrolases (Griffiths et al, 1988).

On the other hand, autophagy is a process by which the cell sequesters its own cytoplasm and organelles forming *autophagosomes*. Fusion of the latter with *endolysosomes* initiates the digestion of its contents forming an *autophagolysosome*.

Lysosomes containing incompletely digested substrates, are known as *residual bodies*. These may either remain in the cytoplasm or may discharge their contents by exocytosis.

Figure 1.1 Biosynthesis of lysosomes

FIGURE OMITTED DUE TO COMPYRIGHT RESTRICTION

Source: Figure 8-71, p 461. Alberts et al: Transport of proteins from the Golgi apparatus to lysosomes. In: Molecular Biology of The Cell. Lackenbach L, Cobert S (eds), Garland Publishing Inc., New York, 1989. Lysosomes can be formed by three ways: phagocytosis, endocytosis, and autophagocytosis. These pathways meet at an intermediate compartment designated as endolysosome. Lysosomal hydrolases and membrane proteins are sorted by different receptors in the Golgi apparatus, then leave in separate transport vesicles to meet in the endolysosome.

2.3 Lysosomal membrane

Various studies have unveiled a great deal of knowledge about the morphology of the lysosomal membrane, its permeability to different molecules, and the factors affecting its structural integrity.

2.3.1 Morphology

Electron microscopic studies of the lysosomal membrane classify it with the thick type of biomembranes; i.e. 9 nm in thickness, resembling the plasma membrane and the membranes of maturing or transface Golgi apparatus (Yamamoto, 1963; de Duve and Wattiaux, 1966; Grove et al, 1968). A characteristic electron-lucent halo, present at the inner side of the membrane, has been described. It is a 7-10 nm layer which separates the membrane from the matrix (Daems and van Rijssel, 1961; Daems et al, 1969; Novikoff,

1973) and contains high concentrations of hydroxyl, vicinal glycol, and α-amino alcohol groups (Neiss, 1984).

The luminal surface of the membrane is protected from the effect of lysosomal proteases by complex oligosaccharides rich in sialic acid. These sialic acid containing residues can be detected by colloidal iron staining techniques (Henning et al, 1973). It was suggested that acting as immobilized polyanions, these sialic acid moieties might also create a Donnan potential for protons to maintain the acidic intralysosomal pH (Reijngoud and Tager, 1977). The majority of the lysosomal membrane proteins are exposed to the cytosolic space (Schneider et al, 1978); this might explain the digestion of lysosomal membranes when exposed to proteases from the outside (Henell et al, 1983a and b). The cytoplasmic surface of the lysosomal membrane possesses receptors that mediate the fusion of lysosomes among themselves or with phagosomes or endosomes to which lysosomal enzymes are provided. This surface was also found to interact with the cytoskeleton responsible for lysosomal movement (Mehrabian et al, 1984; Collot et al, 1984).

2.3.2 Lysosomal membrane permeability

In an attempt to briefly review the modes of transport of molecules across biological membranes, we find that, in general, there are two modes of transport: either simple diffusion or carrier mediated transport *(Fig 1.2)*. The latter can be either passive or active transport (Stryer, 1988). In simple diffusion, the transport is proportional to the concentration of solute. This is not commonly seen in biological membranes except in case of water (H_2O) which reaches equilibrium. The distinction between passive and active carrier mediated transport depends on the change in free energy of the transported material: active transport is coupled with an input of free energy, whereas, passive transport occurs spontaneously. Active transport processes can be driven by a variety of energy sources e.g. ATP, H^+ gradients, Na^+ gradients, and high potential electrons. ATP is one of the most common sources of free energy in active carrier mediated transport systems e.g. H^+ - K^+ ATPase transport system of the stomach, Na^+ - K^+ ATPase and Ca^{2+} ATPase systems found in animal cells, proton pumps found in endocytic vesicles, and F_O-F_1 H^+ ATPase system found in mitochondria. Active transport processes can be coupled with the flow of an ion down its electrochemical gradient: e.g. Na^+ gradients drive the active transport of glucose across the plasma membrane of intestinal and kidney cells. High potential electrons constitute a driving force for the proton pumping assemblies in oxidative phosphorylation.

Passive carrier mediated transport usually involves a protein in the membrane but no metabolic energy is required; a good example is well described in red blood cells. The latter contain an integral membrane protein known as the anion exchange protein which

mediates the exchange of HCO_3^- for Cl^-. There is a tight coupling between the effusion of one anion (HCO_3^-) and the entry of the other (Cl^-); this protein is defined as an antiporter and the obligatory nature of the exchange process results in electro-neutrality of the transport.

Another example of passive transport is seen in what are known as "Gap junctions". These are channels for passive transport clustered in the plasma membranes of opposed cells. They allow ions and most metabolic products such as sugar, amino acids, and nucleotides to flow between the interiors of contiguous cells. However, proteins, nucleic acids and polysaccharides are too large to cross these channels. These gaps are closed by high concentrations of Ca^+ and low pH (H^+); this feature likely serves to protect normal cells from traumatized or dying neighboring cells.

Figure 1.2 **Modes of transport across membranes: Simple diffusion and carrier mediated transport**

FIGURE OMIITED DUE TO COPYRIGHT RESTRICTIONS

source: Figure 6-45, p303. Alberts et al: Membrane transport of small molecules. In: Molecular Biology of The Cell. Lackenbach L, Cobert S (eds), Garland Publishing Inc., New York, 1989.

Several studies have been performed to determine the permeability of the lysosomal membrane for the different end products resulting from the degradation of macromolecules. It was at first thought that the rates of penetration through the lysosomal membrane depended on variables such as molecular size, hydrophylic-hydrophobic nature, and in the case of ions, the ionic field strength. As a result, it was suggested that water soluble

substances such as monosaccharides and other polyols, amino acids, small peptides, and nucleosides, would cross from the lysosomal matrix to the cytosol and vice versa at rates that decrease with increasing molecular size and hydrophylicity (Lloyd, 1973; Reijngoud and Tager, 1977). The permeability of monovalent cations at 37°C was suggested to be as follows $Cs^+ > K^+ > Na^+ > H^+$, and that of anions: Thiocyanates > iodide > acetate > chloride > phosphate > bicarbonate > sulphate (Casey et al, 1978).

Recently, this classical view has been challenged by new evidence that describes the lysosomal membrane as being a selective permeability barrier between the lumen of the lysosomes and the cytoplasm. The passage of substances between both compartments was shown to be controlled or facilitated by carriers and transport systems found in the membrane. Recognition of those transport systems has occurred largely during the 1980's. The first evidence of a carrier mediated transport across the lysosomal membrane was demonstrated in the genetic disorder, nephropathic cystinosis. The evidence consisted of demonstration of saturation kinetics, stereospecificity, competition with structural analogues, temperature dependence, and countertransport. The excessive accumulation of cystine in this disorder was shown to result from a defective lysosomal cystine carrier (Gahl et al, 1982 a,b; Jonas et al, 1982). Since that time, lysosomal transport systems for cationic amino acids (Pisoni et al, 1985;1987a), large neutral and aromatic amino acids (Bernar et al, 1986; Stewart et al, 1988), small neutral amino acids (Pisoni et al, 1987b), and anionic amino acids (Collarini et al, 1988) have been described (*Fig 1.3).*

Lysosomes possess transport systems for sialic acid (Renlund et al, 1986 a;b), and vitamin B_{12} (Rosenblatt et al, 1985). Defects in those transport systems result in Salla disease and in a form of methylmalonic aciduria (cblF), respectively. In 1983, Maguire et al also provided evidence to support the existence of a lysosomal transport system for monosaccharides. A substrate specific facilitated diffusion for glucose has also been reported (Hales et al, 1984).

Another transport system involving a membrane associated enzyme, acetyl-CoA: α-glucosamide-N-acetyl transferase, has been described (Bame et al, 1985). This enzyme is necessary for the transfer of acetyl moieties from acetyl CoA in the cytoplasm, to acceptor glucosamine groups linked in terminal α-linkage to heparan sulfate molecules within the lysosomes. This acetylation step is essential for the degradation of heparan sulphate and, therefore, a deficiency in the transferase results in the accumulation of this mucopolysaccharide in the lysosomes. This disorder is known as Sanfillipo C syndrome (Klein et al, 1978). Finally, a nucleoside transport system has been recently characterized in human fibroblast lysosomes (Pisoni and Thoene, 1989).

Figure 1.3 **Provisional representation of the amino acid transport systems detected for human fibroblast lysosomes.**

FIGURE OMIITED DUE TO COPYRIGHT RESTRICTIONS

source: Figure 1, p124. Christensen HN: Amino acid transport systems of lysosomes: Possible substitute utility of surviving transport system for one congenitally defective or absent. Biosc Rep 8(2): 121, 1988.

2.3.3 Structural integrity of the lysosomal membrane

The integrity of the lysosomal membrane can be assessed by measuring the extent of latency. This can be performed by assaying the activity of β-hexosaminidase, a lysosomal marker enzyme in the presence or absence of agents that disrupts lysosomes e.g. a detergent. The percentage of lysosomes that are broken is represented by the free β-hexosaminidase activity (measured in the absence of a detergent) expressed as a percentage of the total enzyme activity (measured in the presence of a detergent). Many factors such as freezing and thawing, vortexing, waring blender, inadequate osmotic protection, pH changes, high temperature, sonication, detergents, protease, and lecithinase have been found to disrupt lysosomes (de Duve, 1955). It has been shown that lysosomes kept at 0°C remained intact over a five hour period, while increasing incubation temperature (37°C) resulted in an increased rate of lysis of approximately 8% / hour. The lysosomal membrane was also found to break rapidly in pH < 4-5 and pH > 8, with the greatest stability being between pH 6 and pH 7.5 (Rome and Crain, 1981).

2.4 Lysosomal contents

Two different types of contents can be distinguished in the lysosomes (Pfeifer, 1987):

2.4.1 Intrinsic components: matrix and enzymes

2.4.2 Lysosomal food e.g. tracer substances, digestible contents,.etc.

2.4.1 Intrinsic components

The intrinsic components consist mainly of the lysosomal matrix and lysosomal hydrolases.

2.4.1.1 Lysosomal Matrix

It was suggested that the lysosomal matrix is formed of non enzyme anionic substances which produce an acidic intralysosomal pH as a result of a Donnan equilibrium (Reijngoud et al, 1976), and that it is responsible for the protection against harmful substances such as silica and heavy metals.

2.4.1.2 Lysosomal Enzymes

The lysosomes possess a diverse array of enzymes most of which are hydrolases. The lysosomal enzymes found in different tissues vary according to the specific needs of individual tissues to digest specific substances.They are synthesized in ribosomes bound to the endoplasmic reticulum membrane, and the majority of them are converted to negatively charged glycoproteins containing N-linked and sometimes O-linked oligosaccharides chains. The nascent ribosome-associated chains contain cleavable N-terminal signals that lead to co-translational passage of the polypeptide into the lumen of the endoplasmic reticulum (Erickson and Blobel, 1979; Rosenfeld et al, 1982; Proia and Neufeld, 1982). The passage of this polypeptide across the endoplasmic reticulum membrane is paralleled by the acquisition of N-linked high mannose oligosaccharide chains and, by the cleavage of the signal sequence.

The lysosomal polypeptides undergo certain modifications which introduce in their oligosaccharide chains mannose-6-phosphate (Man-6-P), the lysosomal marker. The latter distinguishes lysosomal from secretory glycoproteins and addresses them to their lysosomal destination. In the cis Golgi, this recognition marker is generated through two sequential reactions catalyzed by a phosphotransferase and a phosphodiesterase, respectively. A defect in the enzyme N-acetylglucosaminyl-1-phosphotransferase which catalyses the first step results in the disorders known as I-cell disease (ML II) and Pseudo-Hurler-Polydystrophy (ML III). In these disorders, the cells are deficient in their ability to

equip the newly synthesized enzymes with the Man-6-P marker necessary for their targeting to lysosomes (Kaplan et al, 1977). It is believed that the phosphotransferase enzyme contains a site that recognizes a specific feature of the lysosomal polypeptide and positions it in proximity to the active site where modification of the mannose residue takes place.

Two types of Man-6-P receptor molecules, MPR^{CI} and MPR^{CD}, have been purified by affinity chromatography of solubilized cellular membranes on matrices containing immobilized ligands (Sahagian et al, 1981; Hoflack and Kornfeld, 1985). These receptors differ in their binding properties and in their divalent cation requirements. It was shown by immunocytochemistry that MPR^{CI} receptors are only found in coated vesicles, membranes of the Golgi apparatus and endolysosomes but not in mature lysosomes. Their affinity for the ligands is pH dependent. They bind strongly to lysosomal hydrolases bearing the marker (Man-6-P) at neutral or slightly acidic pH, and release them quantitatively at the high acidic intralysosomal pH.

In I-cell disease, the lysosomes in some cell types such as hepatocytes, Kupffer cells, and leucocytes were found to contain a normal complement of lysosomal enzymes (Owada and Neufeld, 1982; Waheed et al, 1982). This implies that alternate pathways may exist for directing hydrolases to lysosomes. The nature of these Man-6-P independent pathways is unknown. It was suggested that lysosomal enzymes which are present at high concentrations in the serum of ML II and ML III patients could be internalized through other systems which recognize other carbohydrate moeities such as galactose, N-acetylglucosamine, mannose, and L-fucose present in them (Waheed et al, 1982). It has also been reported that two lysosomal enzymes, β-glucocerebrosidase and acid phosphatase, are present at nearly normal levels in ML II and ML III fibroblasts. It is believed that membrane associated lysosomal enzymes, such as β-glucocerebrosidase, reach lysosomes through an unknown pathway which does not require phosphorylation (Granger et al, 1985; Barriocanal et al, 1986). However the normal acid phosphatase content in I-cell disease is an interesting feature since in normal fibroblasts its targeting to lysosomes appears to be Man-6P-dependent (Lemansky et al, 1985). Increased stability of this enzyme in lysosomes of I-cell fibroblasts might explain its accumulation (Nolan and Sly, 1989).

Lysosomal enzymes are all acid hydrolases optimally active near pH 5 which is maintained within the acidic lysosomal matrix. Certain enzymes seem to be primarily membrane bound (an esterase may be found on the outside of intact lysosomes) and are fully expressed in intact lysosomes. However, in vitro the majority of lysosomal enzymes are latent, that is to say, the lysosomal membrane must be disrupted to release them so that

they can react with their substrates in solution. This is termed "structure linked latency" (Dean, 1977).

2.4.2 Lysosomal food

The lysosomal food consi of material taken up into lysosomes, be it digestible or indigestible. For indirect visualization tracer substances such as haemoglobin (Goldfischer et al, 1970), peroxidase (Mori and Novikoff, 1977), and cytochrome C (Kiesewetter and Kugler, 1985) have been used; these are usually taken up by endocytosis into the lysosomal compartment. Other tracers can be directly identified by electron microscopy due to their high electron density i.e. compounds containing metals or due to their particular shape and structure e.g. latex particles (Daems et al, 1969). Ions of metals such as iron and manganese are found normally in lysosomes at higher concentrations than in other organelles; this can be explained by the fact that lysosomes sequester such ions to protect the cell form their deleterious effects.

A lysosomal amino acid pool has been identified (Harms et al, 1981). Lysosomes, isolated from rat liver, were shown to have significant intralysosomal pools of threonine, serine, valine, cystine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, and arginine. These amino acids are transported across the lysosomal membrane into the cytosol, where they can re-enter the metabolic pathways of the cell.

2.5 Intralysosomal pH

Published values for intralysosomal pH vary from < 4 to as high as 6.5 depending on the method of pH measurement. In vitro, the internal pH of lysosomes was found to be about one unit lower than the external medium (Reijngoud and Tager, 1973), whereas in vivo, the pH gradient across the lysosomal membrane is believed to be more than two units (Reijngoud and Tager, 1977; Ohkuma and Poole, 1978). It was controversial for a long time whether this low intralysosomal pH reflected an equilibrium of protons (Donnan type equilibrium) or an energy dependent mechanism, (active transport). According to the first mechanism, a pH difference across the lysosomal membrane is brought about by the presence of fixed negatively charged groups within the lysosomes (Reijngoud and Tager, 1977; Hollemans et al, 1979; 1980). However, recently the concept of an ATP-dependent lysosomal proton pump (Mego et al, 1972; Mego, 1979) has become widely accepted (Reeves and Reams, 1981; Reeves, 1984; Mellman et al, 1986). The lumen is maintained at an acidic pH by a H^+ pump in the membrane that utilizes cytoplasmic ATP *(Fig 1.4)* and functions in an electrogenic (Ohkuma et al, 1982; Harikumar and Reeves, 1983) or electroneutral (Schneider, 1983) manner, and operates most effectively in the presence of

chloride ions. The lysosomal pump strongly resembles other proton ATPases in its sensitivity to inhibitors.

Figure 1.4 **Intralysosomal pH**

FIGURE OMITTED DUE TO COPYRIGHT RESTRICTIONS

Source: Figure 8-69, p459. Alberts et al: Transport of proteins from the Golgi apparatus to lysosomes. In: The Molecular Biology of The Cell. Lackenbach L, Cobert S (eds), Garland Publishing Inc., New York, 1989. The intralysosomal lumen is maintained at an acidic pH ~ 5 by a H^+ pump in the membrane that uses the energy of ATP hydrolysis to pump H^+ into the vesicle

2.6 Metabolic pathways in lysosomes

In 1977, Barrett and Heath reviewed the properties of about 50 known lysosomal enzymes which included proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, and sulfatases. These enzymes were classified by Barrett, in 1984, according to their contribution to the catabolic pathway into four classes: The"proteolytic", "glycanolytic", "nuclease", and "lipolytic" *(Table 1.1)*.

Table 1.1 **The classification of the enzymes involved in the lysosomal catabolic pathways**

TABLE OMITTED DUE TO COPYRIGHT RESTRICTIONS

source: Table 1, p900. Barrett A: Proteolytic and other pathways in lysosomes. Biochem Soc Trans 12: 899, 1984

2.7 Heterogeneity of lysosomes

The heterogeneity of lysosomal morphology contrasts with the relatively uniform structures of most other cellular organelles. Density gradient subcellular fractionation of human diploid fibroblasts on Percoll-colloidal silica, separates two populations of particles containing acid hydrolases which can be considered lysosomal. The denser peak (density 1.11) is relatively free from contaminants and the more buoyant peak (density 1.04) sediments with other organelles such as mitochondria, endoplasmic reticulum, Golgi and plasma membrane. The activities of the following enzymes are used as markers for subcellular organelles: 5'nucleotidase (plasma membrane), succinate-p-iodonitrotetrazolium violet reductase (mitochondria) and β-hexosaminidase (lysosomes).

It has been demonstrated by both biochemical and ultra-structural methods that these two distinct populations did not appear to be due to artifactual trapping of one population by the other nor to release and redistribution of soluble enzyme. Both populations also showed latency. The origin of the buoyant and dense lysosomal organelles was suggested to be the GERL network[1] and the residual bodies, respectively (Rome et al, 1979). Another classification of lysosomes was reported: primary and secondary lysosomes. The term "primary lysosome" was used to designate lysosomes which contain the hydrolytic enzymes and have not yet acquired the substrate for digestion, that is they have not yet participated in a digestive event. These are in the form of small vesicles containing mannose-6-phosphate receptor molecules and carry newly synthesized hydrolases from the Golgi apparatus to endosomes undergoing transformation into lysosomes. The term "secondary lysosomes" was used to describe those lysosomes that have received substrates subsequent to endocytosis or autophagy. They are acid phosphatase rich vacuoles, 400-600 nm in diameter, containing hydrolases which have participated in or are engaged in a digestive event (Sabatini and Adesnik, 1989).

It is important to note that in fact the heterogeneity of lysosomes also reflects the character of material being digested within the lysosomes as well as the wide variety of digestive functions mediated by acid hydrolases. This polymorphism has almost become a hallmark by itself and for this reason, lysosomes are viewed as a heterogenous collection of distinct organelles whose common feature is a high content of hydrolytic enzymes.

---

[1] The name GERL was chosen because of the location of the organelle on the concave (trans) aspect of the Golgi complex, being of an anatomical connection to the Endoplasmic Reticulum and of an apparent role in the genesis of Lysosomes.

3. *Lysosomal Disorders*

Two groups of deficient lysosomal functions have been described: deficiencies of lysosomal enzymes resulting in what is known as "lysosomal storage diseases", and defective egress of products from the lysosomes resulting in so-called "lysosomal transport defects".

3.1 Lysosomal storage diseases

In general, the digestion of macromolecules in the lysosomes is a stepwise process: each step is catalysed by a specific enzyme, and the product of one step forms the substrate for the next reaction in the digestive pathway. Therefore, deficiency of a lysosomal enzyme leads to the accumulation of its substrate(s) in the lysosomes. The storage of these undegraded molecules may result in cell, tissue, or organ dysfunction causing specific clinical syndromes. In man, more than 30 hereditary disorders have been described in which one or more lysosomal enzyme is defective (Tager et al, 1984).

In general, typical clinical features include: psychomotor retardation, coarse facial features, organomegaly, dysostosis multiplex, abnormal joint mobility, and corneal clouding. Simple enzyme assays are available for the diagnosis of the different enzyme deficiencies in peripheral leucocytes and or fibroblasts. Diagnosis is also suggested by the presence of increased urinary excretion of abnormal macromolecules. The genes for several of these enzymes have been cloned and mapped to specific chromosomes *(Table 1.2)* and different mutations have been described.

The deficiency of lysosomal enzymes can be due to several mechanisms *(Table 1.3)*: a) the precursor of the enzyme may not be synthesized e.g. α-Glucosidase deficiency in Pompe disease, early onset form, b) normal amounts of the precursor are synthesized but rapid degradation of the enzyme in lysosomes may occur e.g. Arylsulfatase A deficiency in Metachromatic Leukodystrophy, late onset form, c) the precursor may lack the mannose-6-phosphate recognition marker e.g. Deficient targeting of lysosomal hydrolases to lysosomes in I-cell disease, d) the precursor may have altered physiochemical and / enzymological properties e.g. Pseudodeficiency of Arylsulfatase A, e) the enzyme may be degraded due to the absence of a protective protein required for its stabilization e.g.β-Galactosidase deficiency in Galactosialidosis, f) the deficiency may result due to the absence of a factor required for enzymic activity e.g. absence of factor required for hydrolysis of sulfatide by Arylsulfatase A in a variant form of Metachromatic dystrophy (Tager et al, 1984).

Lysosomal storage diseases are mainly autosomal recessive genetic disorders except for Hunter's syndrome (Iduronate sulfatase deficiency) and Fabry's disease (α-Galactosidase deficiency) which are X-linked.

Table 1.2 **Chromosomal locations of hydrolase genes in humans as determined chiefly from investigations of storage diseases**

TABLE OMITTED DUE TO COPYRIGHT RESTRICTIONS

source: Table 6.5, p 350. Holtzman E: Extensive release. Excessive storage. In: lysosomes. Siekevitz (ed.), Plenum Press, New York, 1989.

Table 1.3 The defects in human lysosomal storage diseases.

TABLE OMITTED DUE TO COPYRIGHT RESTRICTIONS

Table 1.3 (cont)

TABLE OMITTED DUE TO COPYRIGHT RESTRICTIONS

source: Table 6.3, p 346. Holtzman E: Extensive release. Excessive storage. In: Lysosomes. Plenum Press, New York, 1989.

3.2 Lysosomal Transport Disorders: Cystinosis, Sialic Acid Storage Disorders, and an Inborn Error of Vitamin B_{12} Metabolism (cblF)

To date, lysosomal transport disorders include only three known diseases: Cystinosis, Salla disease, and one type of methylmalonic aciduria due an inborn error of vitamin B_{12} metabolism (cblF).

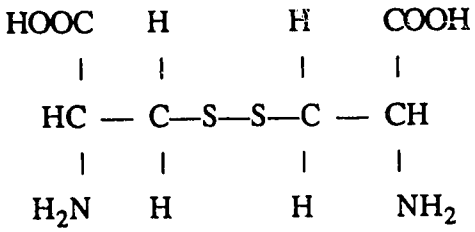
3.2.1 Cystinosis

In this review, emphasis is put on the investigations performed in cystinosis as cystinotic fibroblasts were used in this study, whereas the other two disorders are only briefly described.

3.2.1.1 Definition

Cystinosis is a rare autosomal recessive metabolic disorder, characterized by excessive intralysosomal accumulation of cystine (Schulman et al, 1969). Three forms of the disease have been described: nephropathic cystinosis, benign cystinosis, and intermediate (late onset) cystinosis (Kroll and Lichte, 1973). Patients with nephropathic cystinosis die in their first decade of life due to kidney disease, whereas those with the benign form have abnormal deposits of cystine in many organs but do not develop renal dysfunction. Patients with intermediate cystinosis have, together with the abnormal cystine deposition, a later onset of renal dysfunction.

Cystine is the disulfide of the amino acid cysteine. In the presence of oxygen, cysteine is rapidly oxidised to cystine.



Cystine

Cystine has a molecular weight of 240.3; its two carboxyl groups and its two amine residues have pK_a's of <1, 1.7, 7.48, and 9.02, respectively; its net charge at physiological pH is zero and its pI is 4.60 (Meister, 1965).

3.2.1.2 Localization of stored cystine in cystinosis

The plasma and urine levels of cystine and the intestinal absorption of cystine in cystinotic patients were found to be normal (Seegmiller et al,1968), indicating that the cystine accumulation is an intracellular phenomenon.

The localization of the stored cystine in the lysosomes was determined in several ways: Schneider et al (1967a;b) demonstrated by subcellular fractionation of cystinotic fibroblasts that the bulk of stored cystine co-sedimented in the granular fractions with the lysosomal enzyme acid phosphatase. In 1968, Patrick and Lake showed, by the electron microscopic study of a cystinotic lymph node, that the deposited cystine crystal was surrounded by acid phosphatase staining material all within an intact limiting membrane. In another study, Schulman et al (1970) described the lysosomal site of crystalline cystine deposits in cystinosis by ferritin uptake. Such evidence and many other studies have confirmed the lysosomal localization therefore, the following step was to define the underlying defect in cystinosis.

3.2.1.3 Source of accumulated cystine in cystinotic lysosomes

It was suggested that the possible sources of cystine in the lysosomes of cystinotic cells might include phagocytosed cystine, de novo synthesis via the cystathione synthetase-cystathionase pathway, glutathione degradation, and protein degradation.

Phagocytosis of preformed crystals accounting for intralysosomal cystine accumulation was ruled out based on earlier reports showing that the cystine crystals were absent in both urine and extracellular fluid and that they were soluble in plasma (Seegmiller et al, 1968).

Crawhall et al (1977) found that incubating cystinotic fibroblasts with labelled cystathionine or methionine did not result in the recovery of labelled cystine. De novo cystine synthesis through the cystathione β-synthetase pathway, therefore, did not appear to contribute to the cystine that accumulates in the cystinotic cells.

Thoene et al (1977) demonstrated that (^{35}S)cystine accumulated only when the protein pool was labelled and not when the glutathione pool alone was labelled .They also showed that when cystinotic fibroblasts were depleted from cystine by cysteamine and grown in a cystine deficient medium, the lysosomal cystine reaccumulated from the degradation of endogenous proteins. In another study, it was found that the lysosomal cystine content varied directly with the concentration in the growth medium of cystine rich protein, bovine serum albumin (Thoene and Lemons, 1980).

3.2.1.4 The basic defect in cystinosis

The investigation of the underlying defect in cystinosis was attempted by studying several possibilities. Oshima et al (1976) showed that cystine can reaccumulate in cystinotic cells even in the absence of extracellular cystine; therefore a defective plasma membrane transport accounting for cystine accumulation was ruled out. Another important possibility was abnormal cystine reducing systems. Data from cystinotic fibroblasts confirmed that there were normal amounts of cystine reductase, glutathione reductase, and cysteine glutathione thioltransferase (Kaye and Nadler, 1975). Several enzyme activities have been investigated: cystinotic γ - glutamyltranspeptidase activity was reported normal (Patrick et al, 1979), but arylsulphatase B (Furusho et al, 1971) and β- galactosidase (Scardigli et al, 1977) were found to be reduced in cystinotic fibroblasts. None of the above enzyme findings have been reported by more than one laboratory. Because the cytoplasmic catabolic pathways for cystine appeared normal in cystinosis, investigators suspected that the defect in this disorder might involve the escape of cystine from the lysosome to the cytosol.

3.2.1.5 Lysosomal cystine transport studies

The concept of a lysosomal cystine transport defect was difficult to test experimentally. In 1979, Reeves solved the problem by introducing a technique using methyl esters of amino acids to load the lysosomes, and the rate of egress of labelled cystine could then be monitored. These methyl esters are rapidly taken up into the lysosomes, and are hydrolyzed by the acid hydrolases yielding methanol and the ionized form of the corresponding amino acid.

In case of cystinotic cells, cystine dimethyl ester was found to be a suitable substrate. Two approaches were studied: firstly the use of (^{35}S)cystine dimethyl ester for loading and high voltage electrophoresis for (^{35}S)cystine determination. It was found that (^{35}S)cystine was cleared much slower from cystinotic cells ($t_{1/2}$= 80 to over 500 minutes) than from normal cells ($t_{1/2}$= 42.7± 3.1 minutes). One of the major drawbacks was the possibility of dilution of the specific radioactivity of (^{35}S)cystine by the large endogenous cystine pool of the cystinotic but not the normal cells. This problem was overcome by using a second approach: non radioactive cystine dimethylester for loading and subsequent measurement of non radioactive cystine by the cystine binding protein assay. In 1982, Steinherz et al applied the latter technique and showed that leucocytes from two normal individuals lost half their total cystine in 27 and 67 minutes respectively compared to 366 minutes and infinity in case of leucocytes from two cystinotic patients.

These radioactive and non radioactive experiments were performed on granular

fractions of leucocytes (Gahl et al, 1982a;b). The results were similar to those of whole cell experiments suggesting clearly that there is a defective cystine transport across the lysosomal membrane. It is worth noting that in those cystinotic granular fractions methionine and tryptophan were removed with normal half times suggesting that in cystinosis the defect did not represent a generalized abnormal lysosomal amino acid transport.

3.2.1.6 Lysosomal cystine transport: carrier mediated

The mode of transport of cystine was defined as carrier mediated; this was verified by the demonstration of countertransport and transstimulation. Trans-stimulation, is demonstrated experimentally when tracer amounts of radiolabelled material will cross a membrane at a high rate if there is a considerable concentration of its non radioactive form on the opposite side of the membrane. Therefore if non radioactive cystine loaded lysosomes take up (^{3}H)cystine more rapidly than lysosomes not loaded with cystine this suggests that a carrier for cystine must be present in the lysosomal membrane. It was found that only extralysosomal non radioactive L-cystine but not D-cystine competed with (^{3}H)cystine for uptake into these cystine loaded normal granular fractions (Gahl et al, 1983) establishing the stereospecificity of the carrier for the "L" isomer. In contrast, cystinotic granular fractions took up virtually no (^{3}H)cystine (Gahl et al, 1983). It was also shown that leucocyte granular fractions (^{3}H)cystine uptake was proportional to the level of non radioactive cystine, loading up to 3 nmol 1/2 cystine / unit β-hexosaminidase above which uptake plateaued indicating saturation kinetics.

The cystine carrier was described as being specific for compounds of chain length 6 sulfur or methylene units having an amine group at each end. Sulfur compounds resembling cystine such as cystathione and cysteamine compete for countertransport as they are recognized by the cystine carrier.

Structural specificity and stereospecificity, together with temperature dependence, countertransport and saturation kinetics confirmed that cystine transport across the lysosomal membrane is carrier mediated. The virtual absence of such a transport system in cystinotic patients resulted in defective egress of cystine across the lysosomal membrane (Gahl et al, 1982 a,b; Jonas et al, 1982).

3.2.1.7 Methods of measuring cystine content

Cystine content can be measured in several ways. One of the earliest procedures consisted of the reduction of cystine to cyst(e)ine and its qualitative measurement by the cyanide nitroprusside reaction (Brand et al, 1930). More recently, routine quantitation of

cystine was performed by automated amino acid analyzers (Schneider et al, 1967a; Lee Ply, 1974). Other cystine assays used were ion exchange paper (Schneider et al, 1968), and thin layer chromatography (States and Segal, 1969). One of the most commonly used techniques is the sensitive competitive protein binding assay using an Escherichia Coli cystine binding protein (Oshima et al, 1974).

3.2.1.8 Factors affecting the recovery of cystine from cystinotic cells

Factors such as storage and incubation temperatures of cystinotic fibroblasts, method of fibroblast detachment , cell passage number, cystine, albumin and chloroquine content of the culture medium, and pH of the medium were found to affect the recovery of free cystine from cystinotic cells

It was shown that the recovery of free cystine from cultured skin fibroblasts declined about 50% after 24 hours of storage at the following temperatures -20°C, -70°C, and -196°C and about 25% more following another six days at those temperatures; the decline was slightly greater at -20°C than the other temperatures (Kroll et al, 1974). Increased incubation temperature of cystinotic fibroblasts to 40°C or 43°C produced a 70%-80% decrease in lysosomal cystine content within 24 to 48 hours (Lemons et al, 1984).

The method of fibroblast detachment was found to affect the the recovery of amino acids in general and to a lesser extent cystine. It was demonstrated that scraping the fibroblasts with a "rubber policeman" decreased the recovery of amino acids compared with detaching them by trypsinization (Kroll et al, 1974). Crawhall et al (1977) showed that the length of time in cultivation affected the content of cystine in cystinotic fibroblasts. They found that the cystine content increased with increased cell passage number. The effect of the composition of the culture medium was emphasized in two studies by Thoene and Lemons, 1980;1982. They demonstrated that the cystine content of cystinotic fibroblasts varied with the addition of bovine serum albumin and cystine to the medium; and when placed in growth medium containing chloroquine, cystinotic cells were found to lose their stored cystine: 50% or more disappearing in 2 hours and 80% in 4 hours (States et al, 1983). Chloroquine is a potent lysosomotropic drug which accumulates in lysosomes, raises the lysosomal pH, and inhibits the proteolytic activity of cathepsin B_1 and other lysosomal enzymes. Thoene et al (1977) showed that chloroquine acts as a lysosomal inhibitor of protein catabolism and that reaccumulation of cystine by cystine-depleted cystinotic cells was prevented when the cells were incubated in cystine free medium containing chloroquine. It was also suggested that the increase in intralysosomal pH caused by chloroquine, could affect the solubility of cystine contributing to its marked loss.

The free cystine content of human cystinotic fibroblasts was found to vary with the pH of the culture medium (Ritchie et al, 1981). It was found to be highest at alkaline pH and lowest at acidic pH. When the cells were placed in culture medium at an acid pH (pH 6.3), they lost 50% of their free cystine and >95% over a period of 30 hours.

3.2.1.9 Therapeutic value of of administrating cysteamine in cystinosis

From the initial observation that cysteamine depletes cystine from lysosomes, it was believed at first, that it enters into a sulfhydryl-disulfide exchange reaction with the accumulated lysosomal cystine, generating amino acid molecules small enough to escape via hypothetical pores in that organelle. However, it has since been shown that removal of cystine actually occurs instead in a modified form by the transport system for basic amino acids. The mixed disulfide generated by the sulfhydryl-disulfide exchange, being itself a cationic amino acid, serves as a substrate for the system characterized for transporting basic amino acids. Exodus of ^{14}C-Lysine was shown to be stimulated by the external presence of the mixed disulfide of cysteine and cysteamine (Christensen, 1988). Thus the therapeutic utility of cysteamine acquired a new explanation through its generation of a specifically transportable derivative of cystine.

The follow up of 93 children treated with cysteamine showed that the mean leucocyte depletion was 82% (Thoene et al, 1976). In addition significant improvement of growth was noted during the first year of treatment and every year after. Cysteamine has recently been shown to prevent the development of Fanconi syndrome in a patient treated from 5 weeks of age (Da Silva et al, 1985) but not in one treated from 9 weeks of age (Gradus et al, 1986). Corneal crystal formation can be reversible after using cysteamine eye drops (Kaiser-Kupfer et al, 1987). In utero therapy with cysteamine seems reasonable as cystine accumulation begins early in fetal life, but the risks of teratogenic effects of the free thiol appear prohibitive at this time (Gahl et al, 1989).

3.2.2 Sialic Acid Storage Disorders

The free sialic acid storage disorders include Salla disease and the more severe infantile free sialic acid storage disease (ISSD). These disorders show autosomal recessive inheritance. Patients store excessive amounts of N-acetylneuraminic acid (sialic acid) in their tissues due to impaired free sialic acid transport across the lysosomal membrane. Most of the affected patients were originally from an area in northern Finland called Salla, from whence comes the name "Salla disease". Patients with Salla disease present at 3 - 12 months of age and show psychomotor delay, ataxia and moderate to severe impairment of intelligence, with slightly reduced life span. Patients with ISSD have a more severe clinical

picture and die in the first few years of life. They present at birth with hepatosplenomegaly, dysostosis multiplex, coarse facial features, and severe mental and motor retardation (Ghal et al, 1989).

The intralysosomal localization of sialic acid in Salla disease was first demonstrated by histochemical methods (Virtanen et al,1980). Differential centrifugation of ISSD fibroblasts has also shown that the accumulation of free sialic acid is mainly in the lysosomal-rich granular fraction (Hancock et al, 1980). These findings were further confirmed by electron microscopy which showed enlarged lysosomes containing fibrillogranular material forming multilinear arrays or lamellar stacks (Wolburg-Buchholz et al, 1985).

The underlying defect in Salla disease and ISSD was suggested to be defective transport of sialic acid across the lysosomal membrane based on two observations: 1) the major enzymes involved in the metabolism of sialic acid are normal, 2) the existence of a cytoplasmic but not a lysosomal enzyme for the catabolism of free sialic acid.

Lysosomal transport studies were carried by loading normal fibroblasts with N-acetylmannosamine (ManNac), a precursor of sialic acid (Thomas et al, 1985), and absolute amounts of free sialic acid were measured by high performance liquid chromatography (Silver et al, 1981). Lysosomal-rich granular fractions were prepared by Percoll gradient centrifugation and ManNac-derived sialic acid was found to fractionate with the lysosomal marker enzyme. It was shown that free sialic acid lost from inside lysosome-rich fractions was recovered outside, and that the velocity of this reaction was linear with time through 15 minutes. Failure to demonstrate saturation kinetics was possibly due to inability to load normal lysosomes sufficiently. In Salla disease granular fractions did not exhibit a similar loss over the same period of time. The egress of sialic acid from normal lysosome-rich granular was temperature dependent suggesting the presence of a carrier mediated transport process (Tietze et al unpublished observations as cited in Gahl et al, 1989). When Salla disease, ISSD, and control normal fibroblasts were exposed to (^{3}H)sialic methyl ester, the radioactivity appeared in the lysosomes as free sialic acid. The follow up of this radioactivity demonstrated a prolonged $t_{1/2}$ of sialic acid egress from the mutant lysosomes compared with normal (Mancini et al, 1986).

3.2.3 An inborn error of vitamin B_{12} metabolism: cblF

In general, cobalamins (Cbls) consist of a corrin ring, a central cobalt atom, and various axial ligands. Ten different inherited defects, defined by complementation analysis, are known to impair the pathways of Cbl metabolism in humans (Fenton and Rosenberg, 1989). CblF is a newly described mutation, where the defect appears to be in the transport

mechanism by which Cbl is released from lysosomes. As a result there is impaired synthesis of both methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) and accordingly deficient activity of both methylmalonyl-CoA mutase and N^5-methyltetrahydrofolate:homocysteine methyltransferase *(Fig 1.5)*. One of the two described cblF patients had both methylmalonic aciduria and homocystinuria, while the other only had mild methylmalonic aciduria. Normal serum cobalamin and transcobalamin II concentrations in these patients indicated that the defect is mainly in the release of vitamin B_{12} from lysosomes. The mode of inheritance of the cblF disorder is not yet established due to the paucity of the number of patients.

Cultured fibroblasts from one of the reported cblF patients were found to accumulate unmetabolized, non protein-bound cyanocolabamin (CN-Cbl), suggesting that these cells are deficient in the mediated process by which cobalamin exits from lysosomes. Genetic complementation analysis between the cblF patient fibroblasts and fibroblasts whose complementation groups have been previously determined allows the cblF group to be distinguished from other defects (Rosenblatt et al, 1985; 1986; Watkins and Rosenblatt, 1986). Recently, the EM radioautography of cblF fibroblasts provided morphological evidence that accumulation of vitamin B_{12} occurs within the electron dense lysosomes. When control and cblF fibroblasts were incubated in a medium containing (^{57}Co)-CN-B_{12} and 21% human serum as a source of transcobalamin II, it was found that cblF cells accumulated 4-8 times more radiolabelled cobalamin than control cells. The control and the cblF fibroblasts were then processed for electron microscopy and the radioautographic reaction was quantitated. The findings showed that in the cblF cells 65% of the radiolabel was associated with lysosomes and 8% was associated with electron lucent endosomes. In contrast , in the normal cells 56% of the label was found within the cytoplasm and only 5% was associated with the lysosomes (Vassiliadis et al, 1988).

4. Cystine storage in I-cell disease

I-cell disease and cystinotic fibroblasts were selected for this study as they have been reported to show intralysosomal accumulation of cystine (Tietze and Butler, 1979). I-cell disease (ML II) is an autosomal recessive disease manifested in early infancy by stunted growth, profound psychomotor disturbance, widespread skeletal deformities and death within the first decade (Leroy and Martin, 1975). I-cell fibroblasts and other cells demonstrate a deficiency or a virtual absence of several lysosomal hydrolases. In 1972, Leroy et al performed biochemical studies in six patients with I-cell disease and showed that in their fibroblasts, the activities of several lysosomal enzymes were as follows: β-galactosidase 2% of normal, β-glucosaminidase 8% of normal, β-glucuronidase 7% of

normal, α-galactosidase 10% of normal, and arylsulfatase A 5% of normal, whereas both β-glucosidase and acid phosphatase were not deficient. The deficient enzymes resulted in intralysosomal accumulation of partially hydrolysed molecules which appeared as phase-dense inclusions in the fibroblasts of affected individuals. As a result the cells were termed "Inclusion cells", and subsequently the disease was termed "I-cell disease".

Figure 1.5 Reactions catalyzed by cobalamin coenzymes in mamalian tissues

FIGURE OMITTED DUE TO COPYRIGHT RESTRICTIONS

source: Figure 82-2, p2067. Fenton, Rosenberg: Inherited disorders of cobalamin transport and metabolism. In: The Metabolic Basis of Inherited Diseases. Scriver, Beaudet, Sly, Valle (eds), McGraw Hill, New York, 1989.

The primary biochemical defect in I-cell disease was found to be in the packaging process of the lysosomal enzymes required for their proper targeting to the lysosomes (Hickman and Neufeld, 1972; Hasilik and Neufeld, 1980; Hasilik et al, 1981; Reitman et al, 1981). In normal cells, targeting of the lysosomal hydrolases to the lysosomes is mediated by receptors that bind a Mannose-6-phosphate marker on the enzymes. In the cis Golgi, this recognition marker is generated through the sequential actions of two enzymes; a phosphotransferase and a phosphodiesterase. In I-cell disease N-acetylglucosaminyl-1-phosphotransferase, the enzyme which catalyses the first step, is defective (Nolan and Sly, 1989).

Other biochemical defects have been observed in I-cells. In 1979, Tietze and Butler, found in seven out of ten I-cell disease fibroblasts abnormally high levels of free cystine comparable to those seen in homozygous cystinotic cells. They also demonstrated that the increased cystine content seen in I-cells was unique to cystine and did not reflect a generalized increase in the total free amino acid content of these cells. Jonas et al (1981) showed that in I-cell disease, cystine accumulates in lysosomes similar to what is seen in cystinosis.

The intralysosomal pH in normal, cystinotic , and I-cell fibroblasts were compared (Oude Elferink, Harms and Tager as cited in Kooistra et al, 1984). There was no difference found in the intralysosomal pH between the cystinotic and normal cells ; but in I-cell disease the pH was lower than normal. The significance of these results was not clear. Other theories to explain of the increased cystine in I-cell fibroblasts were suggested: a) abnormal lysosomal membrane integrity in I-cell fibroblasts (i.e. defective lysosomal enzymes required for the integrity of the membrane, abnormal charged groups at the binding site, change in pH and potential gradient), b) blockage of cystine egress by the presence of undigested inclusions (Greene et al, 1985)

More recently, in 1986, Tietze et al suggested, based on (^{35}Scystine) egress studies, that I-cell fibroblasts, like cystinotic cells possess a defective carrier mechanism for cystine transport. They measured the rates of clearance of free (^{35}S cystine) in normal, cystinotic and I-cell intact fibroblasts. They found that the loss of radioactivity occured slowly in the two mutant cell lines compared to the rapid loss from normal cells. They also reported decreased rates of efflux of free cystine from lysosome-rich granular fractions of cystinotic and I-cell fibroblasts. They proposed that this could be due to either defective packaging of cystine carrier glycoprotein lacking the Man-6-P marker, or due to inadequate proteolytic processing of an inactive precursor form of the carrier as a result of low activity of lysosomal proteinases in I-cell fibroblasts.

Another secondary defect in I-cell disease fibroblasts is the high content of sialic acid-containing residues due to the lack of neuraminidase activity. It was suggested that this defect might change the membrane potential (Greene et al, 1985). This altered membrane structure has also been found to confer increased sensitivity to freezing (Sly et al, 1976).

5. Review of the methodology

"The general problems encountered when one tries to isolate subcellular organelles in an undamaged state are mainly : how to break cells without breaking the organelle of interest, how to maintain the organelles intact as they are removed from their normal cellular milieu, and finally how to separate the organelles from each other" (Dean, 1977).

5.1 Cell Harvesting

Two main methods of harvesting fibroblast cell lines were mostly used:

1. Controlled trypsinization (Rome et al, 1979; Harris,1981; Thomas et al, 1985; Renlund et al, 1986a).
2. Scraping with a rubber policeman (Harms et al, 1980; Gieselmann et al, 1983; Mokrasch, 1985; Renlund et al, 1986b)

It has been reported that the method of fibroblast detachment affects the recovery of amino acids in general and to a lesser extent cystine e.g. the recovery of amino acids was decreased when fibroblasts were scraped with a rubber policeman compared with their detachment by trypsinization (Kroll et al, 1974)

5.2 Cell disruption

Several methods of cell lysis have been described in the literature depending on the the type of cells and on the complexity of of their intracellular and extracellular matrices.

I- Homogenization:

Homogenization is the most popular method of cell lysis, however, it has been applied in many different ways according to several variables i.e.

i- Type of pestle used being either tight or loose fitting.

ii- Lysis buffer being hypo-osmotic or iso-osmotic.

iii- Number of strokes.

iv- Type of homogenizer, glass (Gieselmann et al, 1983) or metal (ball bearing homogenizer) (Balch and Rothman, 1985).

The interaction of the above mentioned variables can be examplified in the following techniques:

1- Uniform lysis of cells exposed to hypo-osmotic conditions was done by the use of a loose fitting pestle with 2 to 5 strokes (Mokrasch, 1985).

2- Fibroblasts suspended in isotonic sucrose were broken with 20 strokes by a tight fitting pestle (Gieselmann et al, 1983).

II- Nitrogen cavitation

Cells suspended in 0.25 mol/L sucrose were disrupted by nitrogen cavitation [30 psi (2.0 atm), 10 minutes] (Renlund et al, 1986a).

III- Sonication

Sonication, as a means of cell lysis, has been applied for three 10 second periods (Thomas et al, 1985); however, in general sonication is considered as an inconsistent method of cell lysis.

IV- Finally, cell disruption was done by passing the suspension through the tip of a pipette i.e. drawing and expelling the cell suspension 15 times through a 10 mL glass pipette (internal diameter of the tip 1.45 mm) (Harms et al, 1980; Pisoni et al, 1985; 1987), whereas, Renlund et al (1986b) lysed the cells by passing the cell suspension 50 times through the tip of a 1 mL Eppendorf pipette.

5.3 Methods of isolation of lysosomes

Centrifugal techniques depends mainly on differences in size and / or density of the particles to be separated. Certain functional characteristics of lysosomes have been used to modify their behavior in centrifugation i.e. altering their density. In order to review the methods of the isolation of lysosomes, we classify them into two main categories:

5.3.1 with pre-treatment (alteration of lysosomal density)

5.3.2.without pre-treatment (no alteration of lysosomal density)

5.3.1 Isolation of lysosomes with pre-treatment (alteration of lysosomal density)

The sedimentation coefficients of lysosomes, mitochondria, and peroxisomes overlap, especially since the lysosomal population is extremely heterogenous, with only one common characteristic, the content of acid hydrolytic enzymes. Therefore, pre-treatment of lysosomes has been used in attempts to allow better separation.

a) Triton loading technique

When injected in experimental animals, Triton WR-1399 was deposited in secondary lysosomes and induced a decreased equilibrium density of these organelles causing them to float and so could be collected from the interphase between the 14% and 35% sucrose layers of the gradient (Wattiaux et al, 1963; Leighton et al, 1968). This technique has proven to be effective in purifying lysosomes from many organs other than liver, such as lymphoid tissue, spleen or kidney or in getting other constituents such

peroxisomes and mitochondria free of lysosomes (Dean, 1977). The drawbacks of this method were found to be mainly the swelling of lysosomes and the interference with lipid metabolism (Huterer et al, 1975; Dean, 1977).

b) Iron loading technique

This method consisted of repetitive injections of an iron-sorbitol-citric acid complex which usually increases ferritin synthesis (Arborgh et al, 1974; Glaumann et al, 1975; Hultcrantz and Glaumann, 1982); and since ferritin is metabolized in the lysosomes, it accumulates together with haemosiderin in the lysosomal compartment resulting in a marked increase in the density of the lysosomes. The loaded lysosomes can then be isolated by both differential and isopycnic centrifugation in a continuous sucrose gradient, however, the main drawback lay in the fact that the ferritin loaded lysosomes may have altered functional attributes.

Other techniques such as injection of colloidal gold (Henning and Plattner, 1975) or administration of dextran (Thines Sempoux, 1973) have been described but did not gain general use. In both methods the introduced compounds accumulated in the lysosomes, increasing their densities and allowing their separation from the mitochondria and the peroxisomes.

5.3.2 Isolation of lysosomes without pre-treatment (no alteration of lysosomal density)

a) In 1978, Wattiaux et al described a method for the isolation of rat liver lysosomes without any alteration of their density. The technique consisted of centrifugation in a discontinuous metrizamide gradient. The two encountered drawbacks were the high cost of metrizamide and the occasional aggregation of lysosomes and other organelles (Glaumann et al, 1982).

b) Another technique was described by Yamada et al, in 1984, where lysosomes were isolated from livers of untreated rats. The post-nuclear supernatant was incubated with 1 mmol/L Ca^{2+} which causes swelling of mitochondria and alteration of their isopycnic density. Lysosomes were then separated from the swollen mitochondria by Percoll density gradient centrifugation followed by repetitive washing procedures.

c) Isolation of lysosomes by free flow electrophoresis was described by Harms et al, 1980.

d) Lysosomes from human skin fibroblasts were isolated by differential pelleting followed by Percoll gradient centrifugation and finally repetitive washing procedures (Pisoni et al, 1985).

5.4 Protein determination

Lowry's method is the most widely used protein assay; however, it was found that Percoll causes a background color with the Folin-Ciocalteau reagent, interfering with the procedure (Bensadoun et al, 1976; Rome et al, 1979). Modifications were attempted using Percoll solutions for the preparation of the blank. However, since a Percoll gradient is being analyzed, the accurate concentration of the Percoll in the samples is not known and it is not practical to run a separate control for each sample especially when a large number of samples have to be assayed (Khan et al, 1981). Several methods have been described to deal with interfering substances, for instance, removal of the precipitate formed in the assay by centrifugation (Raja and Klein, 1975) or precipitation of protein before assay (Bensadoun, et al, 1976).

In 1979, Terland et al described another technique recommending the use of the Coomassie blue method of Bradford (1976). However, this procedure did not give satisfactory results due to the development of intense turbidity in the assay tube in the presence of Percoll (Khan et al, 1981).

A method for quantitation of protein in the presence of Percoll (maximum concentration 30%) was developed by khan et al (1981) using a dual wavelength correction. This method was based on monitoring the absorbance at 750 and 420 nm of color developed by protein plus Percoll with Folin Ciocalteau reagent. The difference in the extent of absorbances was used to determine the protein concentration in samples containing protein plus Percoll. Standard curves for Percoll and protein in 0.25 mol/L sucrose were linear.

5.5 Amino acid Analysis

Interest in the determination of free amino acid content in biological fluids is as old as the knowledge of amino acids (Berzelius, 1833; Frerichs, 1854; Fischer and Bergell, 1902 as cited in Deyl et al 1986). Column liquid chromatography was rediscovered in 1941, by Martin and Singe, who were aiming at the separation of amino acids. In 1944, Consden et al, improved the method by introducing paper chromatography of amino acids, and it was soon applied to the analysis of free amino acids in blood and urine (Dent, 1947). Automated sample collectors were introduced into column liquid chromatography in 1950, making the separation easier. The first fully automated amino acid analyzer was constructed by Spackman et al, 1958 (as cited in Deyl et al, 1986). Paper chromatography was slowly overtaken by thin layer chromatography and ion exchangers dominated the area of amino acid analysis in the 1960s and 1970s.

Prolifing of amino acids is of great importance today and has found broad application in clinical practice. The main diagnostic applications of free amino acid profiling have been to urine (Jagenburg, 1959), capillary and venous blood (Emery et al, 1970), serum, plasma (Armstrong and Stave, 1973), and amniotic fluid (Bremer et al, 1981 as cited in Deyl et al, 1986). Amino acid analysis was also performed on other biological fluids such as saliva (Moor and Gilligan, 1951), tears (Fleischmayer and Wiechert, 1963), synovial fluid, cerebrospinal fluid, exudates and transudates (Biserte et al, 1963), feaces (Levy et al, 1969), sweat (Liappis and Hungerland, 1972), sperm and breast milk (Brown-Woodman and White, 1974; Hyanek et al, 1968 as cited in Deyl et al, 1986). In the case of tissues and cell populations, free amino acids were determined in fibroblasts cultures (Holden, 1962), hair (Pollit et al, 1968), nails , teeth, bones, muscles (Curtius et al 1968), red blood cells (Levy and Barkin, 1971), homogenates of liver cells and brain cells (Perry et al, 1971), and leucocytes (Houpert et al, 1976).

5.5.1 Preparation of biological samples for amino acid analysis

Deproteinization of biological samples is an essential step in their preparation for amino acid analysis as the presence of protein has a considerable influence on the determination of free amino acids. Proteins may adhere to ion exchangers in columns causing peak spreading and resulting in an increased column back pressure. Other special sample treatment might need to be done before analysis; for instance desalting in case of high concentrations of salts particularly seen in urine samples (Jagenberg, 1959), extraction of lipidic components as in the case of hyperlipaemic serum or breast milk (Peters et al, 1969), removal of excessive amounts of ammonia in urine samples (Turnell and Cooper, 1984).

Deproteinization can be carried out either by chemical reagents or by physical techniques, and can result in some losses due to the adsorption of amino acids on the protein surface. These losses are particularly important when the amino acids are present in small amounts such as in the case of aspartic acid, asparagine, citrulline, ornithine, cystine and tryptophan. Precipitation of proteins by chemical reagents is the oldest deproteinization technique (Moore and stein, 1948). Sulfosalicylic acid was found to be the most suitable deproteinization reagent. It has been used as a 20% solution (Moore and Stein, 1948), or in the solid form, especially when working with small protein content in the samples. In the latter case, it was suggested to use as little of the precipitating reagent as possible i.e. 20-50 mg sulfosalicylic acid / mL plasma (Mondino, 1970). However, in case of high protein content in the sample as in fibroblast cultures, it was recommended to apply a concentration of sulfosalicylic acid as high as 200 mg / mL (Holden, 1962). Perchloric, trichloroacetic

and picric acids were also used as deproteinizing reagents especially for tissue samples. Another chemical technique of deproteinization was performed using ion exchangers e.g. a cation exchanger in the H^+ form such as chromobead (A). Amino acids are bound to the ion exchanger by shaking the solution with the ion exchange resin. The mixture is then centrifuged, the supernatant containing the proteins is discarded and the ion exchanger bound amino acids are released with 0.1mol/L HCl (Deyl et al, 1986).

Ultrafiltration seems to be the most popular.physical method of deproteinization. Amicon centriflo membrane cones of cF25 - cF50 (molecular weight cut off 25,000 and 50,000 respectively) were used and were found to remove 99.8 ± 0.06% and 99.5 ± 0.31% (mean ± S.D) of plasma protein (Blanchard, 1981). Ultracentrifugation is another physical means used in separating protein from free amino acids. However, it is cumbersome (Cohen and Strydom, 1988) and it is hence, rarely used. For the sake of completeness, a third physical method of deproteinization should be mentioned, that is deproteinization using thermal fixation on cellulose fibres, where the protein fraction is immobilized on cellulose fibres by increased temperature, acetic acid vapour or by drying (Guthrie and Susi, 1963).

5.5.2 Chromatographic separation and profiling of free amino acids

In principle, separation of amino acids is based either on the charge or on hydrophobicity differences and in liquid column chromatography, the type of chromatography used depends on whether one is separating derivatized or underivatized amino acids. Therefore, two possibilities could be applied: Firstly, underivatized amino acids can be separated by cation exchange chromatography followed by a post-column derivatization reaction for detection (it is important to note that with the exception of the aromatic amino acids and few others, most underivatized amino acids cannot be detected by UV absorption unless if very short wave length is used). Secondly, amino acids can be converted into suitable derivatives before separation and then the separation is run in the reversed-phase mode. None of these available chromatographic techniques fit all demands because of the many possible applications of amino acid analysis, and each technique exhibits specific advantages and disadvantages.

5.5.2.1 Post-column derivatization

Usually, separations performed with post-column derivatization are carried out on cation exchangers with a gradient of acidic buffers. The more acidic amino acids (possessing an additional carboxyl group) elute first and those with more than one primary amino group or possessing a guanidyl residue elute at the end of the chromatogram. Then,

the separated amino acids are converted into coloured derivatives e.g. ninhydrin derivatives for spectrophotometric detection, or into fluorescent derivatives e.g. O-phthalaldehyde (OPA) derivatives for fluorescence detection.

Ion exchange chromatography of underivatized amino acids with ninhydrin detection has been the standard method because of the number of compounds separated and detected within the profile i.e. 52 ninhydrin-positive compounds can be separated. Replacement of ninhydrin by fluorescamine (Udenfriend et al, 1972; Stein et al, 1973) promised to enhance the sensitivity of all amino acid analysis, except those with secondary amino groups such as proline and hydroxyproline. Analysis at low pico mole levels were possible (Stein and Udenfriend, 1984). However, the use of another fluorogenic reagent, O-phthalaldehyde (OPA) has superceded the use of fluorescamine, in most laboratories (Benson and Hare, 1979). Post-column derivatization with OPA therefore offers greater sensitivity than the classical ninhydrin procedure. Although OPA does not react with secondary amino acids, it is possible to generate a primary amine from secondary amines by oxidation with sodium hypochlorite (Bohlen and Mellet, 1979). It was shown that the potential limits of detection with OPA are below 100 pmol (Cooper et al, 1984); however, in practice this limit seemed to be at 50-100 pmol for proline, and 5-10 pmol for the other amino acids (Dong and Gant, 1985).

For completeness, two more post column fluorescent reagents should be mentioned. Firstly, a technique, using 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole (Watanaabe and Imai, 1983 as cited in Deyl et al,1986) was described. It demonstrated sensitivity in the picomole range. It was also tested for clinical applicability and was proven to be suitable for the analysis of free amino acids in blood discs. Secondly, a reagent known as Pentane-2,4-dione and formaldehyde has been suggested for amino acid analysis by Kakehi et al, 1985; however, it was found to be much less sensitive than OPA and the color yields of proline and tyrosine were very low.

One often encountered problem in the amino acid profiling of tissues with ion exchange chromatography is the occurrence of one to three fast peaks at the start of the chromatogram. This fraction has been called the "Taurine fraction", representing a complex mixture of more than ten components of which taurine, phosphoserine, phospho ethanolamine, glycerophospho-ethanolamine, hypotaurine, cysteic acid and cysteine sulphinic acid have been characterized (Deyl et al, 1986). These individual highly acidic and highly ionized compounds have very close retention times and ion exchange chromatography fails to separate them adequately under the conditions used for routine amino acid profiling.

5.5.2.2 Pre-column derivatization

Reversed phase chromatography is the preferred separation procedure if the aim is to separate amino acid derivatives. Octadecysilane hydrophobized sorbents have been widely used. A variety of solvent systems are available, mostly containing methanol and acetonitrile as the organic component of the mobile phase (Deyl et al, 1986). Numerous compounds have been used in pre-column derivatization of amino acids, mostly electrophiles capable of reacting with the free α-amino group. The most popular is phenylisothiocyanate (PITC), known as the Edman reagent, which forms phenylthiocarbamyl derivatives of amino acids (Heinrikson and Meredith, 1984). There are several key features of the phenylthiocarbamylation chemistry that encouraged the development of PTC amino acid analysis: a) specificity of PITC has been well characterized with no evidence for the formation of any disubstituted derivatives with tyrosine or histidine, b) reagent interference is minimal, c) PTC derivatives have been detected at the 1 pmol level (Cohen and Strydom, 1988)

Other reagents include 5-dimethyl aminonaphthalene-1-sulfonyl (dansyl) chloride, and its analog 4-dimethylamino-azobenzene-4-sulfonyl (dabsyl) chloride, 2,4 dinitrofluorobenzene, and 4-chloro-7-nitrobenzo-2-oxo-1,3-diazole (NBD-Cl) (Cohen and Strydom, 1988). HPLC analysis of Dansyl amino acid derivatives was judged to be moderate in terms of separation, sensitivity, precision, and time of analysis. However, various unidentified and interfering peaks were often encountered, and it was unknown whether both mono and didansyl derivatives were being formed or whether reaction products were using the extraneous peaks. It was suggested that the use of dansyl derivatives might not be appropriate in various biological applications (Wilkinson, 1978). On the other hand , although the Dabsyl derivatization procedure was found to be simple and rapid, it presented a major drawback : the formation of multiple derivatives with bis-amino acids which eluted from the column at the same time as other monoderivatives.

O-phthalaldehyde (OPA) has been also used in pre-column derivatization of amino acids. In conjunction with a thiol reagent, OPA reacts with primary amino groups to form highly fluorescent isoindole products (Hill et al, 1979). The wide use of OPA in pre-column systems is due to several advantages such as the high fluorescent yield exhibited by its derivatives, the solubility and stability of the derivatives in aqueous buffer, the rapidity of the reaction, and the absence of OPA interference with the reaction. However, OPA has exhibited some major drawbacks, namely the inability to form fluorescent derivatives with secondary amino acids, the formation of double peaks with histidine, lysine, and ornithine; and lastly the instability of the derivatives (Mc Clung and Frankenberger jr, 1988).

A reagent originally introduced as a blocking group in peptide synthesis, FMOC-Cl (9-fluorenyl-methyl Chloroformate), has been also used for amino acid analysis (Einarsson et al, 1983). FMOC-Cl was found to react rapidly with both primary and secondary amino acids forming highly fluorescent derivatives. However, disadvantages, such as the formation of mono or disubstituted derivatives with histidine, and reagent interference, were encountered.

Derivatization of the carboxyl group of amino acids was tried (Yoshida et al, 1985), but did not gain popularity. Phenylthiohydantin (PTH) derivatives were also used and found to offer a rapid HPLC analysis, fairly good resolution, sensitivity, precision and derivative stability. However, the lengthy process of forming PTH derivatives made this amino acid derivatization technique undesirable (McClung and Frankenberger jr, 1988).

6. Objective

The purpose of this study is to develop a method for the measurement of the whole lysosomal amino acid profile in normal fibroblasts, and to test it in cystinotic and I-cell disease fibroblasts where specific abnormalities are expected. Finally, if successful the methodology could be used to screen other candidate genetic diseases.

7. Hypotheses

1- Intralysosomal concentrations of amino acids would be increased if a lysosomal transport defect were present.

2- If the cystine carrier transported amino acids other than cystine i.e. not completely specific, concentrations of these other amino acids would be increased in cystinosis.

3-The cystine transporter may be deficient in I-cell disease lysosomes due to defective targeting. If there are other amino acid transporters in the lysosomes, these may also be affected and increased intralysosomal concentrations of the corresponding amino acids could be observed.

4- The features one might expect in disorders of lysosomal transport are abnormal lysosomal morphology, or abnormal storage of material (particularly localized to lysosomes), and deficiencies of vitamins, minerals, or other essential compounds. Some possibilities could include Menke's disease (copper storage), Chediak Higashi syndrome (azurophilic granules accumulation), Indian childhood cirrhosis (iron storage), and ceroid lipofuscinosis (lipofuscin storage).

Chapter two: Methodology

1- Human Fibroblast Culture

Fibroblast cultures were either started from cell lines purchased from the Human Genetic Mutant Cell Repository (Camden, New Jersey) or from human skin punch biopsies.

1.1 Reagents

1) Minimum Essential Medium (MEM, Gibco Cat. # 410-1100)

Dissolve one package of MEM in 1000 mL deionized water. Add 2 g $NaHCO_3$ and mix until dissolved. Add 10 mL Pen / Gent. Mix well, adjust the pH of the medium to 0.2-0.3 unit below the desired final working pH, as the pH units will usually rise 0.1-0.3 unit upon filtration. Filter under vacuum into a sterile flask through Nalgene 0.2 µ filter. Store at 4°C in two 500 mL sterile bottles.

2) MEM with 20% Fetal Calf Serum (FCS)

To 20 mL MEM (with Pen / Gent) add 5 mL fetal Calf Serum (FCS), 0.25 mL L-Glutamine, and 0.15 mL Fungizone. Mix well.

3) MEM with 10% Fetal Calf Serum (FCS)

To 90 mL MEM (with Pen / Gent), add 10 mL fetal Calf Serum (FCS), 1 mL L-Glutamine, and 0.6 mL Fungizone. Mix well.

4) Penicillin / Gentamycin (Pen / Gent)

Dissolve Penicillin G (Sodium salt , 5 x 10^6 IU), and Gentamycin sulfate (80 mg/mL) in 400 mL sterile deionized water, mix well and store in 10 mL aliquots at -20°C.

5) Fungizone (Gibco #600-5295) 20 mL

Reconstitute Amphotericin B (250 µg / mL) with 20 mL sterile deionized water, and store the vial at -20°C. It may be frozen and thawed several times without effect.

6) Fetal Calf Serum (FCS, heat inactivated, mycoplasma and viral tested, Gibco #230-6140).

Filter 100 mL through 0.22 µ corning filter membrane. Store in 10 mL aliquots in sterile tubes at -20°C.

7) L-Glutamine (200 mmol / L, Sigma G-3126)

Dissolve 2.92 g L-Glutamine in 100 mL deionized water. Filter through Nalgene 0.22 μ filter, and store in 5 mL volumes in sterile tubes at -20°C.

8) Trypsin (Gibco #610-5095) 2.5%

Reconstitute with 20 mL sterile deionized water. Store at -20°C. It may be frozen and thawed several times without effect.

Working Trypsin Solution:

Dilute one part in ten with Hanks Balanced Salt Solution. This solution may be stored frozen. When frozen and thawed more than once, it loses its effectiveness (a yellow or orange colour turns red when it loses its effectiveness).

9) Hanks Balanced Salt Solution (1 L, No Calcium, No Magnesium, Gibco #310-4170)

Store at 4°C once opened.

10) Dimethyl Sulfoxide (DMSO, 5 mL, Sigma D2650)

11) 10% DMSO / MEM

Add 1.0 mL DMSO to 9.0 mL MEM (with 20% FCS, P/G, FGZ, L-Gln). Mix well.

1.2 Procedures

Tissue culture procedures include: cell culture from skin biopsies, cell subculture, cell storage in liquid nitrogen, and thawing cells from liquid nitrogen (Kruse and Patterson, 1973).

1.2.1 Cell culture from skin biopsies

i- Collect a sterile skin biopsy 2 to 3 mm full thickness core of skin in a sterile tube containing MEM with 20% FCS. Start culture within the first five hours.

ii- Transfer biopsy core to a sterile 30 mm petri dish containing a large bead of MEM with 20% FCS.

iii- Using sterile scissors and forceps, cut the skin into very small fragments in a plane perpendicular to the surface.

iv- Add 1 mL MEM with 20% FCS to a sterile T-25 flask to cover the bottom surface of the flask.

v- Transfer the tissue fragments with a sterile pipet to the T-25 flask and carefully position the pieces in the flask to allow growth.
vi- Add 2 mL MEM with 20% FCS to the flask and let it sit at a tilted angle for several minutes to allow pieces to stick to the surface of the flask.
vii- Place the flask in the CO_2 incubator with the cap loose to allow exchange of air in the flask with the 5% CO_2 environment of the incubator.
viii- Examine under inverted microscope after 5 to 7 days without disturbing the fragments.
ix- Epidermal cells should begin to form after 5 days. Add 2 mL of MEM with 20% FCS and incubate further for several days.
x- If growth is progressing well draw off the medium and replace it with about 3 mL of MEM with 10% FCS. Continue the culture at 37°C, and check growth every 2 to 3 days under the microscope.
xi- Increase the amount of medium to about 6 mL during the following week.
xii- Subculture when there is a monolayer of confluent fibroblasts covering at least 50% or more of the flask surface.

1.2.2 Cell subculture[1]

i- Remove the medium from the flask and wash the cells with 3 mL Hanks Balanced Salt Solution.
ii- Prewarm 0.25% trypsin to 37°C and add 1 mL to the T-25 flask .The trypsin should just cover the cells. Incubate the cells at 37°C for 5-7 minutes until they detach from the surface of the flask. Tap and shake the flask to loosen the cells if necessary.
iii- Add rapidly, 3 mL of MEM with 10% FCS to stop the action of the trypsin. Wash the surface of the flask with the mixture of MEM and trypsin to remove any cells left attached to the surface.
iv- Transfer to a large T-75 flask or to 3 small T-25 flasks. Add 12 mL of MEM with 10% FCS to the large flask and 6 mL to each of the smaller flasks. Place the new cultures in the incubator with cap loosened.
v- Change most of the medium next day and replace with fresh MEM with 10% FCS.
vi- Incubate for 2 to 3 days, check under 10 X power for growth.
vii- Feed cultures by adding MEM with 10% FCS every 2 to 3 days.

---

[1] Subculture was done 1 in 3 (T-75) flasks in case of normal fibroblasts and 1 in 2 (T-75) in case of mutant cell lines

1.2.3 Cell storage in liquid nitrogen

i- Proceed with the cell detachment as mentioned above and stop the trypsin action with 5 mL of 10% DMSO / MEM mixture.

ii- Transfer cells to small NUNC ampoules (1.8 mL maximum volume). Refrigerate for 3 hours at 4°C. Cells adjust to quiescence. Freeze at -20°C overnight, then transfer to -70°C for 12 hours and finally, store in liquid nitrogen.

1.2.4 Thawing cells from liquid nitrogen

i- Transfer the ampoule from liquid nitrogen immediately into a 37°C water bath until frozen liquid has just thawed.

ii- Dilute out DMSO by adding MEM with 20% FCS dropwise to a final volume of 6 mL.

iii- Transfer mixtures to a T-25 flask and incubate at 37°C and 5% CO2 overnight. Cells attach to the flask surface within several hours. Next day, change medium completely with fresh MEM with 20% FCS to remove DMSO.

iv- Carry on culture as usual, feeding with fresh MEM with 10% FCS.

2. *Subcellular fractionation of human fibroblasts*

Subcellular fractionation of the human fibroblasts is performed on a self generating Percoll gradient (Gieselmann et al, 1983).

2.1 Reagents

1) Percoll gradient

Percoll solution consists of silica particles coated with non dialysable polyvinyl pyrrolidone (PVP), with a density of 1.1130 ± 0.005 g/mL, and particle size of 15 to 30 nm in size and a mean diameter of 21 to 22 nm (Pharmacia Percoll manual, 1980).

When a solution of Percoll (in 0.25 mol/L sucrose) is centrifuged at > 10,000 x g, the coated silica particles will start to sediment, resulting in an uneven distribution of particles and thus forming a self generating density gradient.

<u>The one step procedure for diluting Percoll</u>

Percoll (from the bottle) may be diluted directly to make a final working solution of known density. The required volume of Percoll (from the bottle) is calculated using the following formula

$$V_0 = V\,(P - 0.1P_{10} - 0.9) / (P_0 - 1)$$

where,

V_0 = volume of Percoll (from the bottle) in mL

V = volume of the final working solution = 30 mL

P = desired density of the final solution = 1.065 g/mL

P_0 = density of Percoll from the bottle = 1.130

P_{10} = density of 2.5 mol/L sucrose[1] = 1.316 g/mL

Therefore, volume of Percoll required = $30\,(1.065 - 0.1)(1.316 - 0.9) / (1.130 - 1)$

$= 30\,(1.065 - 1.0316) / 0.13$

$= 30 \times 0.26 = 7.8$ mL

In order to prepare 30 mL of working solution of Percoll of density 1.065 g/mL in 2.5 mol/L sucrose, to 3 mL (1/10 of final desired volume) of 2.5 mol/L sucrose add 7.8 mL Percoll. Make up 30 mL with deionized water.

---

[1] The working sclution of Percoll is made up with sucrose as the subcellular particles usually aggregate in the presence of salts (Pharmacia manual).

2) Density Marker Beads

Density determination is performed using "Density Marker Beads". These are derivatives of sephadex. There are 10 types, each type is color coded and modified to have a specific density in gradients of Percoll. These beads are used as an external marker and their densities (p= 1.037 - 1.138 g/mL) cover the buoyant densities of the vast majority of cells and organelles to be separated in Percoll. A sample of 25 µL of each colour bead (No 1-4 and 6-10) is layered on top of Percoll gradient (p= 1.065 g/mL). This tube is centrifuged simultaneously with the tube containing the biological samples.

3) Washing buffers

Buffer I: 10 mmol/L Na Phosphate, 0.14 mol/L NaCl, pH 7.4

Dissolve 0.69 g NaH_2PO_4 (monobasic, MW= 137.99) in 500 mL deionized water; solution A. Dissolve 0.71 g Na_2HPO_4 (dibasic, MW= 141.96) in 500 mL deionized water; solution B. To make up the buffer with pH 7.4, add 95 mL of solution A to 405 ml solution B. Dissolve 4.1 g NaCl in 500 mL phosphate buffer pH 7.4.

Buffer II: 0.25 mol/L sucrose, 1 mmol/L EDTA, pH 7.4

Dissolve 42.78 g sucrose (Ultrapure sucrose, MW= 342.30), and 0.186 g EDTA (MW= 372.2) in 450 mL deionized water. Adjust pH to 7.4 with 1N NaOH and qs with deionized water to 500 mL.

4) Isolation Buffer: 0.25 mol/L sucrose, 3 mmol/L imidazole HCl, pH 7.4

Dissolve 42.78 g sucrose and 0.16 g imidazole HCl (MW= 104.5) in 450 mL deionized water. Adjust pH to 7.4 with 1N NaOH and qs with deionized water to 500 mL.

2.2 Procedure

i- When cells are confluent (5 x 10^6 cells / flask) wash the tissue culture flasks[1] thoroughly with ice cold buffers: twice with "Washing Buffer I" (5 mL / flask) and once with "Washing Buffer II" (5 mL / flask).

ii- Detach the fibroblasts in chilled "Isolation buffer" (3 mL / flask) using a rubber policeman.

iii- Collect the cells by centrifugation at 600 x g, for 5 minutes, at 4°C. Resuspend all the pellets in a total volume of 3 mL "Isolation buffer".

---

[1] The average number of tissue culture flasks used per fractionation is 20 (75cm2) flasks

iv- Cell disruption is performed on ice. The homogenizer is cooled prior to use. Break the cell pellet by 20-30 strokes, using the tight fitting pestle B of a glass homogenizer. Pipet 1 mL of the homogenate for total enzyme activities and protein determination.
v- Remove nuclei and broken cells by centrifugation at 600 x g, for 10 minutes at 4°C.
vi- Keep the supernate, subject the pellet to 10 additional strokes, and repeat centrifugation.
vii- Layer the post nuclear supernates on top of 10 mL 25% Percoll gradient (p=1.065). The gradient is underlayered with 0.7 mL 2.5 mol/L sucrose. Centrifuge at 48,200 x g in a JA20 rotor, for 60 minutes at 4°C.
viii- Collect 1 mL fractions from the top of the tube for enzyme and protein assays.

3. Protein determination

Quantitation of protein in the fibroblast homogenates was performed according to a modification of Lowry's method; whereas the protein content in the lysosomal fractions was determined according to a method described by Khan et al (1981).

3.1 Protein determination in fibroblast homogenate: Modification of Lowry

Principle

Protein interacts initially with copper in alkali; then the Cu-protein complex, the tyrosine, and the tryptophan of the protein reduce phosphotungstic and phosphomolybdic acids (Folin Ciocalteau reagent) to tungsten blue and molybdenum blue respectively. The standard curve ranges from 30 µg to 150 µg and the absorbance is measured at 750 nm.

Reagents

1) Bovine Serum Albumin

Dissolve 30 mg Bovine Serum Albumin (Sigma A-4503) in 100 mL deionized water. Store at 4°C no longer than 2 months.

2) Solution A

Dissolve 2 g Potassium Sodium Tartarate, and 100 g Na_2CO_3 in 400 mL deionised water. Add 500 mL 1 mol/LNaOH, mix well, and qs to 1000 mL with deionized water. Shelf life is 6 months at room temperature.

3) Solution B

Dissolve 2 g Potassium Sodium Tartarate, and 1 g $CuSO_4.5H_2O$ in 50 mL deionized water. Add 10 mL 1 mol/L NaOH, mix well, and qs to 100 mL with deionized water. Shelf life is 6 months at room temperature.

4) Solution C

Dilute 1 volume of Folin-Ciocalteau reagent (2N Phenol reagent) with 15 volumes of deionized water just prior to use.

Procedure

i- Prepare Bovine Serum Albumin standards as follows:

***Table 2.1* Bovine serum standard (Lowry's method)**

Serum bovine albumin (mg / tube)	Serum bovine albumin (mL / tube)	Deionized water (mL / tube)
0.00	0.00	1.00
0.03	0.10	0.90
0.06	0.20	0.80
0.09	0.30	0.70
0.12	0.40	0.60
0.15	0.50	0.50

ii- Dilute 50 µL of fibroblast homogenate with 950 µL deionised water. Set up a protein control .

iii- To all tubes add 0.45 mL of Solution A. Mix well.

iv- Incubate all the tubes at 50°C in a water bath for 10 minutes. Remove and cool to room temperature.

v- To all the tubes add 50 µL of Solution B. Mix well, and let stand at room temperature for 10 minutes.

vi- Add 1.5 mL of Solution C to all the tubes and mix vigorously.

vii- Repeat incubation at 50°C for 10 minutes, then cool to room temperature.

viii- Read absorbance at 750 nm against reagent blank.

ix- Plot absorbance versus concentration in mg / tube.

Calculations:

mg (from the graph) x 20 = mg protein / mL

3.2 Protein quantitation in lysosomal fractions in the presence of Percoll

Principle

The method is based on measuring the differences in the extent of the absorbance at 750 nm and 420 nm of the color which develops by protein plus Percoll with Folin-Ciocalteau reagent (Khan et al, 1981).

Reagents

1) 2% Na_2CO_3

Dissolve 10 g of Na_2CO_3 in 500 mL deionized water.

2) 2% Sodium-Potassium Tartarate

Dissolve 2 g of Sodium-Potassium Tartarate in 100 mL deionized water.

3) 1% $CuSO_4$

Dissolve 1 g of $CuSO_4$ in 100 mL deionized water.

These stock solutions can be stored at 4°C for six months. The copper reagent is prepared just prior to use, by mixing 98 parts 2% Na_2CO_3 with 1 part 2% Sodium-Potassium Tartarate and 1 part 1% $CuSO_4$.

4) 1.2 mol/L NaOH

Dissolve 4.8 g of NaOH in 100 mL deionized water.

5) The Folin-Ciocalteau reagent (2N)

Dilute one volume of the reagent with one volume of deionized water just prior to use.

6) 0.5 mol/L sucrose

Dissolve 17.12 g sucrose in 100 mL deionized water.

7) 0.25 mol/L sucrose

Dissolve 8.56 g sucrose in 100 mL deionized water.

Procedure

Use 15 x 120 mm disposable culture tubes.

i-Prepare standard curve of bovine serum albumin (20-100 µg) in 0.25 mol/L sucrose (total sample volume = 500 µL).

Table 2.2 **Bovine serum albumin standards (Khan, 1981)**

Bovine serum albumin (µg / tube)	Bovine serum albumin (µL / tube)	Deionized water (µL / tube)	0.50 mol/L sucrose (µL / tube)
20	20	230	250
40	40	210	250
60	60	190	250
80	80	[illegible]	250
100	100	150	250

ii- Prepare Percoll standards

A 25% Percoll solution is prepared in 0.25 mol/L sucrose (v/v).

Table 2.3 **Percoll standards**

Percoll concentration (%)	25% Percoll solution (µL)	0.25 mol/L sucrose (µL)
5%	100	400
10%	200	300
15%	300	200
20%	400	100
25%	500	0.00

iii- Dilute the samples (the subcellular fractions) with 0.25 mol/L sucrose 1:1, total sample volume = 500 µL

iv- Prepare reagent blanks :

a) 500 µL deionized water, b) 500 µL 0.25 mol/L sucrose

v- Add to all the tubes 500 µL of 1.2 mol/L NaOH and mix well. White turbidity is observed in all tubes containing Percoll.

vi- Heat all the tubes in a boiling water bath for 20 minutes (cover the tubes with marbles to avoid evaporation). The white precipitate disappears.

vii- Add 5 mL of the Copper reagent to all the tubes. Mix well and incubate at room temperature for 20 minutes.

viii- Add 500 µL of the diluted Folin-Ciocalteau to all the tubes. Mix vigorously, and incubate at room temperature.

ix- Read the absorbances at 750 and 420 nm within 60 minutes of the final color development.

Calculations:

Plot the standard graphs of both the Percoll and the bovine serum albumin: m_1, m_2, m_3, m_4, and C_1, C_2, C_3, C_4 are the slopes and ordinate intercepts, respectively, of the straight lines.

Protein concentration (µg / sample) = (A_{750} - α A_{420} + β) / m_1 − α m_3

where,

A_{750} = absorbance rate at 750 nm

A_{420} = absorbance rate at 420 nm

α = m_2 / m_4

β = α C_3 + α C_4 - C_1 - C_2

4. Marker Enzyme Assays

Marker enzyme activities: β-hexosaminidase for lysosomes, succinate dehydrogenase for mitochondria, and 5'nucleotidase for plasma membranes are assayed in fibroblast homogenates and subcellular fractions.

4.1 β-hexosaminidase enzyme (Lysosomal marker):

Principle

The assay is performed by adding the sample i.e. a homogenate of fibroblasts or a subcellular fraction, to the buffered substrate, p- nitrophenyl-β-D-N-acetyl glucosaminide. β-hexosaminidase enzyme in the sample hydrolyses the nitrophenyl derivative and p-nitrophenol is released. The latter is measured colorimetrically at 400 nm at an alkaline pH (Pisoni et al, 1985).

The lysosomal membrane integrity is determined by measuring the enzyme latency i.e. the enzyme activity in the presence and absence of a detergent such as Triton X-100. The percentage of broken lysosomes is represented by the free β-hexosaminidase activity (measured in the absence of the detergent), expressed as a percentage of the total enzyme activity (measured in the presence of the detergent) (Rome and Crain, 1981).

Reagents:

1) 0.034 mol/L Citric acid - 0.11 mol/L Phosphate Buffer pH 4.9

Dissolve 4.13 g Citric acid (anhydrous MW= 197.13) and 7.18 g Na_2HPO_4 (MW= 141.96) in 450 mL deionized water. Adjust pH to 4.9 with 1N HCl and q.s. with deionized water to 500 mL. The buffer can be stored at 4°C for 3 months.

2) 4 mmol/L p-Nitrophenyl β-D-N-acetyl glucosaminide pH 4.9

Dissolve 0.137 g p-Nitrophenyl β-D-N-acetyl glucosaminide (MW= 342.3) in 100 mL citric acid - phosphate buffer pH 4.9. Keep frozen in aliquots of 10 mL each at -20°C. Do not freeze and thaw more than twice.

3) 0.8 mol/L Glycine / NaOH buffer pH 10.4

Dissolve 30.03 g Glycine (MW= 75.07) in 450 mL deionized water. Adjust pH to 10.4 with 10N NaOH and q.s. with deionized water to 500 mL.

4) 10 mmol/L p-Nitrophenol (Stock standard solution)

Dissolve 0.139 g p-Nitrophenyl (MW= 139.11) in 100 mL glycine / NaOH buffer pH 10.4.

5) p-Nitrophenol (Working standard solution)

Dilute 0.5 mL stock solution with 50 mL glycine / NaOH buffer

6) 0.1% Triton X-100

Prepare a 10% solution of Triton X-100 and and use a dilution. Dissolve 10 g Triton X-100 in 100 mL deionized water

Procedure:

i-Prepare standards

***Table 2.4* p-Nitrophenol standards**

Working standard (µmol / tube)	Working standard (mL / tube)	Glycine / NaOH buffer (mL / tube)
0.00	0.00	2.00
0.01	0.10	1.90
0.02	0.20	1.80
0.04	0.40	1.60
0.05	0.50	1.50
0.10	1.00	1.00
0.20	2.00	0.00

ii- Prepare two sets of tubes . Label one set with "T". Add 50 µL sample to all the tubes

iii- Add 20 µL 10% Triton X-100 to the labeled set of tubes.

iv- Add 450 µL substrate to all the tubes. Incubate at 37°C for 15 minutes.

v- Stop the reaction by adding glycine / NaOH, 1.48 mL to the "T" labeled tubes and 1.5 mL to the other set of tube; total volume = 2 mL

vii- Read absorbance at 400 nm against substrate blank.

Calculations:

One unit of β-hexosaminidase activity is defined as 1 nmol of p-nitrophenol formed per minute at 37°C (Pisoni et al, 1985).

Plot a standard curve.

The reading from the graph = µL p-nitrophenol / Tube / 15 minutes.

The reading from the graph x 20 /15 = µL p-nitrophenol / min / mL at 37°C

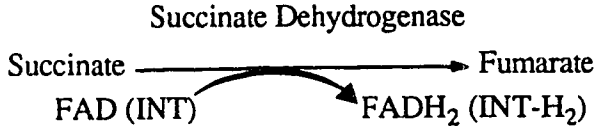
Therefore,

IU / mL = nmol p-nitrophenol / min / mL at 37°C

4.2 Succinate dehydrogenase (Mitochondrial marker):

Principle

The assay is carried out by adding the sample i.e. a homogenate of fibroblasts or a subcellular fraction, to the substrate, sodium succinate, in the presence of p-iodophenyl-3-p nitrophenyl-5-phenyl tetrazolium chloride (INT). Formazan is extracted by ethyl acetate and its absorbance is measured at 490 nm (Pennington, 1961).



Reagents:

1) 50 mmol/L Potassium Phosphate Buffer, pH 7.4

- Solution A:

Dissolve 0.689 g KH_2PO_4 (monobasic) in 100 mL deionized water

- Solution B:

Dissolve 0.870 g K_2HPO_4 (dibasic) in 100 mL deionized water

To make up Potassium Phosphate Buffer pH 7.4 add 19 mL of Solution A to 81 mL of Solution B and mix well.

The substrate consists of :

50 mmol/L Potassium Phosphate Buffer pH 7.4	100 mL
25 mmol/L Sucrose	0.856 g
50 mmol/L Sodium Succinate	1.350 g
0.1% INT	0.100 g

The substrate is stable for 2 weeks at -20°C. It is preferred to add the INT just prior to use.

Procedure:

i- Add 100 µL sample to 900 µL substrate.

ii- Shake the reaction mixture gently at 37°C for 15 minutes.

iii- Stop the reaction with 1 mL 10% Trichloroacetic acid.

iv- Extract formazan by adding 4 mL ethyl acetate. Vortex for 30 seconds.

v- Centrifuge at 800 x g for 10 minutes at room temperature.

vi- Remove an aliquot of the supernatant and measure absorbance at 490 nm versus substrate blank.

Calculations:

The molar extinction coeffecient of formazan in ethyl acetate at 490 nm is 20.1 x $(10)^3$

1 mol/L $\varepsilon = 20.1 \times (10)^3$

1 mmol/L $\varepsilon = 20.1$

$OD = \varepsilon C l$

where,

ε = extinction coeffecient

C = concentration

l = length = 1 cm

Therefore,

$C = OD / \varepsilon l$

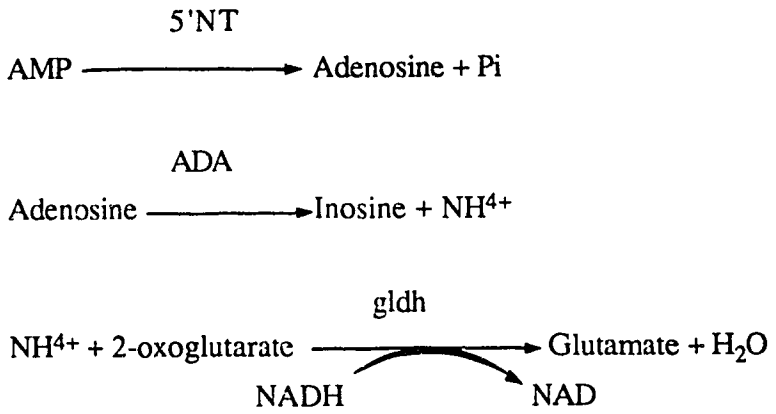
$C = OD/20.1 \times 4/1 \times 1/15$ µmol/min/4 mL

$C = OD/20.1 \times 4/15 \times 1/mg$ µmol/min/mg protein

4.3 5'nucleotidase enzyme (plasma membrane marker):

Principle

5'nucleotidase catalyses the hydrolysis of adenosine monophosphate (AMP) to give adenosine and inorganic phosphorus. Adenosine is deaminated by adenosine deaminase (ADA) to produce ammonia, which in turn reacts with 2-oxoglutarate and NADH, which is oxidised to NAD (Arkesteijn, 1976) as follows:



The rate of formation of NAD is followed automatically on a Multistat III microcentrifugal analyzer by measuring the decrease in absorbance at 340 nm which is directly proportional to the 5'nucleotidase activity. β-glycerophosphate is added to inhibit alkaline phosphatase activity.

5. Amino acid Analysis

5.1 Principle

Amino acid content of the fibroblasts homogenate and the lysosomal fractions is measured in protein-free-filtrates by High Pressure Liquid Chromatography on a cation exchange column. Continuous post-column derivatization of the amino acids is achieved with o-phthalaldehyde (OPA) in conjunction with a mercaptan under basic conditions. At ambient temperature, the reaction is generally complete within 30 seconds. The fluorescent derivatives, 1-alkylthio-2-alkylsubstituted isoindoles exhibit optimum excitation at 340 nm and emission at 455 nm (Yakymyshyn and Audette, 1990; Yakymyshyn, 1991).

5.2 Reagents

1) 0.24 mol/L Lithium Citrate Eluent (LI 275) Buffer, pH 2.75

2) 0.64 mol/L Lithium Citrate Eluent (LI 750) Buffer, pH 7.50

3) 0.30 mol/L Lithium Hydroxide Column Regenerant (LI Regen)

4) 0.27 mol/L Lithium Citrate Diluent , pH 2.2

5) 10 mmol/L β- Thienyl-alanine stock internal standard

6) Working Internal standard / buffer mixture:

Dilute 0.60 mL stock β-Thienyl-alanine standard with 100 mL Lithium Citrate diluent, pH 2.2.

7) 10 mmol/L L-Glutamine:

Dissolve 14.6 mL L-Glutamine in 10 mL Lithium Citrate diluent, pH 2.2

8) 2.5 mmol/L L-Tryptophan and 1.25 mmol/L L-Asparagine:

Dissolve 51.0 mg L-Tryptophan and 18.8 mg L-Asparagine in 100 mL Lithium Citrate diluent, pH 2.2

9) Physiological Calibration Standard, stock solutions , 2.5 mmol/L

Beckman STD #338088

Beckman AN^+ #338156

Beckman B^+ #338157

10) 250 µmol/L Working Calibration Standard

Dissolve 1 mL of Beckman STD, 1 mL Beckman Standard AN^+, 1 mL Beckman Standard B^+, 1 mL Tryptophan / Asparagine Standard, and 0.25 mL L-Glutamine in 10 mL Lithium Citrate diluent, pH 2.2

11) o-Phthalaldehyde Fluorimetric Reagent

12) Sulfosalicylic acid 30 mg/mL sample

13).Column Cation Exchange : 3 mm x 150 mm, 7 µm

14).Millipore Milex Filters, 0,45 µm pore size

15) Amicon membrane cones cF50 (molecular weight cut off is 50,000)

5.3 Instrumentation

Instrumentation consists of a Perkin-Elmer HPLC adapted with the Pickering Labs Amino Acid Application. The Perkin-Elmer modular instrument consists of a 4-Solvent Titanium LC410 Biopump providing the continuous gradient of lithium citrate to the column at a rate of 0.30 mL / min, an ISS-100 automated sample injector fitted with a 20 mL sample loop, a Series10 HPLC isocratic pump for continuous delivery of o-phthalaldehyde at a rate of 0.20 mL / min for postcolumn derivatization at ambient temperature, an LS-5 luminescence spectrometer to measure fluorescence of the amino acid derivatives,1-alkylthio-2-alkylsubstituted isoindoles, at their excitation and emission optima of 340 nm and 455 nm, respectively, with slits of 10 and a fixed scale setting of 2.0. An LCI-Laboratory computing integrator provides a hard copy of the resultant chromatograms with calculations of each constituent amino acid. A calibration standard of known concentration and a control sample are run with each set of samples to ensure accuracy and precision of results. The analytical detection limit is as low as 1 pmol for individual amino acids.

5.4 Preparation of the samples for amino acid analysis

i- Pool the lysosomal fractions (highest β-hexosaminidase activity). Wash the fractions with ultrafiltration using Centriflo membrane cones cF50 (molecular weight cut off 50,000). The membrane cones should be soaked in deionized water for at least one hour prior to use. Filter the samples through the cones by centrifugation at 3000 rpm in a Sorvall

centrifuge at 4°C for 30 minutes. Wash the retentate with 1.5 mL phosphate buffered saline and repeat the filtration. Resuspend the retentate in 500 µL phosphate buffered saline and disrupt the lysosomes by 0.1%Triton X-100, and immediately acidify with sulfosalicylic acid (30 mg / mL sample) and repeat the filtration for the last time. Store the filtrates at -70°C.

ii- At time of analysis thaw and filter the samples through a 0.45 µm pore size millipore HV filter (CAT#SJHV004NS) to remove any residual protein.

iii- Dilute 19.5 µl filtrate with 5 µl 0.27 mol/L lithium citrate buffer pH 2.2, containing β-thienylalanine as internal standard.

5.5 Procedure

i- The equivalent of 19.5 µL of sample in a total volume of 20 µL is injected onto a High Speed Lithium Cation Exchange Column, 3 mm x 150 mm, 5 µm (Pickering Labs).

ii-The amino acids are separated using a continuous gradient of 0.24 to 0.64 mol/L lithium citrate pH 2.75 to 7.50, respectively. Regeneration of the column is performed with 0.30 mol/L lithium hydroxide. Run time for a complete amino acid profile is 136 minutes. Composition of the buffer gradient used for the separation of amino acids is given in *(Table 2.5)*.

***Table 2.5* Composition of the buffer gradient used for the separation of amino acids**

Step	Time	Flow	% A LI 2.75	% B LI 7.50	% C	% D LI Regen	Curve
0[1]	20.0	0.30	100	-	-	-	-
1	9.0	0.30	100	-	-	-	-
2	37.0	0.30	65.0	35.0	-	-	1.0
3	44.0	0.30	-	100	-	-	1.0
4	6.0	0.30	-	100	-	-	-
5	30.0	0.30	-	94.0	-	6.0	2.5
6	5.0	0.30	-	80.0	-	20.0	4.0

Calculations

Concentration of a specific amino acid =

Amino acid concentration in the calibration standard X $\frac{\text{Peak height of the amino acid in the sample}}{\text{Peak height of the amino acid in the calibration standard.}}$

[1] Step "0" is the equilibration step following regeneration prior to injection

In contrast to peak heights, peak areas did not give a linear response with different concentrations of various amino acids.

It is important to note that cystine content has been conventionally reported as "1/2 cystine" because routine subnanomolar quantitation of cystine was performed by ion exchange chromatography with postcolumn identification by ninhydrin staining. This procedure measures total cystine plus cyst(e)ine and cannot differentiate the two and therefore the amount of disulfide has been expressed as half cystine.

6. Electron microscopy

The purity of the isolated lysosomal fractions was judged by assaying marker enzyme activities and by electron microscopy.

Procedure:

6.1 Preparation of samples

a- whole fibroblast

A cell pellet of normal fibroblasts (GM00010) is fixed overnight at 4°C in 4% gluteraldehyde solution in cacodylate buffer, in eppendorf tubes.

b- lysosomal fractions

Pool the lysosomal fractions into two groups the upper fractions, the light lysosomes (p = 1.049-1.054), and the lower fractions, the heavy lysosomes (p = 1.096-1.107). Fix the samples with 1% glutaraldehyde-1% formaldehyde in 0.1mol/L cacodylate for one hour at room temperature. Centrifuge the samples at 35,000 x g for 30 minutes at 4°C. Remove the supernate, rinse and store the pellets overnight at 4°C in 0.1 mol/L cacodylate buffer in eppendorf tubes (Oliver et al, 1989).

6.2 Specimen processing:

i- Fix the samples (a and b) in 1% OsO_4 for two hours at 4°C, with the pellet remaining at the bottom of the ependorff tubes. Rinse the pellets once with cold cacodylate buffer, and then with deionized water for 5 minutes each.

ii- Cut the the eppendorf open with a razor, remove the pellet, cut it into 3 mm^3 portions, and place in deionized water.

iii- Dehydrate the portions in a graded series of ethanol solutions, then rinse three times in propylene oxide.

iv- Place the portions in a 50 : 50 mixture of propylene oxide: epon for 30 minutes, and then in pure epoxy resin (Epon 812) for 2 hours.

v- Place the portions in embedding capsules with fresh epoxy resin and cure them at 60°C for 48 hours.

In case of the heavy lysosomal fraction, where the collected material can be extremely small, dehydrate the pellet and infiltrate it with epon while still in the ependorff tube. Cut the tube open and scrape the bottom to remove the material. Place the scrapped material in an embedding capsule filled with epon. Centrifuge at 2000 x g for 30 minutes.

vi- Remove the various blocks from the capsules and thick section them. Mount the thick (1 μm) sections on glass slides, stain with Toluidine Blue, and examine with the light microscope.(The block consisting of the extremely small material was only thin sectioned to avoid sectioning away the material).

vii- Select a block from each specimen, further trim, and thin section (100 nm) for electron microscopy examination. Mount the thin sections on grids and contrast them with Uranyl Acetate and Lead Citrate.

viii- Examine the grids with the electron microscope (Hitachi 600).

Chapter Three: Results

1. Tissue culture

It was observed that mutant cell lines, cystinotic and particularly, I-cell fibroblasts, grew much slower compared to normal fibroblasts, and that their subculture was only possible at a ratio of one to two flasks. It was also found that I-cell fibroblasts were very sensitive to freezing, and when salvaged from liquid nitrogen, they required a significantly longer period of time than controls to reach confluency.

2. Subcellular fractionation of human fibroblasts and marker enzyme assays

The subcellular fractionation of human skin fibroblasts was based on the method described by Gieselmann et al (1983); however, some modifications were made. Several lysis techniques were attempted: sonication, passing the cell suspension through a pipette tip (1.45 mm in diameter) 15 times, and homogenization in a glass homogenizer. The degree of cell lysis was assessed by examination under light microscope. It was found that sonication resulted in inconsistent lysis, and passing cell suspensions through the pipette tip was not disruptive enough to break the cell membranes. Homogenization was found to be the preferred lysis technique. However, it was observed that the number of strokes needed to be varied between different cell lines to achieve the same degree of disruption. Mutant cell lines, especially, I-cell fibroblasts must be subjected to gentler homogenization i.e.10 strokes instead of 20. This also seemed to apply to ageing normal fibroblasts. This conclusion was arrived at from two sets of data: 1) loss of β-hexosaminidase latency with excessive homogenization of mutant cell lines, indicating that the stroking is too strong and disrupts the lysosomal membrane integrity e.g. β-hexosaminidase activity measured in the absence and presence of Triton X-100 in the I-cell fibroblast (GM02013B) homogenate was 39.5 IU/mL and 61.1 IU/mL, respectively, which indicates that the lysosomal membrane integrity was conserved only to the extent of 35%, 2) high β-hexosaminidase activity in the pelleted broken membranes and nuclei indicates the presence of unbroken cells e.g. β-hexosaminidase activity in the control fibroblast homogenate and in pelleted broken membranes and nuclei was 79.6 and 151.6 IU/mL, respectively, in the absence of Triton X-100 and 312.0 and 448.2 IU / ml, respectively in its presence.

Centrifugation of the postnuclear supernates layered on a 25% Percoll gradient yielded two peaks of lysosomal marker enzyme activity (β-hexosaminidase). The activity recovered in the lower density fractions (p= 1.049 - 1.054) was found to be between 20-50% of that recovered in the higher density region (p= 1.096 - 1.107). β-hexosaminidase activity in I-cell disease fibroblasts was significantly lower than control values (10 - 17% of

normal) i.e. β-hexosaminidase activity in normal and I-cell fibroblast homogenates in the presence of Triton X-100 was 434.7 and 72 IU / ml, or 6.86 and 1.18 μmol/hr/mg protein, respectively.

Lysosomal membrane integrity was conserved to the extent of 75 - 85% in most of the granular fractions as judged by the latency of β-hexosaminidase activity. The latter was determined by the difference in β-hexosaminidase activity in subcellular fractions of normal human fibroblasts (GM00010) in the presence and absence of Triton X-100 (*Fig 3.1*).

The heavy lysosomal population sedimenting from human skin fibroblasts, was found to be relatively free of enzyme activities specific to other intracellular organelles. The marker enzymes succinate dehydrogenase for mitochondria and 5'nucleotidase for plasma membranes were present to a significant degree only in the buoyant fractions near the top of the Percoll gradient (*Fig 3.2*).

3. Electron microscopy

Results of the electron microscopic examination of the whole fibroblast pellet *(micrograph a)* reveals a tightly packed arrangement of cells which show little evidence of freeze artifact. Cells show pleomorphic nuclei (N) and cytoplasm revealing features typical of fibroblast cells, namely dilated rough Endoplasmic Reticulum (ER) cysterna, lipid droplets, mitochondria (M) and few lysosomes (L) in a filamentous matrix. Cells showed secondary lysosomes, and a few forming lysosomes with contents of low electron density were noted surrounded by tiny vesicles probably arising from Golgi Apparatus.

Sections of the upper lysosomal fractions *(micrograph b)* showed a mixture of mitochondria, ER, ribosomes, and lysosomes, both primary and secondary. Whereas in the lower lysosomal fractions *(micrographs c, d)* very few structures were noted ("solid arrow" points to intact lysosomes whereas "empty arrow" points to disrupted lysosomes). Most of these structures had characteristics of primary lysosomes: i.e. very electron dense contents surrounded by a single limiting membrane. The remainder of the structures showed lamellar appearance and membranous interiors typical of secondary lysosomes.

4. Protein Determination

The harvest of twenty confluent 75 cm^2 flasks (5.0 x 10^6 cells / flask) generally, yielded about 4 mg protein / mL homogenate sample. Problems were encountered in determining the protein concentration of the gradient fractions. When the modified Lowry method was used, there was an overestimation of the protein content due to the presence of Percoll which forms a colour with the Folin reagent. The Bio-rad assay was also tried but did not give satisfactory results due to the intense turbidity which immediately formed

when Percoll was present in the samples. Precipitating the protein with deoxycholate then redissolving it in NaOH was also found unsuccessful due to the formation of turbidity in the samples containing Percoll. Finally, the method described by Khan et al, (1981), for the determination of protein in the presence of Percoll was used. In this method, the Percoll colour interference is corrected for. Standard curves for protein and Percoll at absorption 750 nm and 420 nm were determined and were found linear i.e. r = 0.989 - 0.998 (*Fig 3.3-3.4*). The differences in the extent of absorbance at 750 and 420 nm of the color developed by protein and Percoll with the Folin reagent are calculated according to certain constants (see methodology). The protein content of the lower and higher density lysosomal fractions ranged from 0.95 to 2.0 mg / ml, and 0.10 to 0.70 mg / ml, respectively.

5. Amino acid analysis

5.1 Preparation of samples for amino acid analysis

Three main problems are encountered in the preparation of the samples for amino acid analysis: a) how to maximally concentrate the lysosomal fractions, b) how to eleminate Percoll from the fractions, and c) how to reduce contamination from amino acids outside the lysosomes.

a) In an attempt to concentrate the gradient fractions for a satisfactory recovery of amino acids, it was necessary to reduce the gradient volume. One third of the gradient volume described by Gieselmann et al (1983) was used while keeping the appropriate column height to ensure the density gradient. This modification has significantly improved the recovery of amino acids in general, and to greater degree the recovery of amino acid cystine. Methods such as lyophilysation, and concentration in a speedvac concentrator were attempted but were found unsatisfactory due to the losses of samples.

b) Separating Percoll from the lysosomal fractions was an important step in the preparation of the samples for amino acid analysis in order not to destroy the column of the HPLC system. Several techniques were tried and the fragility of lysosomes was a constant problem. Lysosomes were separated from the coated silica particles by high speed centrifugation in a swing rotor (at 100,000 x g for 2 hours) or angle head rotor (at 100,000 x g for 90 minutes). The biological particles formed a delicate membrane above a hard Percoll pellet. It was rather difficult to pipet this fragile membrane of biological material without disrupting the lysosomes and losing some of their content. This was assessed by measuring β-hexosaminidase activity in the supernate and in the pelleted biological membrane which were 92.6 and 59.1 IU/mL, respectively. A considerable amount of activity was found in the supernate denoting disruption of lysosomes and loss of latency.

Another trial was done where Percoll was separated by one step of ultrafiltration: lysosomes were disrupted by 0.1% Triton-X-100, acidified with solid sulfosalicylic acid (30 mg / mL sample), and then subjected to ultrafiltration through membrane cones.

c) Finally, to ensure that the filtrate represents the intralysosomal contents, a method which combines several washings of the the granular fractions with phosphate buffered saline (3 volumes buffer to 1 volume sample) together with ultrafiltration through membrane cones, was used. The first filtrate, designated as wash 1, represents two components: the amino acids in solution surrounding the lysosomes and those which might have been released from disruption of lysosomal membranes. Lysosomes are disrupted with Triton X-100, acidified with sulfosalicylic acid, and subjected to a last step of ultrafiltration. The last filtrate is reported as wash 3 and is expected to represent only the intralysosomal contents. The major drawback of these methods was an increased loss of contents from fragile lysosomes during the repeated washing steps. The effect of the washing procedure on amino acid content of I-cell disease lysosomal fractions is shown in *(Table 3.7)*.

In the preliminary trials of amino acid analysis, it was rather difficult to detect cystine in cystinotic cell lines. An important observation was made with regard the recovery of cystine: immediate acidification of samples with sulfosalicylic acid, before storing the samples, gave a better recovery of cystine.

5.2 Problems encountered in amino acid analysis

Typical amino acid chromatograms of normal, cystinotic and I-cell fibroblast homogenates and their lysosomal fractions are given in *(Fig 3.7 to 3.10)*. The system has a high sensitivity with an analytical detection limit as low as 1 picomole for individual amino acids. A considerable number of amino acid peaks are resolved except those of secondary amino acids (proline and hydroxyproline), and the run time for a complete amino acid profile is 136 minutes. One of the major drawbacks observed in this study when running the fibroblast homogenate and gradient fractions, was the occurrence of a shift in the retention times at the beginning of the run compared with the calibration standard *(Fig 3.5a)*. A standard mixture of amino acids was added to the reagent blanks and a similar shifting of retention times was noted *(Fig 3.5b)*. This suggests that the shift in the retention times is due to the conditions of the matrix of the samples. When the pH of the samples injected on the column was tested, it was found within expected ranges i.e.pH < 2. Along with the shift in retention times, incomplete resolution of the acidic amino acid peaks at the beginning of the run was found to occur; these amino acids co-eluted in one big peak. The results of such peaks are reported as "not available" (N/A).

A much larger peak in the position of γ-amino-butyric acid (GABA) was observed in lysosomal amino acid profiles compared with whole fibroblast homogenates *(Fig 3.7 and 3.8)*. GABA content in normal (GM00010) fibroblast homogenate and lysosomal fractions was found to be 0.11 and 46.2 nmol / mg protein, respectively. To clarify whether this was real or artifact due to contamination, different reagent blanks were run, as follows: 1) double distilled water, 2) suspension buffer (0.25 mol/L sucrose and 3 mmol/L imidazole HCl), 3) Percoll gradient made up in double distilled water, 3) phosphate buffered saline, 4) 0.1% Triton X-100, 5) sulfosalicylic acid (30 mg/mL double distilled water), and finally, 6) to test contamination that might arise from membrane cones, a blank of double distilled water was filtered through the membrane cones. A large GABA peak was observed to elute only in the Percoll blank see *(Fig 3.6)*. The reagent blanks showed background amino acids such as glutamic acid, glycine, traces of ornithine, hydroxylysine, histidine, arginine, and other contaminants such as urea and ammonia.

Two other amino acids seemed relatively enriched in the lysosomal fractions compared to whole fibroblast homogenate: glycine and hydroxylysine *(Fig 3.7 and 3.8)*. The average glycine content of normal (GM00010) fibroblast homogenate and lysosomal fractions were found to be 4.26 and 90.20 nmol / mg protein, respectively. There was 1.33 and 32.66 nmol / mg protein hydroxylysine in normal (GM00010) fibroblast homogenate and lysosomal fractions, respectively.

5.3 Amino acid content of Normal, Cystinotic , and I-cell disease fibroblast homogenates

The amino acid content of normal fibroblast homogenates is given in *(Table 3.2)*. The findings of this study show about a ten fold increase in the cystine level in the cystinotic fibroblasts (GM00090A) (7.60 nmol 1/2 cystine / mg protein) *(Table 3.3)*, compared to normal cell lines (GM00010) (0.58 nmol 1/2 cystine / mg protein). However, the cystine content of two other cystinotic cell lines (line# 717 and 1324) showed approximately a five fold increase i. e. 2.30 and 2.80 nmol 1/2 cystine / mg protein. The cystine peak in control samples is noted to be broad *(Fig 3.7a)* and unlike that of the standard amino acid mixture *(Fig 3.5a)*. This could raise some doubt as to the presence of cystine at all.

The amino acid content of I-cell disease fibroblast homogenate is given in *(Table 3.4)*. The cystine content of the I-cell disease whole fibroblast homogenate (GM02013B) was found to be lower than reported values: 1.08 nmol 1/2 cystine / mg protein, and that of line #454 was slightly higher than control values : 0.80 nmol 1/2 cystine / mg protein. The results also demonstrated that in cystinotic and I-cell disease fibroblast homogenates, the

increase in cystine level was unique; other amino acid concentrations were not significantly different from controls.

5.4 Amino acid content of high density lysosomal fractions of normal, cystinotic, and I-cell disease fibroblasts

A comparison of amino acid contents of normal (GM00010) and cystinotic (GM00090A) high density lysosomal fractions is given in *(Table 3.5)*. A significantly high cystine content was found in cystinotic (GM00090A, Line# 1324, and #717) lysosomes (9.20 - 15 nmol 1/2 cystine / mg protein) compared to the control samples, where there was no detectable cystine. Findings of this study showed approximately two fold enrichment of cystine in the cystinotic high density lysosomal fractions when compared with the crude homogenate.

Cystine content in cystinotic fibroblast (line#1324) and heavy lysosomal fractions was compared to that of three other amino acids (valine, leucine, lysine) *(Table 3.6)*. These amino acids showed stable and reproducible peaks.

The amino acid content of lysosomal fractions of I-cell disease fibroblasts are given in *(Table 3.7)*. Only traces of cystine were detected.

Figure 3.1

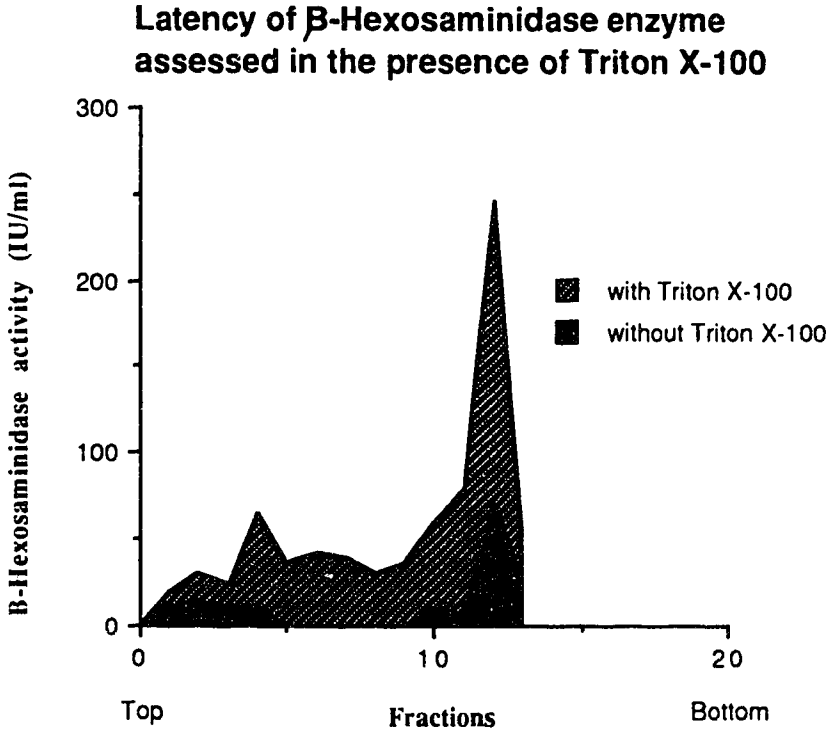
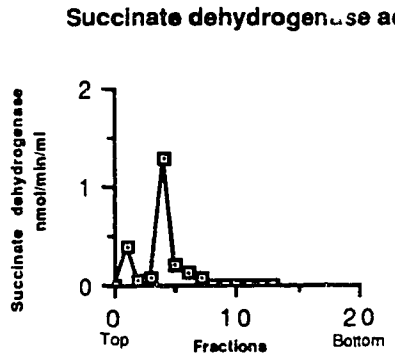
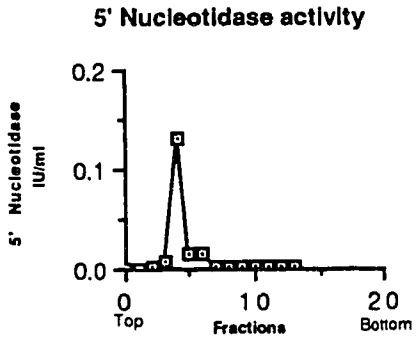
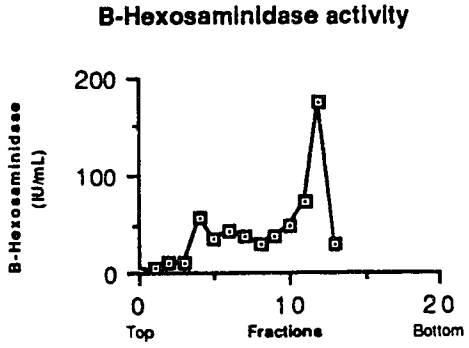


Figure 3.2 Marker enzyme assays

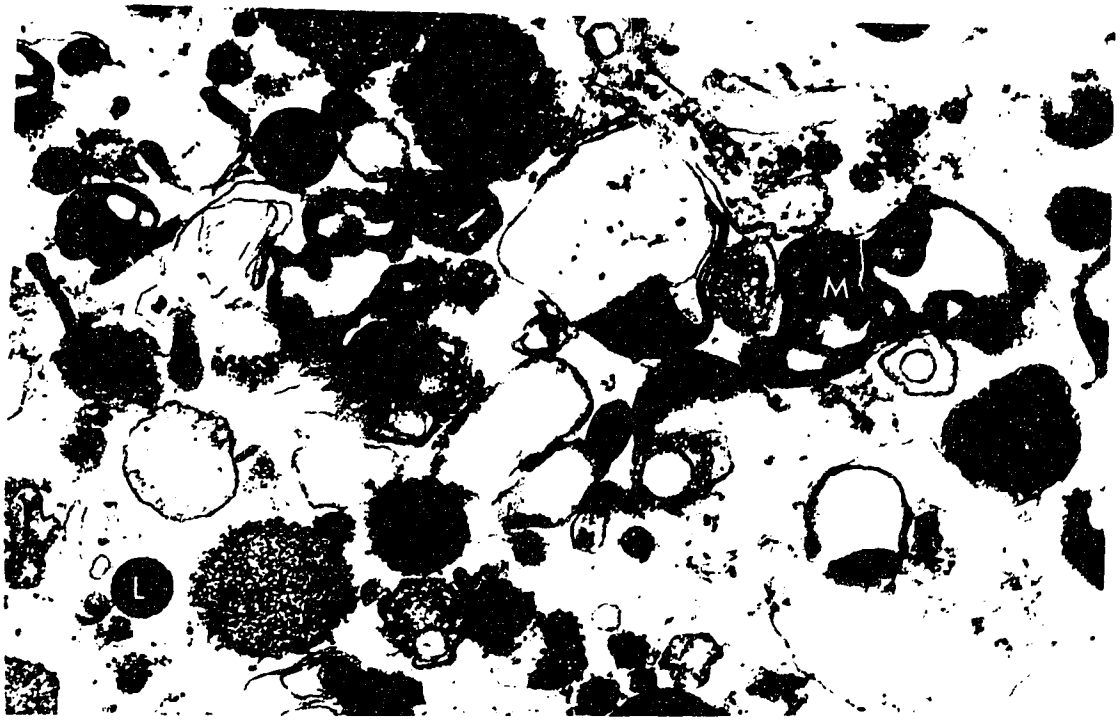




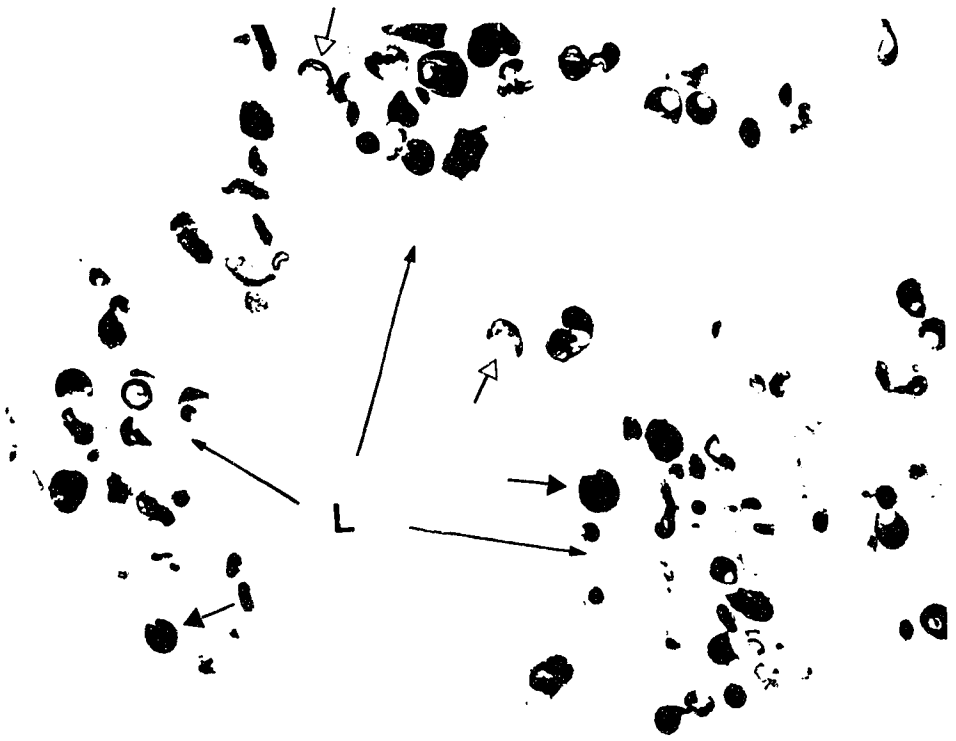


a

b



c



d

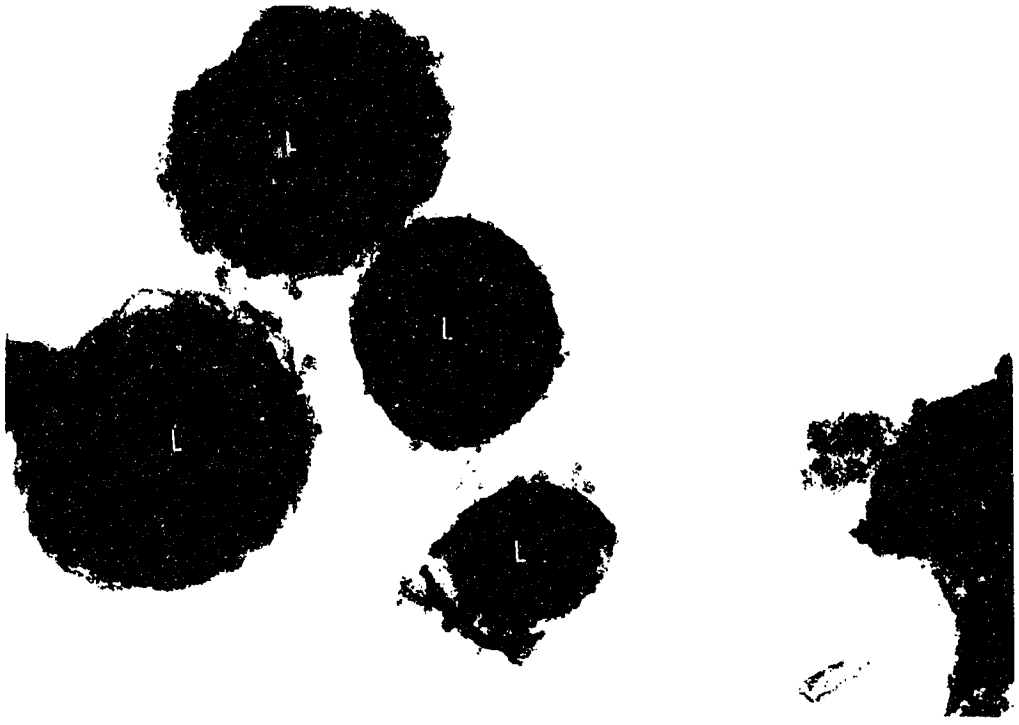
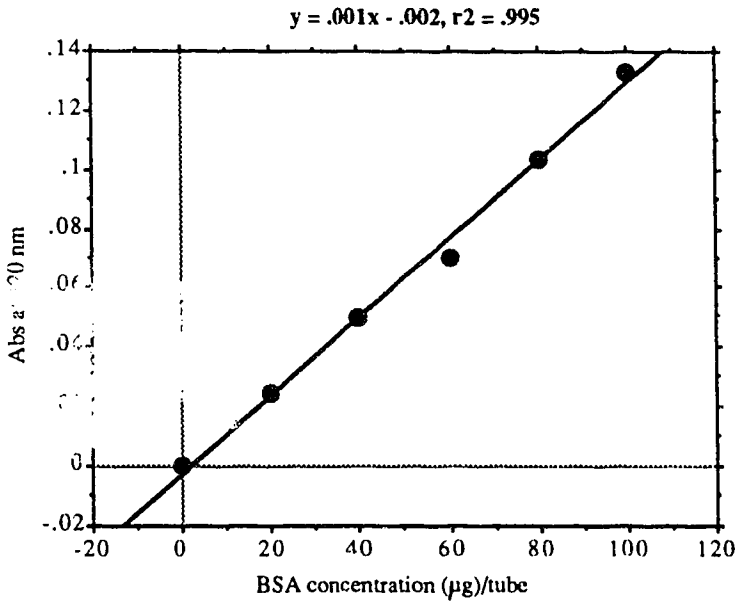


Figure 3.3 **Bovine serum albumin (BSA) standard curves of absorption at 420 nm (a) and 750 nm (b).**

(a)



(b)

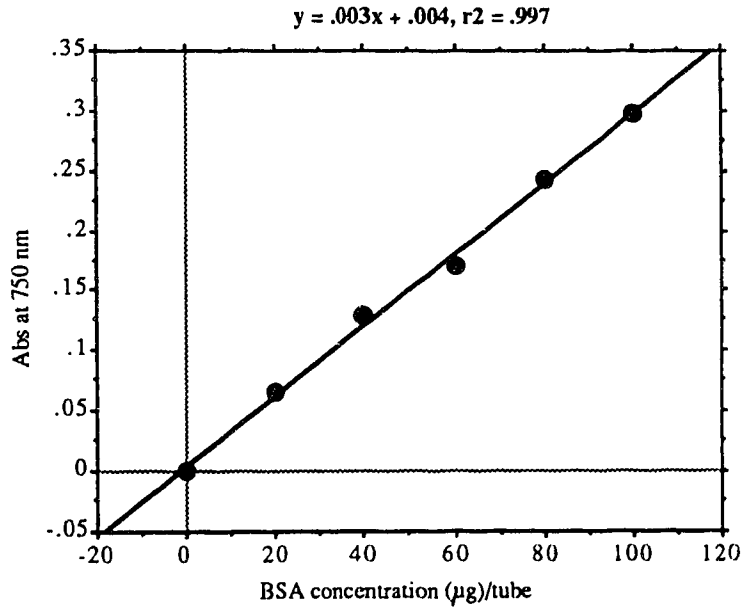
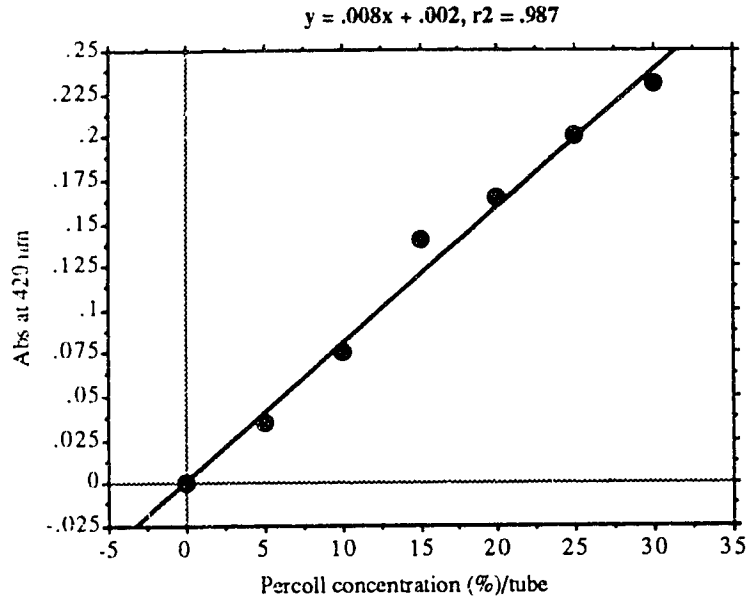


Figure 3.4 **Percoll standard curves of absorption at 420 nm (a) and 750 nm (b)**

(a)



(b)

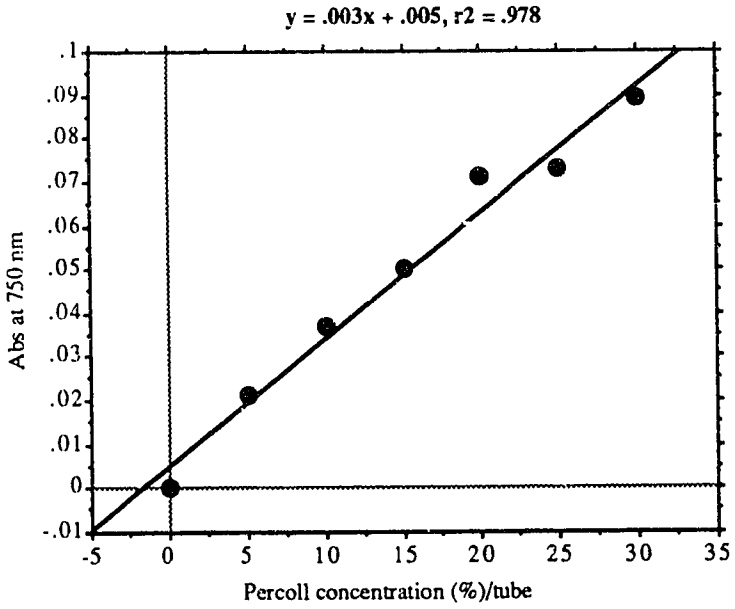


Table 3.1 **Identification of the amino acid peak number (Legend for figures 3.5 to 3.10)**

Peak #	Amino acid	Peak #	Amino acid
1	Taurine	15	Isoleucine
2	Aspartic	16	Leucine
3	Threonine	17	Internal standard
4	Serine	18	Tyrosine
5	Asparagine	19	Phenylalanine
6	Glutamic	20	β-Alanine
7	Glutamine	21	GABA
8	Glycine	22	Tryptophan
9	Alanine	23	Ammonia
10	Citrulline	24	OH-Lysine
11	AABA	25	Ornithine
12	Valine	26	Lysine
13	1/2 Cystine	27	Histidine
14	Methionine	28	Arginine

Figure 3.5 Calibration standard (a). Calibration standard with reagent blank (b)

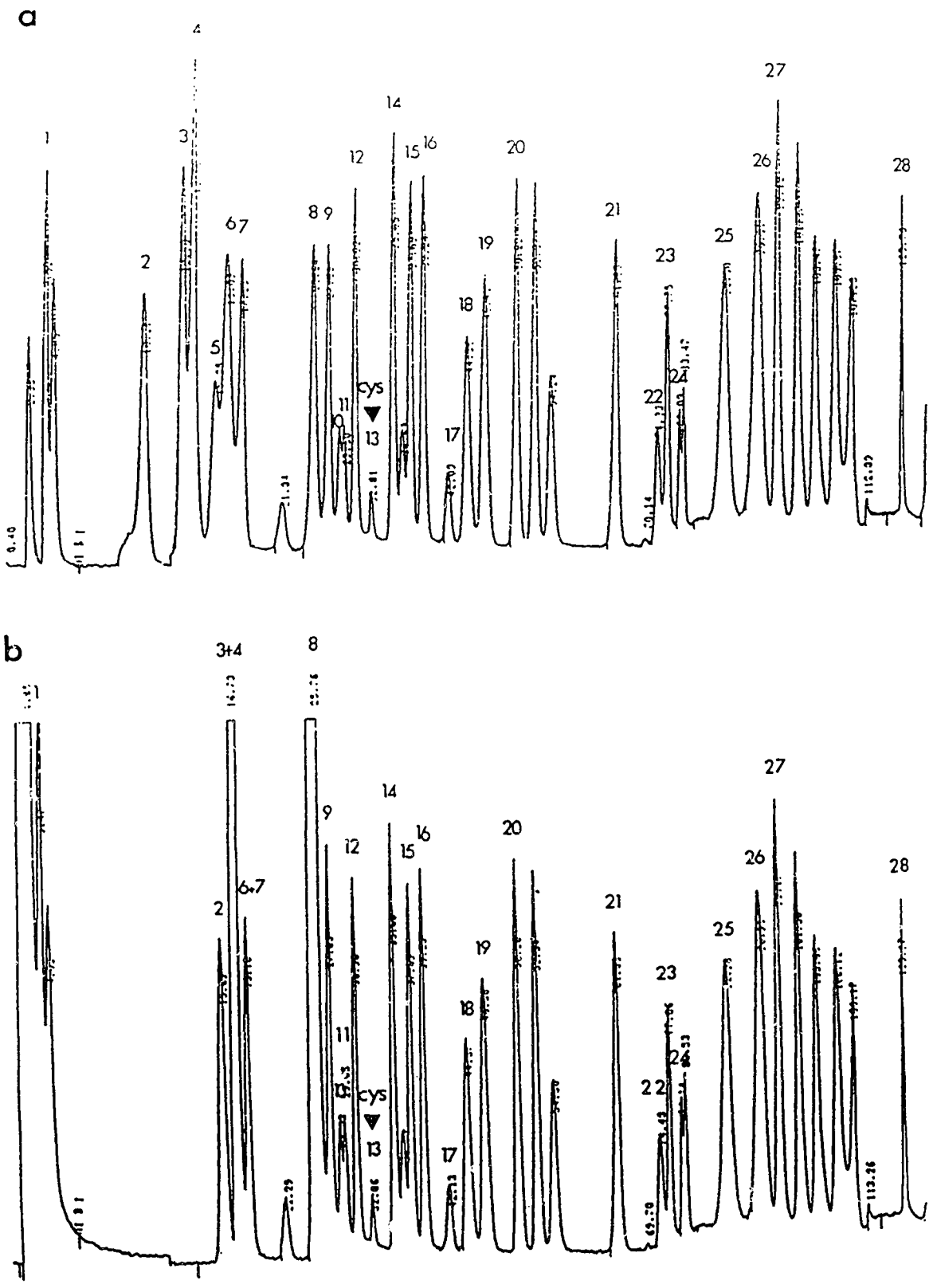


Figure 3.6 **Percoll blank**

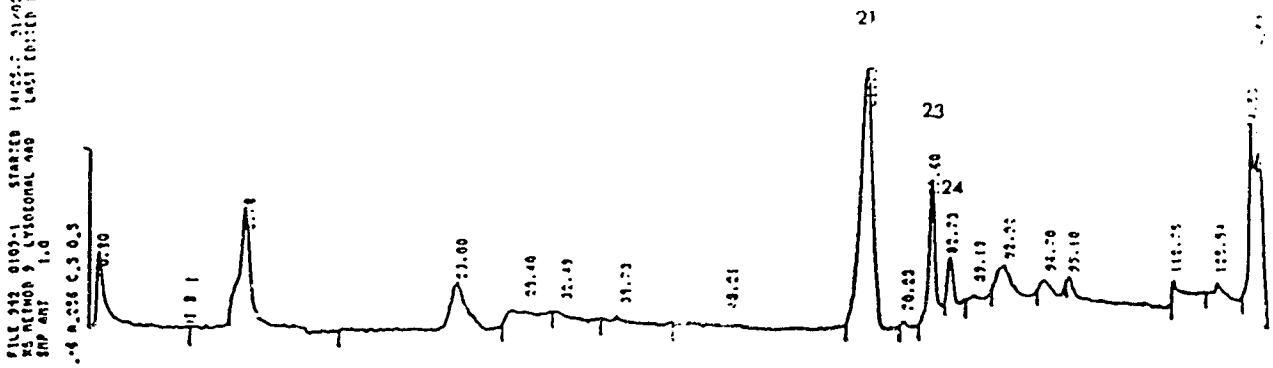


Figure 3.7 Comparison of amino acid profiles in normal (a) and cystinotic (b) fibroblast homogenates

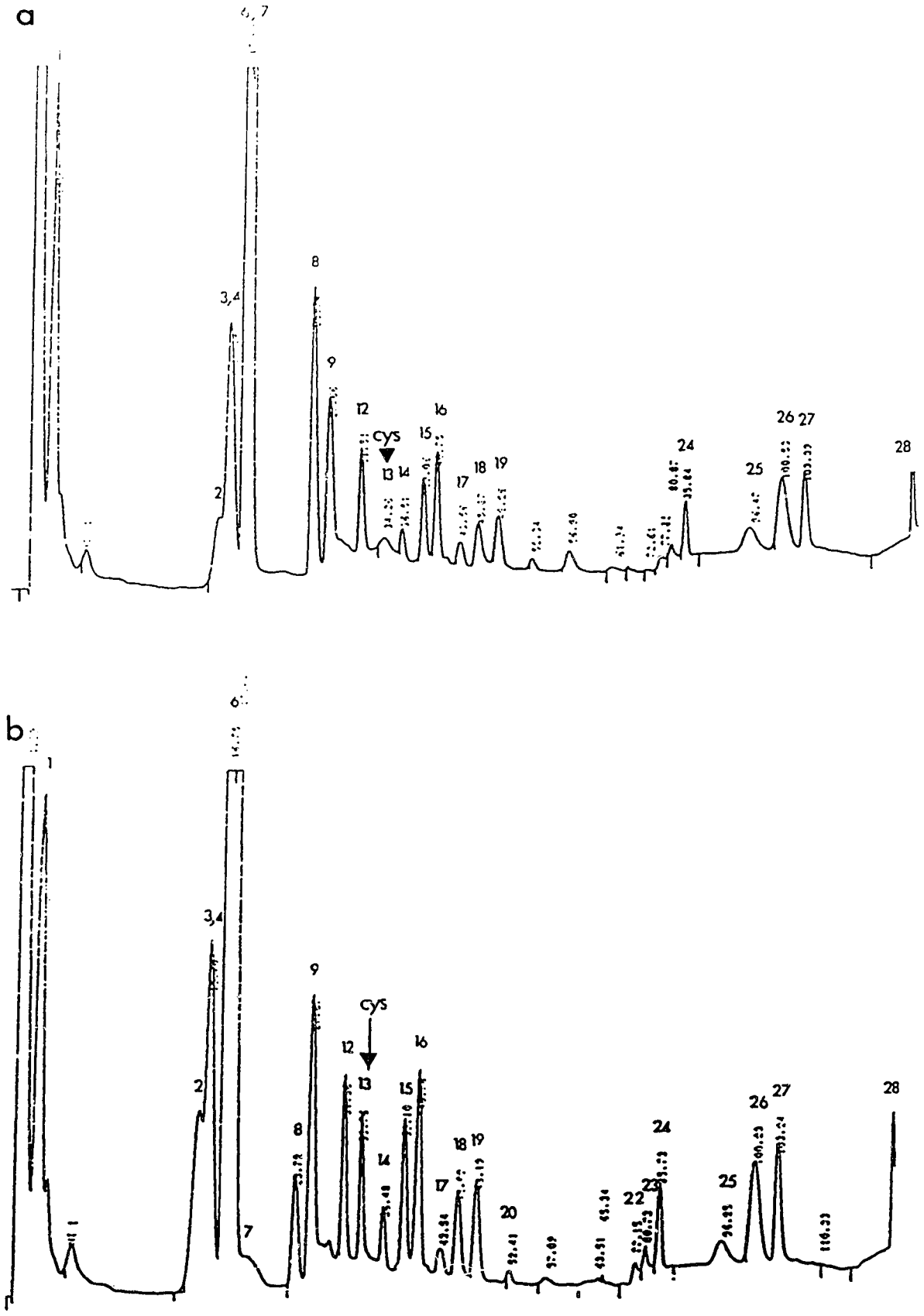


Figure 3.8 Comparison of intralysosomal amino acid profiles in normal (a) and cystinotic (b) fibroblasts

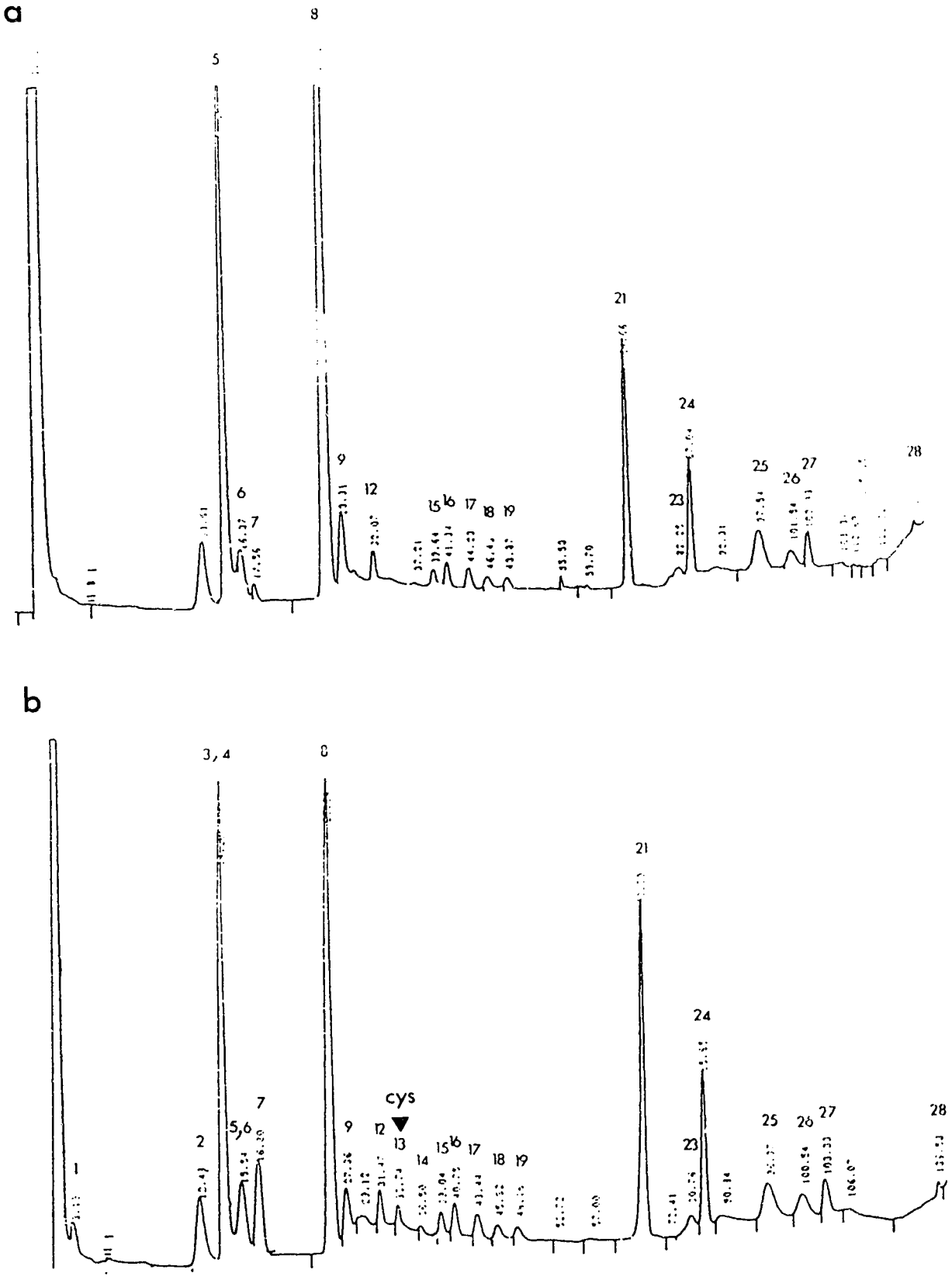


Figure 3.9 **Amino acid profile of I-cell disease fibroblast homogenate**

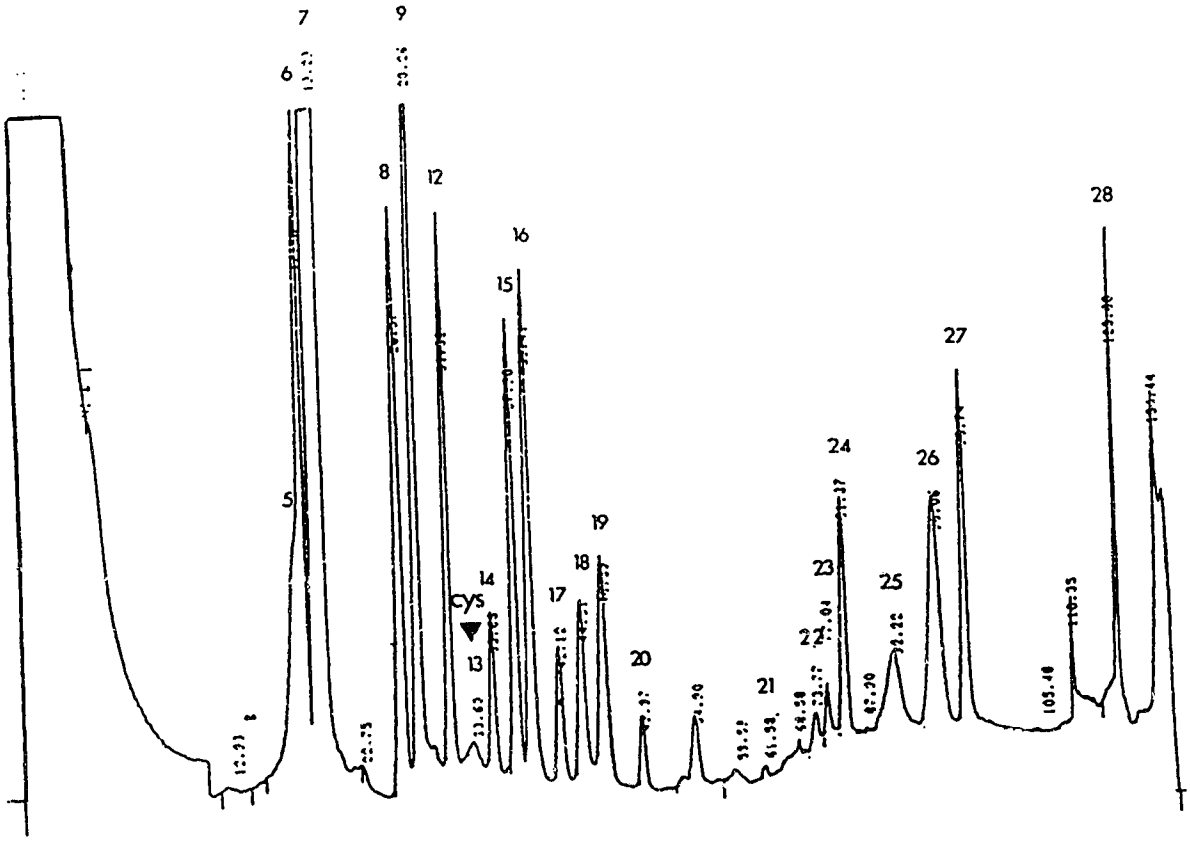


Figure 3.10 Comparison of intralysosomal amino acid profiles of non-washed (a) and washed (b) fractions of I-cell disease fibroblast

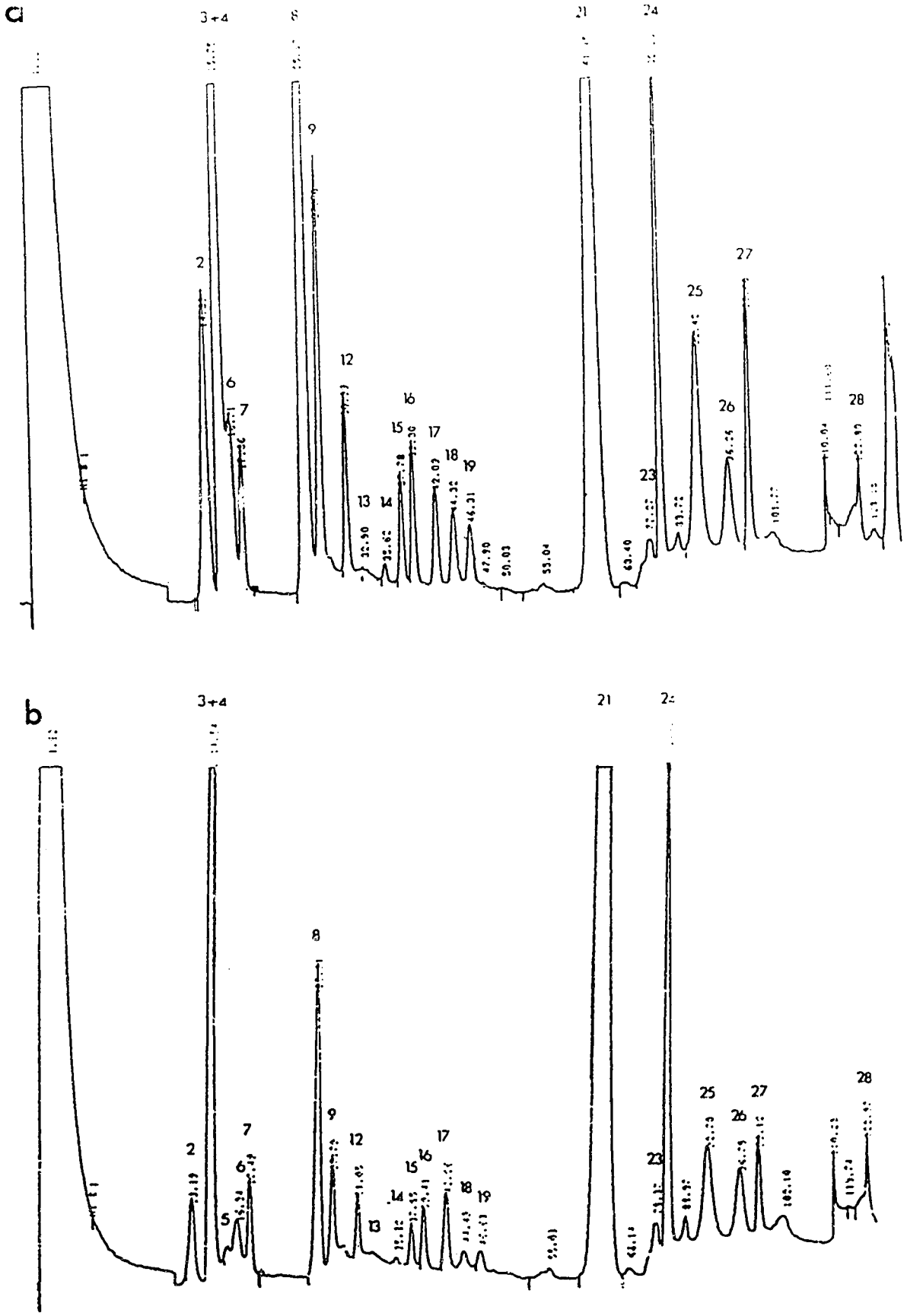


Table 3.2 **The free amino acid content (nmol/mg protein) of normal fibroblast homogenates**

Amino Acid	GM00010 N=2		Control Normal FBB, N=2		Reported[1] Normal values N=3, mean (range)
Aspartic	0.68	1.24	N/A	N/A	5.10 (2.60 - 7.00)
Threonine	N/A[3]	N/A	N/A	N/A	2.2 0(2.10 - 2.50)
Serine	N/A	N/A	N/A	N/A	2.4 0(2.10 -2.80)
Proline[2]	-	-	-	-	3.8 0(3.40 - 4.20)
Glycine	3.01	5.50	4.07	3.77	5.40 (4.50 - 7.20)
Alanine	2.20	4.20	4.42	4.95	4.00(3.3 0- 4.90)
Valine	1.10	1.50	1.55	1.55	1.50 (1.10 - 2.10)
1/2 Cystine	**0.53**	**0.63**	**0.67**	**0.73**	**NM[4]**
Methionine	0.24	0.50	0.56	0.51	2.60(0.70 - 4.60)
Isoleucine	0.88	1.40	1.37	1.38	0.80 (0.62 - 1.10)
Leucine	1.10	1.70	1.83	1.89	2.00 (1.70 - 2.50)
Tyrosine	0.66	1.10	1.08	1.20	1.40(1.20 - 1.60)
Phenylalanine	0.64	1.00	0.96	1.04	0.63 (0.42 -0.90)
Lysine	1.20	1.45	1.50	1.82	1.75 (1.50 - 2.00)
Histidine	0.73	1.00	0.96	1.10	1.80 (1.50 - 2.00)
Arginine	1.40	1.76	1.66	2.34	4.00 (3.50 - 4.40)

[1] Tietze and Butler, 1979

[2] Proline and hydroxyproline are not detected by this system

[3] N/A results not available due to co-elution

[4] NM not measurable at the instrumental sensitivity

Table 3.3 **The free amino acid content (nmol/mg protein) of Cystinotic fibroblast homogenates**

Amino acid	Cystinotic FBB GM00090A[1]		Cystinotic FBB # 1324	Cystinotic FBB # 717
Glycine	1.50	1.15	2.40	2.50
Alanine	3.20	3.60	2.74	2.68
Valine	1.44	1.84	0.96	1.17
1/2 Cystine	**5.40**	**7.60**	**2.80**	**2.30**
Methionine	0.34	0.43	0.30	0.35
Isoleucine	1.10	1.53	0.87	1.18
Leucine	1.40	1.94	1.33	1.70
Tyrosine	0.92	1.22	0.70	0.76
Phenylalanine	0.93	1.16	0.64	0.76
Lysine	1.34	1.60	1.10	1.40
Histidine	0.97	1.07	0.81	1.08
Arginine	1.80	2.02	1.10	1.51

[1] The data represent two sets of measurements for cystinotic cell line GM00090A

Table 3.4 **The free amino acid content[1] (nmol/mg protein) of I-cell disease fibroblast homogenates**

Amino acid	I-cell FBB (#454)	I-cell FBB (GM02013B)	I-cell FBB (GM013211F)
Glycine	1.94	1.49	5.15
Alanine	3.80	1.90	7.67
Valine	1.68	1.93	1.88
1/2 Cystine	**0.80**	**1.01**	**N/D**
Methionine	0.43	0.60	0.59
Isoleucine	1.46	1.68	1.56
Leucine	1.65	2.16	1.88
Tyrosine	1.00	1.18	1.30
Phenylalanine	1.00	1.13	1.15
Lysine	1.39	2.00	1.93
Histidine	0.97	0.90	1.31
Arginine	1.54	1.72	1.99

[1] The values represent one set of measurement for each cell line

Table 3.5 **Comparison of free amino acid content (nmol/mg protein) of high density lysosomal fractions of normal (GM00010) and cystinotic (GM00090A) fibroblasts**

Amino acid	Normal GM00010 N= 3 mean ± SD	Cystinotic GM00090A N= 3 mean ± SD
Glycine	90.20±37.3	48.38±25.2
Alanine	9.40±3.6	8.82±5.82
Valine	2.74±1.95	3.28±0.11
1/2 Cystine	**N/D**[1]	**9.30±0.7**
Methionine	0.40±0.35	0.45±0.02
Isoleucine	1.81±1.14	2.00±0.57
Leucine	2.30±1.35	2.73±0.33
Tyrosine	1.90±1.87	2.11±0.42
Phenylalanine	1.40±1.30	1.31±0.30
Lysine	3.50±1.53	3.00±0.71
Histidine	3.13±1.80	2.65±0.64
Arginine	1.60±0.50	1.46±0.91

[1] N/D non detectable

Table 3.6 **Ratio of cystine content in relation to valine, leucine, and lysine content in cystinotic fibroblast (line# 1324) homogenate and high density lysosomal fractions.**

Amino acid ratio	Cystinotic homogenate	Cystinotic lysosome (no wash)	Cystinotic lysosome (wash 1)	Cystinotic lysosome (wash 3)
cystine / valine	2.92	1.12	1.92	4.50
cystine / leucine	2.10	1.30	2.47	3.36
cystine / lysine	2.54	1.23	1.77	1.91

Table 3.7 **The effect of the washing procedure on the free amino acid content (nmol/mL sample) in high density lysosomal fractions of I-cell disease fibroblasts (GM02013B)**

Amino acid	Lysosome (no wash)	Lysosome (wash 1)	Lysosome (wash 3)
Glycine	12.9	4.5	13.1
Alanine	1.53	0.4	0.9
Valine	0.92	0.22	0.59
1/2 Cystine	**N/D**	**N/D**	**trace**
Methionine	0.18	0.06	0.07
Isoleucine	0.58	0.16	0.32
Leucine	0.78	0.23	0.44
Tyrosine	0.60	0.14	0.29
Phenylalanine	0.49	0.13	0.24
Lysine	1.08	0.18	0.67
Histidine	0.81	0.31	0.59
Arginine	0.93	0.17	0.58

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

UNIVERSITY OF ALBERTA

THE RELATIONSHIP BETWEEN BIOLOGICAL/ENVIRONMENTAL FACTORS AND SENSORIMOTOR AND LANGUAGE DEVELOPMENT OF PRETERM INFANTS AT PRESCHOOL AGE

BY

CARLA SOARES ATTANASIO

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF OCCUPATIONAL THERAPY

EDMONTON, ALBERTA

SPRING, 1991

ISBN 0-315-66607-X

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: CARLA SOARES ATTANASIO

TITLE OF THESIS: THE RELATIONSHIP BETWEEN BIOLOGICAL/ENVIRONMENTAL FACTORS AND SENSORIMOTOR AND LANGUAGE DEVELOPMENT OF PRETERM INFANTS AT PRESCHOOL AGE.

DEGREE: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: SPRING, 1991

Carla Soares Attanasio

203, 10730-83 AVENUE

Edmonton, Alberta

T6E 2E4

Date: April 19th, 1991

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

THE UNDERSIGNED CERTIFY THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS

ENTITLED **THE RELATIONSHIP BETWEEN BIOLOGICAL/ENVIRONMENTAL FACTORS AND SENSORIMOTOR AND LANGUAGE DEVELOPMENT OF PRETERM INFANTS AT PRESCHOOL AGE**

SUBMITTED BY **CARLA SOARES ATTANASIO**

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **MASTER OF SCIENCE**

Joyce Magill-Evans

Joyce Magill-Evans, Ph. D.

Christy Nelson

Christy Nelson, M. Sc.

Sharon Warren

Sharon Warren, Ph. D.

Date: March 26, 1991

ABSTRACT

This study examined the relationship of gestational age and maternal education, and the sensory, motor, and language development (as measured by the Miller Assessment for Preschoolers) of premature infants at 4 years of age.

The sample consisted of 24 preterm children who had been diagnosed as neurologically normal at 18 months of age. Maternal education and gestational age information was obtained for each child. The Miller Assessment for Preschoolers (MAP) was administered when the child was 4 years of age (M age = 4.6). The children were scored on five MAP performance indices (Foundations, Coordination, Verbal, Non-Verbal and Complex Task indices) and the total score was determined.

Maternal education and gestational age did not explain a significant amount of the variation in any performance index and the total score when using multiple regression analysis. Gestational age alone as a continuous variable was significantly correlated with the Non-Verbal index. When two gestational age groups (<32 weeks; 32-36 weeks) were compared on the performance indices and total score, the observed frequencies on the Foundation index differed significantly from the expected frequencies. Maternal education was not significantly related to any of the dependent measures. The significance of these findings for occupational therapists is discussed.

ACKNOWLEDGEMENTS

I would like to thank all individuals who contributed to the accomplishment of this thesis. I am really grateful for their cooperation and support.

My advisor, Joyce Magill-Evans, for sharing her knowledge with me and for her thoughtful comments during the thesis process;

My ex-advisor, Shelly Lane, for her patience, dedication and moral support which were very important on the beginning of my studies;

My committe member, Sharon Warren, for her precious contribution in the methodology and statistical analysis;

My committee member, Christy Nelson, for her expert opinion which enhanced my work;

Annette Kudge, for her prompt help with subject recruitment and provision of supplementary data.

My family in Brazil, for their love, financial and moral support, and constant stimulation in completing this project.

Last but not least, I acknowledge CAPES, the Brazilian Federal Agency, for its financial support through what this thesis could be accomplished.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

CHAPTER I

INTRODUCTION

Within the last twenty years, significant advances in perinatal care have resulted in an improved survival rate for preterm infants less than 37 weeks gestational age (Scott, 1987). A major concern persists, however, regarding the long term prospects of these children. Follow-up studies investigating the outcome of preterm children show that 25 to 64% of these children present neurodevelopmental deficits or learning problems at 3 to 10 years of age (Calame et al., 1986; Hunt, Tooley, & Harvin, 1982; Nickel, Bennett, & Lamson, 1982; Vohr, Coll, & Oh, 1988). In particular, studies that examined the relationship of neurological status of the preterm infant in the first two years of life and their later developmental outcome revealed that, although the majority of these infants were classified as neurologically abnormal and suspicious, a significant percentage (14 - 40%) of the infants who were delayed had been classified as neurologically normal (Stewart et al., 1989; Vohr et al., 1988). These findings raise the question of why some apparently neurologically normal preterm infants develop well while others do not. Authors who have investigated the outcome of the normal preterm babies (Drillien, 1972; Stewart et al., 1989; Vohr et al., 1988) reported that factors such as social environment and younger gestational age may play a role in the development of these babies, a position supported in other reports (Largo et al., 1989; Sameroff, 1986; Siegel, 1982; Smith, Somner, & Tetzchner, 1982; Zubrick, Macartney, & Stanley, 1988). In fact, younger gestational age associated with low birth weight (LBW) has been shown to have a significant correlation with developmental outcome of preterm infants (Largo et al., 1989; Zubrick et al., 1988).

The objective of this study was to investigate how preterm infants who were diagnosed neurologically normal at 18 months were performing on a measure of sensory, motor, language and behavioral development at four years of age. As well, this project also investigated the possible influences of gestational age and maternal education on the performance areas mentioned above. This age for assessment was chosen because, according to Drillien (1972), three years old is the "minimum age for making a reasonably confident assessment about handicaps other than severe physical, mental and neurological defects" (p.564). Thus, reliable assessment of developmental status should be possible by four years of age. As there is a high percentage of normal

preterm infants showing developmental problems at later ages, the study of these children has become important. The detection of possible problems before school age could prevent the adverse consequences associated with late identification of deficits. According to Miller (1982), the consequences of late identification include emotional problems associated with school failure and reduced effectiveness of therapeutic treatment at later ages. Moreover, preschool motor, perceptual-motor, and cognitive abilities are reported to predict school performance (Sell, Gain, Gluckman, & Williams, 1985; Solan & Mozlin, 1986). Thus, early identification of deficits in these areas could be of great benefit for these children and have implications for the practice of occupational therapy.

Objectives

A. General Objective:

1. Evaluate the performance of preterm infants (diagnosed neurologically normal at 18 months) at 4 years of age on the MAP.

B. Specific Objectives:

1. Evaluate the relationship of gestational age to the five indices of performance and the total score of the MAP at 4 years of age;

2. Evaluate the relationship of maternal education to the five indices of performance and the total score of the MAP at 4 years of age.

Definitions of Terms:

1. Neurologically normal: For the purpose of this study, "neurologically normal" was used to describe preterm infants who have no known neurological deficits at 18 months of age. It is recognized that there may be neurological deficits but our instruments may not be sensitive enough to detect them.

2. Low birthweight: Infants whose weight at birth was below or equal to 2,500 grams.

CHAPTER II

LITERATURE REVIEW

Early Development of Preterm Infants

The premature infant (< 37 weeks gestational age) provides an unique opportunity to investigate neurophysiological and behavioral development in its early stages. In addition, the study of these functions also provides better understanding of the "normal" development of the preterm infant, who accounts for 7 to 10% of all live births in the United States (Gorski, 1984).

Several authors have investigated developmental characteristics of the preterm baby (Dargassies, 1966; Ferrari, Grosoli, Fontana & Cavazzuti, 1983; Howard, Parmelee, Kopp, & Littman, 1976; Palmer, Dubowitz, Verghote, & Dubowitz, 1982; Piper, Kunos, Willis, & Mazer, 1985). These studies have shown that premature infants have significant differences in neurological and behavioral development at 40 weeks gestational age (GA) when compared to full-term infants. However, studies of subsequent motor, neurobehavioral, and neurological development of preterm infants during the first two years of life provide inconclusive results. Palisano (1986) found no significant differences between preterm and fullterm infants in gross and fine motor function at 12, 15 and 18 months corrected age, contrary to Forslund and Bjerre (1985) who found significant differences in both functions at 9 and 18 months of age. Ungerer and Sigman (1983) reported that at 13.5 months no difference in fine motor function was found between preterm and fullterm groups; however, the preterm infants were performing at lower levels in the area of gross motor function.

The differences between the results of these studies do not seem to be explained by birth weight or gestational age. Palisano (1986), who reported no significant differences between preterm and fullterm infants, had a preterm sample ranging from 29 to 32 weeks GA and weighing < 2500 grams. On the other hand, Forslund and Bjerre (1985) who found significant differences, had a preterm sample with a wider range of age (27 to 34 weeks GA) and heavier infants (< 3200 grams). Interestingly, the results may be associated with the medical and neurological status of these infants. In Palisano's study, the sample included only healthy preterm infants, while in the other studies (Forslund & Bjerre, 1985; Ungerer & Sigman, 1983), the sample contained both healthy and sick preterm infants.

Some authors (Forslund & Bjerre, 1985; Gorga, Stern & Ross, 1985; Gorga,

Stern, Ross, & Nagler, 1988) have measured primitive reflexes in preterm babies as a means of assessing the maturation of the central nervous system. Gorga et al. (1985) found that, at 3 and 6 months corrected age, the fullterm group (n = 15) was more mature than the two groups of healthy (n = 38) and sick (n = 97) preterm infants in the area of reflex responses and inhibition. However, in another report by Gorga et al. (1988) using a smaller sample, there were no significant differences between healthy preterm (n = 12) and fullterm infants (n = 12) during the first year of life. Forslund and Bjerre (1985) found significant differences between fullterm and sick preterm groups in balance and righting reactions at 9 months. However, no significant differences were reported in dorsiflexion footsole, palmar grasp, moro, and plantar grasp reflex. The contradictory findings between Gorga et al. studies seem to be attributable to differences in some inclusion criteria in the second study (Gorga et al.,1988) and a smaller sample size with more equal groups. Another possible explanation for the differences among the three studies mentioned above (Gorga et al., 1985, 1988; Forslund & Bjerre, 1985) might be that Forslund and Bjerrer (1985) used different measurement instruments from the Gorga et al. studies.

Divergent findings are also reported for preterm infants in the neuromotor behavioral area. Gorga and associates (1985) found similar patterns of development between fullterm and healthy preterm infants on the following examinations during the first year: head control, development of upper extremities, and sitting. Differences in the two groups were reported in muscle tone and standing and walking measures. Although Gorga and colleagues (1985) report some similar findings in a later study (Gorga et al., 1988), the results relating to muscle tone and sitting measures during the first year were different. Forslund & Bjerre (1985) also examined aspects of neuromotor behavior. The authors found no significant differences between fullterm and sick preterm groups in muscle tone and walking measures, contradicting the Gorga et al. study (1985).

Results of these studies are inconclusive and do not permit us to trace a sequence of development for these premature babies. Piper et al. (1985) clearly expressed the present state of preterm infant research:

> Until more information on the ontogeny of neurological development [of the preterm babies] becomes available and the long-term outcome of these infants is known, the predictive value of these neonatal differences may be debated. While the neurological functioning of healthy preterm infants clearly is different from that of normal fullterm infants, it is difficult to say whether this difference is

indicative of delay or merely represents a variation in the pattern of neurological development (Piper et al., 1985, p. 603).

Later Development of Preterm Infants

Several studies have investigated the long term outcome of preterm children (Calame et al., 1986; Largo et al., 1989, 1990; O'Mara & Johnston, 1989; Rubin, Rosenblatt, & Balow, 1973; Siegel et al., 1982; Ungerer & Sigman, 1983; Zubrick et al., 1988) and found that many of the children have neurodevelopmental or learning problems. In general, preterm infants have significantly lower scores than fullterms on a wide range of measures. However, the findings among the studies are not consistent.

Vohr et al. (1988) found that preterm infants at 2 years chronological age were significantly delayed in language ability compared to fullterms. This finding is supported by Calame et al. (1986) and Michelsson, Lindahl, Parre, and Helenius (1984) who conducted studies with 8 and 9 year-old children, respectively. However, Siegel et al. (1982) did not find any significant difference in verbal performance on the Reynell Developmental Language Scale between the preterm and fullterm group at 2 years corrected age. It is possible that differences in the results of these studies could be related to the inconsistent use of age correction for prematurity. As this is an important procedure when comparing young preterm and fullterm infants (Ungerer & Sigman, 1983), the Siegel and associates study (1982) may provide more reliable results than the Vohr et al. study. It may be possible that differences in language development only appear at later ages (see Calame et al., 1986; Michelsson et al., 1984), when more complex linguistic structures are required.

Regarding visual-motor development, Calame et al. (1986) reported that preterm infants had significantly lower scores than fullterm infants. Although this result is supported by other studies (Klein, Hack, & Breslau, 1989; Ungerer & Sigman, 1983), McDonald, Sigman, and Ungerer (1989) did not find any significant difference between the two groups in visual-motor measures at five years of age. As this study is a follow up of Ungerer and Sigman's (1983), it is possible that divergent findings on these reports are attributable to the fact that different measurement instruments were used in the initial and follow-up study.

More consistent results were found in the motor area. Several authors (Calame et al., 1986; Klein et al., 1989; Michelsson et al., 1984, & Siegel, 1982) reported that preterm infants score significantly lower than fullterm infants in fine and gross motor

abilities at 5 to 9 years.

Regarding behavioral development, Walker (1989) and McDonald et al. (1989) found that preterm infants presented more behavioral problems, such as temper tantrums and dependence, than did fullterm children. This finding was not supported by O'Mara and Johnston (1989). However, the latter authors used only the Behavioral Screening Questionnaire for measuring behavior which they felt was not sensitive enough to evaluate characteristics such as irritability, attention span, and restlessness.

Thus, although preterm infants are reported as more developmentally delayed than fullterm infants, agreement concerning the abilities affected has not been reached except in the motor area. The contradictory findings in these studies seem to be attributable to different methodological procedures, such as different measurement instruments, different age at the time of evaluation, and presence of different medical abnormalities. According to Scott (1987), adverse medical abnormalities have been correlated with different outcomes. Thus, this could be one of the major confounding variables when evaluating the child's outcome.

A few studies have investigated the outcome of neurologically normal preterm infants in order to reduce possible confounding variables (Drillien, 1972; Stewart et al., 1989; Vohr et al., 1988). However, only Vohr et al. (1988) provided a fullterm control group. In their study, they documented that preterm infants were significantly delayed in comparison with the fullterm group on language measures. Although Drillien (1972) and Stewart et al. (1989) did not include any control children born at term, they stated that 30% and 14.2%, respectively, of their normal preterm children presented deficits at 3 and 4 years of age, respectively, in the areas of neurological and cognitive development. Thus, some neurologically normal preterm infants are lagging behind normal development. However, no consensus has yet been found concerning the abilities affected.

Gestational Age

Preterm infants are frequently described as at risk due to the fact that they are born with immature systems. This condition is thought to be a threat to children's normal development. As the brain and body systems mature during the last two months of gestation, a change in environment from the uterus to special care nurseries may jeopardize motor, cognitive, emotional, and social functions (Short-DeGraff, 1988).

In general, preterm infants have significantly lower scores on language and

perceptuo-motor measures than fullterm children (Calame et al., 1986, Siegel, 1982, Ungerer & Sigman, 1983; Zubrick et al., 1988). Moreover, when considered as two separate groups, preterm infants who are less than 32 weeks GA at birth have less favorable scores in language, neurological, intellectual (Largo et al., 1989), school performance, attention, and social behavior (Grigoroiu-Serbanescu, 1984) than preterm infants born between 32 and 36 weeks GA. The question concerning these findings is if prematurity by itself is significantly correlated with outcome. In general, investigators have shown that gestational age in association with other factors, particularly LBW and socio-economic status (SES), are significantly correlated with academic performance, cognitive, language, and motor development (Kitchen et al., 1980; Siegel et al., 1982; Zubrick et al., 1988). When considering its effect independently of other variables, most studies report that prematurity alone appears to have no relationship with the abilities mentioned above (Cohen, Parmelee, Sigman, & Beckwith, 1988; Drillien et al., 1980; Rubin et al., 1973; Siegel, 1982). However, Largo et al. (1989) and Grigoroiu-Serbanescu (1984) who investigated the outcome of younger and older preterm infants, found that gestational age was predictive of outcome at 5 to 7 years. Thus, it seems that the independent effect of prematurity on later outcome is still controversial and may be more predictive when examined in groups of younger and older preterm infants at later stages of development.

When looking at the effects of gestational age on the development of preterm infants diagnosed neurologically normal, Vohr et al. (1988) found that gestational age made a significant independent contribution to language development at 2 years. However, Stewart et al. (1989) found that neurodevelopmental status at 1 year was a better predictor of the child's sensory and cognitive functions at 4 years than was gestational age. Differences in these studies seem to be attributable to methodological procedures such as different age at the time of the evaluation and use of different measurement instruments. As only a few studies have been done, the impact of gestational age on the development of normal preterm infants can not be adequately predicted at this time.

Most of the information is still inconclusive. Further investigation is needed in this area in order to determine the effect of prematurity on the later outcome of preterm children.

Maternal Education

In an attempt to examine the relationship between social environment and child development, maternal education has been frequently investigated in association with SES (Rickards et al., 1988; Sameroff, 1986; Siegel, 1982). According to Hunt (1986), "the environmental component of the parent education is conceptualized as an array of parenting styles that determines the physical and social environment of the infant and child" (p.45). As well, McGillicuddy-DeLisi (1982) reported that parents with higher education-income differed from parents with lower education-income in the manifestation of some characteristics such as positive feedback and awareness of the child's readiness for knowledge. Parush and Clark (1988) in their validation study of a sensory developmental questionnaire for mothers of newborns also found that "the more formal education a mother had had, the more knowledgeable she was concerning the sensory capacity of the newborn, and the more she perceived herself as influential over her child's development" (p.14).

Several studies have investigated the effects of mother's educational level on long-term outcome of preterm infants. Scott (1987), in a review of follow-up studies examining the effects of prematurity on later childhood, found that maternal education was a powerful predictor of infant IQ level. The same result, in association with SES, was reported by Sameroff (1986), Rickards et al. (1988) and Klein et al. (1985). Robertson et al. (1990), investigating the school performance of preterm LBW infants, found that maternal education was the primary predictor of reading, spelling, and arithmetic achievement. Additional studies (Blackman, Lindgren, Hein, & Harper, 1987; Siegel, 1982) investigating outcome of preterm infants at preschool age reported a significant correlation between low maternal education ($\underline{M}$ = 13.3; $\underline{SD}$ = 1.9) (Blackman et al., 1987) and perceptuo-motor and cognitive problems. Thus, it appears from these investigations that a low level of maternal education is associated mainly with cognitive deficits.

Although these studies are in agreement concerning the relationship of maternal education and outcome, they do not report the children's neurological status during the first year of life. Follow-up of neurologically normal preterm babies would provide more information about the effects of prematurity and environmental influences on the development of these children, eliminating some possible confounding variables related to medical abnormalities.

In summary, several studies support a strong relationship between maternal education and cognitive ability. However, as this delay in cognitive function may be

related to other unhealthy conditions during the first year, additional research with normal preterm babies would provide a better evaluation of the impact of this variable (maternal education) on the child's development. Separating maternal education and the influences of SES is difficult. However, an evaluation of SES goes beyond the scope of this project and should be investigated in future studies.

Conclusion

Based on the literature review, a series of conclusions can be drawn about preterm development. The developmental sequence of preterm infants is not clearly defined. Although preterm infants are different from fullterm infants in their developmental process, it is not clear if this difference represents a developmental problem or if it is a normal pattern of development for these immature infants.

Studies investigating the long term outcome of premature children report that differences between some preterm and fullterm infants are present at later years. However, there is no agreement concerning the specific abilities jeopardized. This may be due to differing methodological procedures between the studies such as the inclusion or exclusion of infants who present with medical abnormalities (e.g., low Apgar scores, respiratory problems, infections). Studies with normal preterm infants would eliminate one major confounding variable, neurologic abnormality, and provide a better understanding of the infants' development.

It is also possible that exposure to different risk factors may affect different developmental functions. Some authors mention that risk factors such as complications associated with prematurity itself and maternal education may play a role in the outcome of these infants. Although maternal education is significantly correlated with the infant's cognitive development, the significant association of prematurity with later outcome has yet to be confirmed. Some studies have reported the significant effect of gestational age in association with birth weight on later development. Thus, it might be possible that the variables of maternal education and gestational age can predict later outcome.

In conclusion, there is still much work to be done in the area of preterm infant development. Although it is not confirmed that preterm birth is associated with later problems, preterm infants without neurological problems are still showing deficits at later ages. The studies mentioned here presented possible factors, or group of factors, which can influence the outcome of these infants. Investigation of these factors may help us to understand the dynamic process of these infants' development.

Hypotheses

Based on the review of the literature, it is expected that:

1. Some preterm children will score below the normal range on the total score of the MAP at 4 years of age;

2. Gestational age will be predictive of the Verbal index of the MAP;

3. Maternal education at birth will be predictive of the Complex Task and Non-Verbal indices of the MAP;

4. Gestational age and maternal education will be predictive of the Complex Task, Coordination, Verbal and Non-Verbal indices of the MAP.

CHAPTER III

METHOD AND PROCEDURES

Introduction

The current project follows preterm children involved in an project entitled "Impact of preterm birth on the neuromotor development of the premature infants" (Piper, Byrne, Darrah, & Watt, 1989), conducted from 1985 to 1987. The project studied 75 preterm children from birth to 18 months of age.

Study Design

This was a historical cohort study. The sample was "identified from the past records and followed forward from that time up to the present" (Fletcher, Fletcher, & Wagner, 1988, p.99). This design provides information about the possible association between exposure to a risk factor and developmental outcome (Fletcher et al., 1988; Lilienfeld & Lilienfeld, 1980).

The variables considered in this study are:

-independent:

Gestational age - The specific gestational age as well as two groups of gestational age (<32 weeks GA; 32-36 weeks GA) will be considered for multiple regression analysis and chi-square analysis, respectively.

Maternal education - Number of years of education at present and two groups of maternal education (≤12 years of education; >12 years of education) will be used for multiple regression and chi-square analysis, respectively.

-dependent:

The total score and the five performance indices of the MAP: Foundations, Coordination, Verbal, Non-Verbal and Complex Task indices, which are described later in the measurement instrument section. The category scores are reported in percentiles.

Sample

The subjects of this study had been classified as premature infants (less than 37 weeks GA) and were cared for in the neonatal intensive care unit of the University of Alberta Hospital. Infants with congenital abnormalities were excluded. From the initial sample of infants in the Piper and associates (1989) study, 45 were identified as

neurologically normal at 18 months by a experienced pediatric neurologist who used the neurological examination of the Collaborative Perinatal Project (Hardy et al.,1979). From this group of 45 children, 24 were evaluated in the present study. Twenty-one children were not included due to age (too young or too old for the age range established for the project), having moved out of town, or not being available during the six month period of the study.

Eighteen of the 24 subjects in this project were also part of an ongoing study conducted by Lane and Holdgrafer (1990). The families of these children were contacted after the research assistant for the Piper et al. (1989) project obtained permission to release their names. The parents were sent a letter (see appendix A) explaining the study, as well as a consent form (see appendix B). Approximately one week later, parents were contacted in order to see if they had any questions and to explain the objectives of the studies. For the ones who were still interested in participating, an appointment for the child's evaluation was made. On the day of the evaluation, parents were free to ask any questions about the study and the consent form was signed.

The six subjects who were not part of the Lane and Holdgrafer study were recruited using the same procedure. A different consent form (see appendix C) and a letter explaining the purpose of the study were sent (see appendix D).

Data Collection

This study used the following data already collected from Piper et al. (1989) study :

1. Birth weight;
2. Gestational age: This information was collected through mother's report of the last menstrual period and confirmed by ultrasound or by the Dubowitz technique (Dubowitz , Dubowitz, & Goldberg, 1970);
3. Neurological examination of the collaborative perinatal project: This examination included a comprehensive neurological and developmental evaluation of the child at 18 months. The neurological evaluation included information about the child's reflexes and motor development. The medical evaluation provided information about child's health condition. This information was used to ensure that only neurologically normal children were included in the sample.

The present study collected the following additional information:

1. Maternal education (see appendix E) - This information was collected from the

mother on the day of the evaluation.

2. Performance of the children on the total score and the five performance indices of the MAP.

Training in the administration of the MAP was conducted to ensure that the instrument was used consistently. The same occupational therapist who collected the data for the two studies (the present one and Lane and Holdgrafer's study) administered the MAP and scored three children, while the second occupational therapist (supervisor) only scored their responses. A coefficient of 85% agreement was achieved.

The fact that the data for the present study and the Lane and Holdgrafer study were collected at the same time by the same evaluator helped to control for possible observer bias during the evaluation. The Lane and Holdgrafer study included both neurologically normal preterm children and neurologically suspicious preterm children. The evaluator did not know the neurological status of the children during the evaluation and, therefore, did not know which children were part of this study and which were part of the Lane and Holdgrafer study.

The MAP was administered in the same order and at the same place for all subjects.

The Measurement Instrument - The Miller Assessment for Preschoolers

The Miller Assessment for Preschoolers (MAP) was developed by an occupational therapist, Lucy Miller, to provide a comprehensive, reliable, and valid screening instrument for identifying children with moderate to severe preacademic problems (Miller, 1982). The MAP is intended for children ranging from 2 years 9 months to 5 years 8 months. It consists of 27 items measuring sensorimotor, cognitive, and combined abilities. Sensory and motor abilities are measured by: 1) Foundation index - assesses sense of position and movement, sense of touch and basic movements such as flexion, extension, rotation, and movement patterns; and 2) Coordination index - evaluates gross, fine, and oral motor coordination. Cognitive abilities are measured by: 1) Verbal index - measures language expression and comprehension, sequencing, association, and memory; and 2) Non-Verbal index - assesses memory, sequencing, visualization, and mental manipulation in activities not requiring verbal use. Combined abilities, which are measured by the Complex Task index, evaluate skills which involve sensory, motor, and cognitive abilities. In addition to these tests, a "behavior during testing assessment" measures the child's

attention, social interaction, and sensory reactivity. This form is completed after test administration.

The MAP takes 30 minutes to administer. The children receive a total score which classifies them as within normal limits, should be watched, or in need of further evaluation. In addition, they receive a score for each performance index which provides information about the child's abilities in different areas of development.

Reliability and validity information are provided in the manual. Inter-rater reliability coefficients ranged from 84 to 99%. Test-retest reliability ranges from 80 to 94% except for the Coordination index (72%). Content validity is presented through an specification table (which examines the content of the MAP in relation to the behaviors purportedly assessed), factor analysis, and the relationship between the tests and age-groups, and each test and index with the MAP total score. Criterion-related validity was determined by comparing the MAP with the Southern California Sensory Integration Tests, Denver Developmental Screening Test, Weschler Preschool and Primary Scale of Intelligence, and Illinois Test of Psycholinguistic Ability. Although significant correlations were found, the author recommends caution in the interpretation of the results due to the small sample size (N=30). Construct validity was established with 90 children with preacademic problems. The MAP correctly identified 75% of the sample. In addition, predictive validity studies conducted by Miller (1987, 1988) have shown that the MAP is a good instrument for predicting academic achievement. Daniels (1990) found that it discriminates between some diagnostic groups (children with speech-language, psychiatric, neurological, or motor problems).

Limitations of the MAP include long directions to be followed in some items. Children with language comprehension problems may have some difficulty in understanding some instructions. Some items, such as "stamp", may be difficult to score if the child's response is fast. As the assessments were videotaped in this study, the child's performance was verified later for more precise information. The MAP has not been normed in Canada and it is possible that Canadian children may have slight differences in development from American children. However, the MAP has been used in Canadian studies (e.g., Daniels, 1990).

In conclusion, although the MAP has not been used in studies of preterm infants, it appears to be a valid and reliable screening instrument and an useful test in detecting preacademic problems. According to Deloria (1985), the MAP is one of the few instruments for preschoolers which precisely follows the recommendations of the

Standards for Educational and Psychological Tests (APA, 1974).

Ethical Procedures

The parents of the preterm infants who agreed to the evaluation, signed a consent form (see appendix C) and retained a copy of the covering letter and the consent form. Confidentiality was ensured in this project by identifying the children only by code numbers. Their names or other information which might identify them were not reported.

Data Analysis

All variables were summarized using descriptive statistics. Additional information regarding birthweight, behavior during the assessment, number of singleton or multiple births, number of suspect children, and current maternal education was described.

A correlation matrix was done on the five MAP indices to determine if any were highly correlated and could be eliminated from the regression analysis to reduce the number of dependent variables.

Multiple regression analysis was used to test the correlation between gestational age and maternal education (independent variables) and each of the five performance indices and the total score of the MAP separately (dependent variables) and to determine which variable contributed most to the variance in the dependent variables.

Chi-square analysis was used to test the statistical significance of the relative risk associated with the two gestational age groups of infants (<32 weeks GA; 32-36 weeks GA) in the five performance indices and total score (normal; below normal). Chi-square analysis was also used to test the differences between the two maternal education groups (≤12 years of maternal education; >12 years of maternal education) in the five performance indices and total score (normal; below normal). As mentioned, the MAP uses a scoring system in which the item scores are classified as child within normal limits, should be watched or in need of further evaluation. For the chi-square analysis, the "within normal limits" classification was considered "normal". The "should be watched and in need of further evaluation" classifications were considered "below normal", as children scoring in these areas had performances below most children of their age and were likely to have future problems.

The alpha level of the tests was set at 0.05.

CHAPTER IV

RESULTS

CHARACTERISTICS OF SAMPLE

The sample consisted of 24 of the 45 preterm children who were classified as neurologically normal at 18 months of age in the Piper et al. (1989) study. Gender, gestational age, years of maternal education, mother's age at birth, and birth weight are reported in Table 1. There were more female (70.8%) than male infants in the sample despite the fact that the Piper et al. study had approximately equal number of males (n = 25) and females (n = 20). Gestational age ranged from 27 to 34 weeks with birth weight as low as 870 grams and as high as 3,390 grams. Three sets of twins were included. Seventeen subjects (70.8%) were enrolled in day care programs at least part of the day. The mean age at the time of the assessment was 4 years 6 months (SD = 3 months), ranging from 4.0 to 5.0 years. Approximately half of the mothers had an educational level greater than 12 years (58.3%). There was only one teenage mother in the sample (Table 1).

TEST RESULTS

1. MAP Scores

The subjects' mean scores by performance index and total score are reported in Table 2. As can be seen, most of the children, 14 of 23 (60.9%), are within the normal range for the total score. The frequencies of the MAP scores of the 24 infants are illustrated in Figure 1. Only two children (8.3%) were classified as "in need of further evaluation" and that was only for the Complex-Task index. One child had uncompleted scores in the Foundation, Verbal, and Total score performance indices due to behavioral problems. As the number of missing items in each of these indices exceeded one, the child's scores on these indices were not used as they were not considered reliable scores.

TABLE 1

Gender, Gestational Age, Years of Maternal Education, Maternal Age at Birth, and Birth Weight of Sample.

CHARACTERISTIC	N=24	
		MEAN (SD)
GENDER		—
MALE	7 (29.2%)	
FEMALE	17 (70.8%)	
GESTATIONAL AGE		31.3 (2.6)
27 WEEKS	2 (8.3%)	
28 WEEKS	4 (16.7%)	
29 WEEKS	2 (8.3%)	
30 WEEKS	1 (4.2%)	
32 WEEKS	6 (25.0%)	
33 WEEKS	1 (4.2%)	
34 WEEKS	8 (33.3%)	
MATERNAL EDUCATION		14 (2.1)
12 YEARS	10 (41.7%)	
13 YEARS	3 (12.5%)	
14 YEARS	1 (4.2%)	
15 YEARS	1 (4.2%)	
16 YEARS	7 (29.2%)	
18 YEARS	2 (8.3%)	
MOTHER'S AGE AT BIRTH		28.8 (4.5)
19 - 22 YEARS	3 (12.5%)	
23 - 27 YEARS	6 (25.0%)	
28 - 32 YEARS	10 (41.7%)	
33 - 36 YEARS	5 (20.8%)	
BIRTH WEIGHT		1785 (580)
750 - 1000 gm	2 (8.3%)	
1001 - 1500 gm	7 (29.2%)	
1501 - 2500 gm	14 (58.3%)	
> 2500 gm	1 (4.2%)	

TABLE 2

MAP Percentile Scores by Performance Index

CATEGORIES	MEAN (SD)	RANGE	Nº OF CHILDREN WITHIN NORMAL RANGE
FOUNDATION[a†]	44.1 (24.6)	(18-99)	20 (87.0%)
COORDINATION[b]	37.5 (24.8)	(8-99)	13 (54.2%)
VERBAL[c†]	46.2 (34.5)	(7-99)	11 (47.8%)
NON-VERBAL[d]	57.4 (29.9)	(14-99)	22 (91.7%)
COMPLEX-TASK[e]	39.6 (34.5)	(4-99)	14 (58.3%)
TOTAL SCORE[f†]	32.7 (19.2)	(7-74)	14 (60.9%)

Note. Performance within normal limits: [a] ≥ 29. [b] ≥ 33. [c] ≥ 48. [d] ≥ 30. [e] ≥ 31. [f] ≥ 26.
[†]N=23.

TABLE 3

Correlation Between Performance Indices of the MAP

	Foundation	Coordination	Verbal	Non-Verbal	Complex Task
Foundation	1				
Coordination	.12	1			
Verbal	-.24	.21	1		
Non-Verbal	.05	-.21	.13	1	
Complex Task	.37	.36	.33	.17	1

2. Correlation matrix for elimination of dependent variables

A correlation matrix was calculated to determine if there were any highly significant correlations among the dependent variables: Foundations, Coordination, Verbal, Non-Verbal, and Complex Task indices (see Table 3). The correlation coefficients ranged from .05 to .37 and were not significant. None of the indices could be excluded from the regression analyses.

3. Multiple regression analyses for correlation between independent and dependent variables.

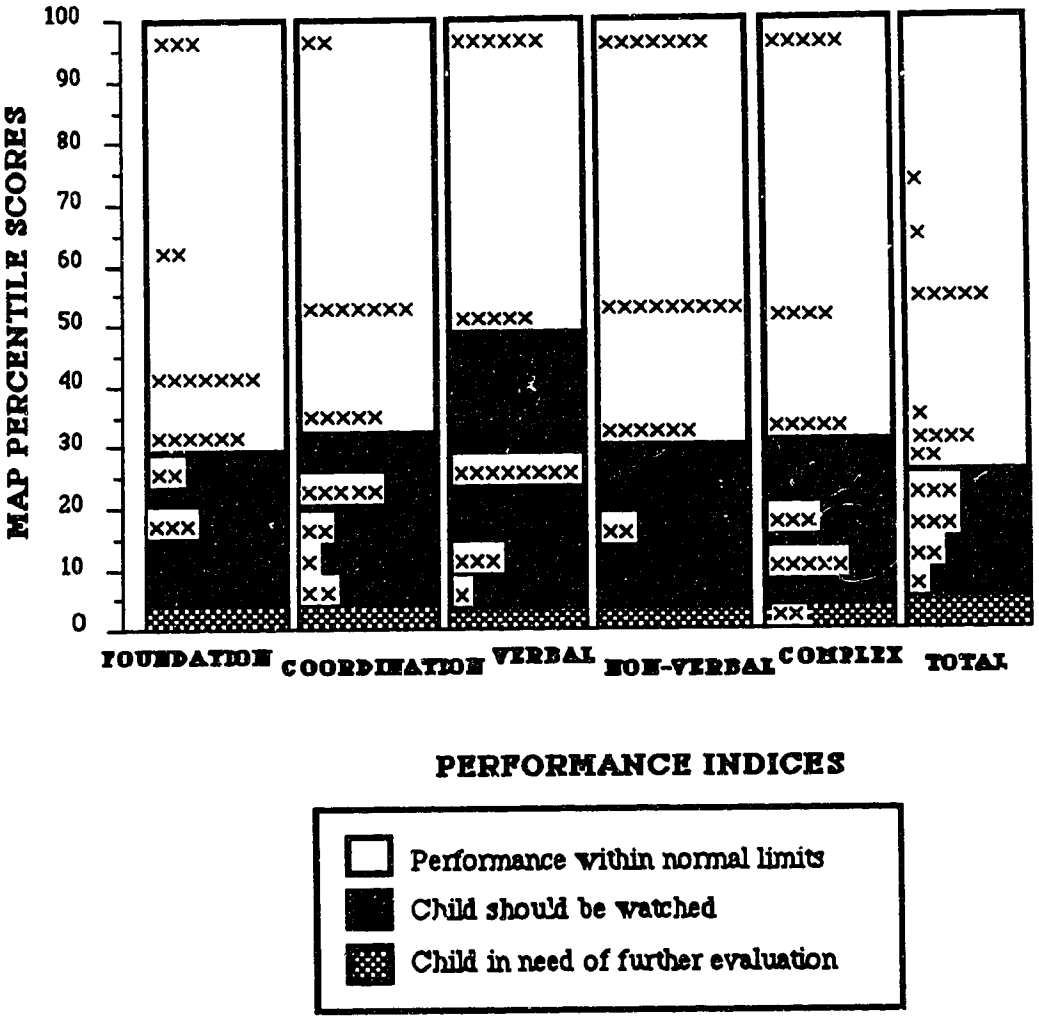
Multiple regression analysis was performed to determine if the independent variables together would be significantly correlated with the dependent variables. Maternal education and gestational age did not explain a significant amount of the variation in any performance index and the total score. Maternal education and gestational age accounted for 1% of the variance in Foundation scores, $F(2, 20) = 0.10$, $p = .91$); none of the variance in the Coordination scores, $F(2, 21) = 0.05$, $p = .95$; 6% of the variance in the Verbal scores, $F(2, 20) = 0.62$, $p = .55$; 17% of the variance in Non-Verbal scores, $F(2, 21) = 2.20$, $p = .14$; 7% of the variance in the Complex Task scores, $F(2, 21) = 0.81$, $p = .46$; and 8% of the variance in the Total scores, $F(2, 20) = 0.87$, $p = .43$.

When gestational age was examined alone as an independent variable, it explained a significant amount (17%) of the variation in the Non-Verbal scores, $F(1, 22) = 4.35$, $p = .05$. Gestational age was not significantly correlated with the other performance indices and the total score. The F tests varied from .02 to .77, with p values ranging from .38 to .96.

Maternal education alone did not explain a significant amount of the variation in any dependent measure. The F tests varied from 0 to .77, with p values ranging from .38 to .96.

FIGURE 1

Frequency of Performance Indices Scores



4. Chi-Square Analysis for Differences Between Gestational Age Groups and Maternal Education Groups.

Two gestational age groups (< 32 weeks; 32-36 weeks) were compared on the performance indices and total score (see Table 4). This cut-off is based on previous work reported in the literature (Largo et al., 1989; Piper, Byrne, & Pinnel, 1989; Piper, Darrah, & Byrne, 1989). The performance indices (Foundation, Coordination, Verbal, Non-Verbal, and Complex Task) and the total score were classified into two categories: normal performance and below normal performance, according to the cut-off established for each index in the MAP manual. A chi-square analysis was performed for each dependent measure. The observed frequencies varied significantly from the expected frequencies only in the Foundation index, $X^2(1, N = 23) = 6.47$, $p = .01$. All the children born at 32-36 weeks GA scored in the normal range on the Foundation test while 3 of the children born below 32 weeks GA (37.5%) scored below normal. The observed frequencies did not vary significantly from the expected frequencies for the other indices: Coordination $X^2(1, N = 24) = 1.14$, $p = .28$; Verbal $X^2(1, N = 23) = 1.06$, $p = .30$; Non-Verbal $X^2(1, N = 24) = 0.15$, $p = .70$; Complex-Task $X^2(1, N = 24) = 0.05$, $p = .83$; and total score $X^2(1, N = 23) = 1.81$, $p = .18$.

Two maternal education groups (12 years or less of education; above 12 years of education) were compared on the performance indices and the total score using the same classifications as described above for identifying normal and below normal groups. The observed frequencies did not vary significantly from the expected frequencies in any performance index score and total score (see Table 5). Chi-square results ranged from .05 to 1.56. Children born of more educated mothers did not do better on the MAP than children born of less educated mothers.

TABLE 4

Comparison of Gestational Age Groups by Performance Indices Categories

PERFORMANCE INDICES	GESTATIONAL AGE GROUPS	
	< 32 WKS (n=9)[A]	32-36 WKS (n=15)
FOUNDATION †		
NORMAL	5 (62.5%)	15 (100%)
BELOW NORMAL	3 (37.5%)	0 (0%)
COORDINATION		
NORMAL	4 (44.4%)	10 (66.7%)
BELOW NORMAL	5 (55.6%)	5 (33.3%)
VERBAL		
NORMAL	3 (37.5%)	9 (60%)
BELOW NORMAL	5 (62.5%)	6 (40%)
NON-VERBAL		
NORMAL	8 (88.9%)	14 (93.3%)
BELOW NORMAL	1 (11.1%)	1 (6.7%)
COMPLEX-TASK		
NORMAL	5 (55.6%)	9 (60%)
BELOW NORMAL	4 (44.4%)	6 (40%)
TOTAL SCORE		
NORMAL	3 (37.5%)	10 (66.7%)
BELOW NORMAL	5 (62.5%)	5 (33.3%)

Note. [A] = n varies from 8 to 9.

† significant

df = 1

TABLE 5

Comparison of Maternal Education Groups by Performance Indices Categories

PERFORMANCE INDICES	MATERNAL EDUCATION GROUPS	
	12 YEARS OR LESS (n = 10)[a]	ABOVE 12 YEARS (n = 14)
FOUNDATION		
NORMAL	8 (88.9%)	12 (85.7%)
BELOW NORMAL	1 (11.1%)	2 (14.3%)
COORDINATION		
NORMAL	7 (70%)	7 (50%)
BELOW NORMAL	3 (30%)	7 (50%)
VERBAL		
NORMAL	5 (55.6%)	7 (50%)
BELOW NORMAL	4 (44.4%)	7 (50%)
NON-VERBAL		
NORMAL	10 (100%)	12 (91.7%)
BELOW NORMAL	0 (0%)	2 (8.3%)
COMPLEX-TASK		
NORMAL	5 (50%)	9 (58.3%)
BELOW NORMAL	5 (50%)	5 (41.7%)
TOTAL SCORE		
NORMAL	4 (44.4%)	9 (64.3%)
BELOW NORMAL	5 (55.6%)	5 (35.7%)

Note. [a] = n varies from 9 to 10.

df = 1

CHAPTER V

DISCUSSION

DEVELOPMENTAL OUTCOME AS MEASURED BY THE MAP

The present study has shown that 14 (60.9%) of 23 children born prematurely were functioning within the normal range according to the MAP total score. This proportion is similar to that reported in other studies of normal preterm infants using other developmental measures (Drillien, 1972; Stewart et al., 1989; Vohr et al., 1988).

On the five performance indices, 74 to 92% of the children were doing well, except on the Verbal index where only 47.8% (n = 11) were within the normal range. English was not the first language for three of the children who scored below average and this may have affected their scores. Miller (1982) reported that the number of children who did not speak English fluently was small in the normative sample. Thus, caution is suggested when interpreting the scores of these children as they may not represent adequately the performance of this group. Exclusion of these three children from the Verbal index would increase the proportion of children scoring in the normal range on this index to 55%, making it more similar to the percentages on the other indices.

As part of the Lane and Holdgrafer study, the language development of 18 of the subjects was assessed and 17 (94.4%) of these children were performing within normal limits (G. Holdgrafer, personal communication, January 29, 1991). Ten children were classified the same on both the MAP and the measures used in the Holdgrafer and Lane study (9 normal, 1 below normal). There were disagreements between the MAP and the Holdgrafer and Lane classifications on the remaining 8 children. Differences between the results in the two studies appear to be due to the measurement instruments used. The MAP is a screening instrument and is not designed to provide an indepth assessment of all aspects of language. Several tools were used to evaluate expressive and receptive aspects of the children's language performance in the Lane and Holdgrafer study while the MAP had only four subtests. Miller (1982) reported that the fewer the items in each performance index, the less reliable the final score. Although reliability and validity information in the manual is within acceptable limits, when comparing the Verbal index with the Illinois Test of Psycholinguistic Ability for construct validity, the correlation was low (.14). Thus, caution should be used when interpreting the verbal scores in this study and in

occupational therapy practice. Also, the items used for the language evaluation in the MAP assess functions such as memory and sequencing, in addition to language abilities. Possible dysfunction in memory and sequencing areas might have decreased the verbal score and these areas should be further assessed.

On the other MAP indices, two children were in need of further evaluation and that was on the Complex-Task index. This index measures the child's combined sensory, motor, and cognitive abilities and may be more sensitive than the other indices in measuring the integration of these senses. As this is a complex function, one would expect some preterm infants to have more difficulty in the execution of tasks on this index.

Behavior during testing was assessed in the areas of sensory reactivity, social interaction, and attention. Of the 24 children, 2 (8.3%) presented moderate dysfunction in the area of sensory reactivity, becoming uneasy when touched; one of the two also presented moderate dysfunction in the area of social interaction. Three (12.5%) other children showed severe and moderate dysfunction in the area of social interaction, refusing to go along with examiner and being quite demanding during the examination; one of these children also manifested moderate dysfunction in the attention area, needing rewards quickly to proceed to next task and being easily diverted during testing. From this group of five children, two had low total scores ("child should be watched"). One of these two subjects' total score and 4 performance indices scores were below average. No relationship was observed between the other children who presented behavioral problems and their scores.

The proportion of subjects with attention deficits is close to that reported in Li, Sauve, and Creighton's study (1990) which found that 2 to 8% of their preterm children had inattention, hyperactivity or distractibility at 3-4 years of age. On the other hand, this study's results are lower in comparison to Astbury, Orgill, and Bajuk's study (1987), which reported the same problems, as well as impulsivity, for 40% of their preterm subjects. Astbury and associates mentioned that their results were also higher than the 3% estimate provided by the American Psychiatric Association (1980). Thus, in general, this study supports the low rate of behavioral problems in preschool preterm children reported in the literature.

GESTATIONAL AGE

The hypothesized relationship between gestational age and the Verbal index was not found in this study. This result does not support the Vohr et al. (1988) study

which found that normal preterm infants were significantly delayed in language ability at 2 years of age. The differences between the present and Vohr et al. (1988) study seem to be attributable to different measurement instruments and age at evaluation. It is also possible that normal preterm infants at younger ages have language deficits which disappear when preterm infant development catches up with "normal" development (Ungerer & Sigman, 1983).

A significant correlation was found between gestational age and the Non-Verbal score. This finding was statistically significant and was not due to chance alone as it accounted for a high percent (17%) of the variance. This result is supported by Towen's theory of development (1980) which proposes that the extrauterine environment can enhance or retard preterm development. Although the Non-Verbal index measures functions such as sequencing, memory, and performance of mental manipulation, the five sub-tests (Puzzle, Figure-Ground, Object Memory, Block Tapping, and Sequencing) contain a heavy visualization component. This function is reported to be still developing until the last month of gestation (Friedman, Jacobs, & Werthman, 1981). It is possible that the children who were born at a younger GA might have their visual ability negatively affected by the extrauterine environment. Although some authors (Ramey, Zeskind, & Hunter, 1981) argue that the lack of stimulatory environment may jeopardize these infants' development, others (Short-DeGraff, 1988) report that the stimulus bombardment in the neonatal intensive care unit (NICU) may not be appropriate in terms of sensory experiences. Sehnal and Palmeri (1989) report that the environment of the NICU may be overstimulating in part due to the bright lights required for the services. Both conditions, lack of stimulation and overstimulation, are seen as not providing the normal course of development as it would be experienced in uterus. Van Holf-van Duin and Mohn (1984) report that neurons of the visual pathway during fetal life are still immature and that they fully develop by nine months of age. Thus, it is possible that if the immature system of the preterm infant is not provided with an adequate setting for development, non-verbal aspects may be affected. However, it must be remembered that only two of the children had below average Non-Verbal scores.

When gestational age was examined as a nominal variable with groups of younger (<32 weeks) and older (32-36 weeks) preterm infants, the results were different. While all of the children born between 32-36 weeks GA were in the normal range for the Foundation index, 62.5% of the children born below 32 weeks GA were in this category. This result was statistically significant. For the other indices, there

were no significant differences between the expected and observed frequencies.

The significant difference between the two gestational age groups on the Foundation index was unexpected. As mentioned before, the Foundation index measures the child's sense of position and movement, tactile perception, and basic components of movement. All children who were born below 32 weeks had at least two items with below average performance in the area of sense of position and movement, and 63.5% had one item related to tactile perception with below average performance. It appears that the major areas affected in the Foundation index were position and movement. Again, according to Towen's theory of development (1980), it is possible that a non-optimal extrauterine environment might have affected development in this area. In the seventh month of fetal development, some functions such as muscle tone and behavioral reflexes are still developing (Short-DeGraff, 1988; Williams, 1983). As these infants still do not have adequate musculature and poor motor control to stimulate themselves, it is possible that this period of inactivity may affect their later development. Some authors (Kramer & Pierpoint, 1976) even suggest intervention techniques such as rocking and water beds to compensate for the hypothesized sensory deprivation. As well, the additional equipment frequently attached to these infants in NICU may prevent parents from carrying and stimulating their babies.

MATERNAL EDUCATION

The hypothesized relationship of maternal education and the Complex-Task and Non-Verbal indices was not found in this study. This finding does not support the literature which reported a significant correlation between low maternal education and perceptuo-motor and cognitive problems (Blackman et al., 1987; Klein et al., 1985; Rickards et al., 1988; Sameroff, 1986; Scott, 1987). Possible explanations for the discrepancy between these studies and the present one might be the neurological status of the sample, age at evaluation, different areas of development investigated, and the range of maternal education. In this study, only children who were diagnosed neurologically normal were included in the sample. In the other studies, some did not mention the neurological status of the preterm infants and others included children with medical abnormalities. It is possible that low maternal education has less effect on the development of preterm children without neurological problems, and that its effect is observed when the infant's medical problems make him/her more vulnerable to environmental effects. As well, the majority of the studies mentioned above were

conducted with children above five years of age. This age may be the minimum age at which the influence of maternal education on child's development becomes apparent. It is also possible that the areas most affected by maternal education are the child's school achievement and IQ, as mentioned in some reports reviewed previously. These areas were not investigated in this project. The fact that all of the mothers had a minimum of 12 years of education might reflect a bias in the sample towards a higher level of maternal education. It is possible that 12 years of education may be sufficient to prepare the mothers for raising their children. Thus, this fact might have also influenced the lack of relationship between maternal education and the children's outcome.

When maternal education was used as a nominal variable in a chi-square analysis (>12 years of education and ≤12 years of education), no significant differences were found between the expected and observed frequencies. This finding adds some support to the results of the multiple regression analysis, which found no relationship between maternal education and the total score and performance indices. However, there are some limitations concerning the chi-square analysis. According to Norman and Streiner (1986), for the chi-square, there should be at least 5 subjects in each cell for the expected frequency or the results are considered inaccurate. In this study, all the performance indices and the total score have some cells with expected frequencies below 5. Thus, the results should be interpreted with caution.

The fourth hypothesized relationship of this study was that gestational age and maternal education would be significantly correlated with the Complex Task, Coordination, Verbal, and Non-Verbal indices of the MAP. This result was not found. This study failed to show a significant relationship between the two independent variables, when treated as continuous data, and each of the 5 performance indices and the total score.

LIMITATIONS

The high prevalence of female (70.8%) to male infants in this sample is unusual. The original sample (Piper et al., 1989) included 44.5% female infants. In other follow-up studies (Calame et al., 1986; Klein et al, 1989; Largo et al., 1990; Ungerer & Sigman, 1983), the ratio of females to males was usually from 38 to 53%. This finding raises the question of whether the disproportionate number of girls could have biased the results.

Some authors reported significant differences between male and female infants

(Grigoroiu-Serbanescu, 1984; Rubin et al, 1973). These authors found that females were doing better than males in school placement. Interestingly, the results of this study showed that 68.8% of females were performing within normal range while 57.2% of males were classified in this area. It is possible that this study may be evaluating a sample which includes a predominantly lower risk group (female). However, other authors (Caputo, Goldstein, & Taub, 1981; Cohen & Parmelee, 1983) have reported no significant differences between males and females. Until further investigation is done in this area, no assumptions can be made concerning the effect of a possible gender bias on the results. A more equal ratio of male and female infants in future studies would eliminate gender as a possible confounding variable.

The results should be taken with caution prior to replication due to a number of limitations including a small sample size. Particularly in the chi-square analysis, there were only 8 subjects in the gestational age group "below 32 weeks". A small change in the number of subjects in the "normal" and "below normal" groups would make a difference in the results. In addition, there were three sets of twins in this study. It is possible that the presence of twins who had the same GA but different birth weights and mothers with the same level of education may have contributed to the failure of this study to find the predicted correlation between gestational age and maternal education and the dependent variables. However, when the twins were excluded from the data analysis of the study, the only significant correlation found was between gestational age and the Non-Verbal index ($F(1, 16) = 6.04$, $p = .02$). Excluding the twins did not alter the results. Other limitations of the study include no control group of neurologically suspect children or normally developing children (MAP normative data allows some comparison to normally developing children).

CHAPTER VI

SUMMARY AND CONCLUSIONS

Few studies have examined the development of preterm children who were diagnosed neurologically normal in the first years of life (Drillien, 1972; Stewart et al., 1989; Vohr et al., 1988). This study investigated the outcome of preterm infants considered neurologically normal at 18 months of age in order to determine their developmental outcome at 4 years of age, and to examine the relationship of biological/environmental factor on outcome in the absence of medical abnormalities. A considerable percentage of the children in this study had MAP scores which indicated developmental delays. It would appear that a neurologically normal status at 18 months does not mean that these children's development will keep pace with their peers. Early identification of these children is important due to the adverse consequences, such as emotional problems associated with school failure and reduced effectiveness of therapeutic treatment at later ages (Miller, 1982).

Professionals, such as occupational therapists, who work in early intervention and in NICU should pay attention especially to the area of position and movement and areas which involve a combination of sensory, motor, and cognitive abilities. Soltesz and Brockway (1989) report that therapeutic handling helps the infant to experience sensorimotor stimulation which improves the child's body schema. They mention that rolling, carrying, lifting, and place the infant in side-lying, among others, are beneficial techniques to improve coordination, muscle tone, and increase flexor patterns. Sehnal and Palmeri (1989) suggest environmental adaptations in NICU to facilitate infants' responses which therapists might consider. Some adaptations include placing toys in the child's line of vision in isolette and avoiding loud noises and abrupt movements. According to these authors, procedures such as these, would help the infant to decrease hypersensitivity to movement and sounds and increase visual responsiveness.

This study failed to demonstrate a significant relationship between gestational age and maternal education, and the five performance indices and the total score of the MAP. However, a significant correlation was found between gestational age and decreased performance on the Foundation index. The Foundation index measures the child's sense of position, movement and touch, and movement patterns such as flexion, extension, and rotation. According to this study, preterm infants who were

born at less than 32 weeks GA may be at-risk in areas requiring abilities involving tactile and kinesthetic activities. Ayres (1972) reported that children who have problems in integrating these sensations do not have a good perception of body parts and their movements. Consequently, they may have difficulty doing some activities such as manipulation of objects, positioning in seats, moving body parts freely and protecting from falling. Follow-up programs for infants less than 32 weeks GA would help to detect possible problems in these areas as soon as they emerged and provide appropriate intervention.

The significant relationship found between gestational age and low scores in the Non-Verbal index also provides useful information. If the development of the very premature infants can be affected by the extrauterine environment, the efficacy of early intervention techniques needs closer investigation as it could provide positive results for this population.

This study failed to show a significant relationship between gestational age and the other performance indices and the total score. No significant association was found between maternal education and the performance indices and the total score. Although no relationship is reported for these variables, it is not possible to affirm that there is no relationship between them due to the small sample size. The study needs to be replicated with a larger sample. It is possible that the lack of relationship between maternal education and the dependent variables may have been due to factors such as, a sample of neurologically normal infants who may not be affected by maternal education level, the limited range of maternal education, age at evaluation, and the areas of development chosen for investigation in the present study.

Future Directions

Replication of this study with a larger sample size, and consequently, a wider range in the level of maternal education, is needed to confirm the results of this study. As well, the inclusion of more environmental and biological factors would permit a better analysis of the effects of both variables in child development. Further follow-up as the children in this study start school will provide information about later outcome of neurologically normal preterm infants and will allow examination of the predictive validity of the MAP with this population. Finally, the use of more indepth measures in the areas in which performance was low (non-verbal, position and movement abilities) would provide additional information as to whether these areas are the ones m likely to be affected by gestational age.

REFERENCES

American Psychiatry Association (1980). Diagnostic and Statistical Manual of Mental Disorders, III. Washington: American Psychological Association.

American Psychological Association (1974). Standards for educational and psychological tests. Washington: American Psychological Association.

Astbury, J., Orgill, A., & Bajuk, B. (1987). Relationship between two-year behaviour and neurodevelopmental outcome at five years of very low-birthweight survivors. Developmental Medicine and Child Neurology, 29, 370-379.

Ayres, A. J. (1972). Sensory integration and the child. Los Angeles: WPS.

Blackman, J. A., Lindgren, S. D., Hein, H. A., & Harper, D. C. (1987). Long-term surveillance of high-risk children. American Journal of Diseases of Children, 141, 1293-1299.

Calame, A., Fawer, C. L., Claeys, V., Arrazola, L., Ducret, S., & Jaunin, L. (1986). Neurodevelopmental outcome and school performance of very-low-birth-weight infants at 8 years of age. European Journal of Pediatrics, 145, 461-466.

Caputo, D. V., Goldstein, K. M., & Taub, H. B. (1981). Neonatal compromise and later psychological development: A 10-year longitudinal study. In S. L. Friedman & M. Sigman (Eds.), Preterm birth and psychological development (pp. 353-386). New York: Academic Press.

Cohen, S. E. & Parmelee, A. H. (1983). Prediction of five-year Stanford Binet in preterm infants. Child Development, 54, 1242-1253.

Cohen, S. E., Parmelee, A. H., Sigman, M., & Beckwith, L. (1988). Antecedents of school problems in children born preterm. Journal of Pediatric Psychology, 13, 493-508.

Daniels, L. (1990). The Miller Assessment for Preschoolers: Analysis of score patterns for children with developmental delays. Canadian Journal of Occupational Therapy, 57, 205-210.

Dargassies, S. A. (1966). Neurological maturation of the premature infant of 28 to 41 weeks' gestational age. In F. Falkner (Ed.), Human development (pp. 306-325). Philadelphia: Saunders.

Deloria, D. J. (1985). Review of Miller Assessment for Preschoolers. In J. V. Mitchell, Jr. (Ed.), The ninth mental measurements yearbook (pp. 975-976). Highland Park, NJ: Gryphon Press.

Drillien, C. M. (1972). Aetiology and outcome in low-birthweight infants.

Developmental Medicine and Child Neurology, 14, 563-574.

Drillien, C. M., Thomson, A. J. M., & Burgoyne, K. (1980). Low-birthweight children at early school-age: A longitudinal study. Developmental Medicine and Child Neurology, 22, 26-47.

Dubowitz, L. M. S., Dubowitz, V., & Goldberg, C. (1970). Clinical assessment of gestational age in the newborn infant. Journal of Pediatrics, 77, 1-10.

Escalona, S. K. (1982). Babies at double hazard: Early development of infants at biologic and social risk. Pediatrics, 70, 670-676.

Ferrari, F., Grosoli, M. V., Fontana, G., & Cavazzuti, G. B. (1983). Neurobehavioral comparison of low risk preterm and fullterm infants at term conceptional age. Developmental Medicine and Child Neurology, 25, 450-458.

Fletcher, R. H., Fletcher, S. W., & Wagner, E. H. (1988). Clinical epidemiology - The essentials. Baltimore: Williams & Wilkins.

Forslund, M. & Bjerre, I. (1985). Growth and development in preterm infants during the first 18 months. Early Human Development, 10, 201-216.

Friedman, S. L., Jacobs, B. S., & Werthmann, M. W. (1981). Sensory processing in preterm and fullterm infants in the neonatal period. In S. L. Friedman & M. Sigman (Eds.), Preterm birth and psychological development (pp. 159-178). New York: Academic Press.

Gorga, D., Stern, F. M., & Ross, G. (1985). Trends in neuromotor behavior of preterm and fullterm infants in the first year of life: A preliminary report. Developmental Medicine and Child Neurology, 27, 756-766.

Gorga, D., Stern, F. M., Ross, G., & Nagler,W. (1988). Neuromotor development of preterm and full-term infants. Early Human Development, 18, 137-149.

Gorski, P. A. (1984). Infants at risk. In M. J. Hanson (Ed.), Atypical infant development (pp. 57-80). Baltimore: University Park Press.

Grigoroiu-Serbanescu, M. (1984). Intellectual and emotional development and school adjustment in preterm children at 6 and 7 years of age. Continuation of a follow-up study. International Journal of Behavioral Development, 7, 307-320.

Hardy, J. B., Drage, J. S., & Jacobson, E. C. (1979). The first year of life: The collaborative perinatal project of the national institute of neurological and communicative disorders and stroke. Baltimore: John Hopkins University Press.

Howard, J., Parmelee, A. H., Kopp, C. B., & Littman, B. (1976). A neurological comparison of preterm and fullterm infants at term conceptional age. Journal of Pediatrics, 88, 995-1002.

Hunt, J. V., Tooley, W. H., & Harvin, D. (1982). Learning disabilities in children with birth weights <1500 grams. Seminars in Perinatology, 6, 280-287.

Hunt, J. (1986). Developmental risk in infants. In B. K. Keogh, (Ed.), Advances in special education: Developmental problems in infancy and the preschool years, Vol. 5. (pp. 25-60). London: JAI Press.

Kitchen, W. H., Ryan, M. M., Rickards, A., McDougall, A. B., Billson, F. A., Keir, E. H., & Naylor, F. D. (1980). A longitudinal study of very low birth weight infants. IV: An overview of performance at eight years of age. Developmental Medicine and Child Neurology, 22, 172-188.

Klein, N. K., Hack, M., Gallagher, J., & Fanaroff, A. A. (1985). Preschool performance of children with normal intelligence who were very low-birth-weight infants. Pediatrics, 75, 531-537.

Klein, N. K., Hack, M., & Breslau, N. (1989). Children who were very low birth weight: Development and academic achievement at nine years of age. Developmental and Behavioral Pediatrics, 10, 32-37.

Kramer, L. I. & Pierpoint, M. E. (1976). Rocking waterbeds and auditory stimuli to enhance growth of preterm infants. Pediatrics, 88, 297-299.

Lane, S. J. & Holdgrafer, G. (1990). [Evaluation of language, perceptual motor skills, and behavior in 3-4 year old children born premature]. Unpublished raw data.

Largo, R. H., Pfister, D., Molinari, L., Kundu, S., Lippi, A., & Duc, G. (1989). Significance of prenatal, perinatal and posnatal factors in the development of AGA preterm infants at five to seven years. Developmental Medicine and Child Neurology, 31, 440-456.

Largo, R. H., Graf, S., Kundu, S., Hunziker, U., & Molinari, L. (1990). Predicting developmental outcome at school age from infant tests of normal, at-risk and retarded infants. Developmental Medicine and Child Neurology, 32, 30-45.

Li, A. K. F., Sauve, R. S., & Creighton, D. E. (1990). Early indicators of learning problems in high-risk children. Developmental and Behavioral Pediatrics, 11(1), 1-6.

Lilienfeld, A. M. & Lilienfeld, D. E. (1980). Foundations of epidemiology (2nd ed.). New York: Oxford University Press.

McDonald, M. A., Sigman, M., & Ungerer, J. A. (1989). Intelligence and behavior problems in 5-year-olds in relation to representational abilities in the second years of life. Developmental and Behavioral Pediatrics, 10, 86-91.

McGillicuddy-DeLisi, A. V. (1982). The relationship between parents' beliefs about

development and family constellation, socioeconomic status, and parents' teaching strategies. In L. M. Laosa & I. E. Siegel (Eds.), Families as learning environments for children. (pp. 261-300). New York: Plenum Press.

Michelsson, K., Lindahl, E., Parre, M., & Helenius, M. (1984). Nine year follow-up of infants weighing 1500 grams or less at birth. Acta Paediatric Scandinavica, 73, 835-841.

Miller, L. J. (1982). Miller Assessment for Preschoolers. Littleton, CO: Foundation for Knowledge in Development.

Miller, L. J. (1987). Longitudinal validity of the Miller Assessment for Preschoolers: Study I. Perceptual and Motor Skills, 65, 211-217.

Miller, L. J. (1988). Longitudinal validity of the Miller Assessment for Preschoolers: Study II. Perceptual and Motor Skills, 66, 811-814.

Nickel, R. E., Bennett, F. C., & Lamson, F. N. (1982). School performance of children with birth weights of 1000 grams or less. American Journal of Diseases of Children, 136, 105-110.

Norman, G. R. & Streiner, D. L. (1986). PDQ Statistics. Toronto: B. C. Decker.

O'Mara, L. & Johnston, C. (1989). Mothers' attitudes and their children's behaviors in 3-year-olds born prematurely and at term. Developmental and Behavioral Pediatrics, 10, 192-197.

Palisano, R. J. (1986). Use of chronological and adjusted ages to compare motor development of healthy preterm and fullterm infants. Developmental Medicine and Child Neurology, 28, 180-187.

Palmer, P. G., Dubowitz, L. M. S., Verghote, M., & Dubowitz, V. (1982). Neurological and neurobehavioral differences between preterm infants at term and full-term newborn infants. Neuropediatrics, 13, 183-189.

Parush, S. & Clark, F. (1988). The reliability and validity of a sensory developmental expectation questionnaire for mothers of newborns. American Journal of Occupational Therapy, 42, 11-16.

Piper, M. C., Kunos, I., Willis, D. M., & Mazer, B. (1985). Effect of gestational age on neurological functioning of the very low-birthweight infant at 40 weeks. Developmental Medicine and Child Neurology, 27, 596-605.

Piper, M. C., Byrne, P. J., Darrah, J., & Watt, M. J. (1989). Gross and fine motor development of preterm infants at eight and 12 months of age. Developmental Medicine and Child Neurology, 31, 591-597.

Piper, M. C., Byrne, P. J., & Pinnell, L. E. (1989). Influence of gestational age on

early neuromotor development in the preterm infant. American Journal of Perinatology, 6(4), 405-411.

Piper, M. C., Darrah, J., & Byrne, P. J. (1989). Impact of gestational age on preterm motor development at 4 months chronological and adjusted age. Child: Care, Health and Development, 15, 105-115.

Ramey, C. T., Zeskind, P. S., & Hunter, R. (1981). Biomedical and psychosocial interventions for preterm infants. In S. L. Friedman & M. Sigman (Eds.), Preterm birth and psychological development (pp. 395-415). New York: Academic Press.

Rickards, A. L., Ryan, M. M., & Kitchen, W. H. (1988). Longitudinal study of very low birthweight infants: Intelligence and aspects of school progress at 14 years of age. Australian Paediatric Journal, 24, 19-23.

Robertson, C. M. T., Etches, P. C., & Kyle, J. M. (1990). Eight-years school performance and growth outcome of preterm small-for-gestational age infants: A comparative study with subjects matched for birthweight or for gestational age. Journal of Pediatrics, 116, 19-26.

Rubin, R. A., Rosenblatt, C., & Balow, B. (1973). Psychological and educational sequelae of prematurity. Pediatrics, 52, 352-363.

Sameroff, A. J. (1986). Environmental context of child development. Journal of Pediatrics, 109, 192-200.

Scott, D. T. (1987). Premature infants in later childhood: Some recent follow-up results. Seminars in Perinatology, 11, 191-199.

Sehnal, J. P. & Palmeri, A. (1989). High-risk infants. In P. N. Pratt & A. S. Allen (Eds.), Occupational therapy for children (2nd ed.). St. Louis: C. V. Mosby.

Sell, E. J., Gaines, J. A., Gluckman, C., & Williams, E. (1985). Early identification of early problems in neonatal intensive care graduates. American Journal of Diseases of Children, 139, 460-463.

Short-DeGraff, M. A. (1988). Motor development. In M. A. Short-DeGraff (Ed.), Human development for occupational and physical therapists (pp. 445-477). Baltimore: Williams & Wilkins.

Siegel, L. S. (1982). Reproductive, perinatal, and environmental variables as predictors of development of preterm (< 1501 grams) and full term children at five years. Seminars in Perinatology, 6, 274-279.

Siegel, L. S., Saigal, S., Rosenbaum, P., Morton, R. A., Young, A., Berenbaum, S., & Stoskopf, B. (1982). Predictors of development in preterm and full-term infants: A model for detecting the at risk child. Journal of Pediatric Psychology, 7,

135-148.

Smith, L., Somner, F. F., & Tetzchner, S. V. (1982). A longitudinal study of low birth weight children: Reproductive, perinatal, and environmental precursors of developmental status at three years of age. Seminars in Perinatology, 6, 294-304.

Solan, H. A. & Mozlin, R. (1986). The correlations of perceptual-motor maturation to readiness and reading in kindergarten and the primary grades. Journal of the American Optometric Association, 57, 28-35.

Soltesz, M. J. & Brockway, N. F. (1989). The high-risk infant. In J. S. Tecklin (Ed.), Pediatric physical therapy (pp. 40-67). Philadelphia: J. B. Lippincott.

Stewart, A. L., DeL. Costello, A. M., Hamilton, P. A., Baudin, J., Townsend, J. Bradford, B. C., & Reynolds, E. O. R. (1989). Relationship between neurodevelopmental status of very preterm infants at one and four years. Developmental Medicine and Child Neurology, 31, 756-765.

Towen, B. C. L. (1986). Very low birth weight. European Journal of Pediatrics, 145, 460.

Towen, B. C. L. (1980). The preterm infant in the extrauterine environment. Implications for neurology. Early Human Development, 4, 287-300.

Ungerer, J. A. & Sigman, M. (1983). Developmental lags in preterm infants from one to three years of age. Child Development, 54, 1217-1228.

Van Holf-van Duin, J. & Mohn, G. (1984). Vision in the preterm infant. In H. F. R. Prechtl (Ed.), Continuity of Neural Functions from Prenatal to Posnatal Life (pp. 93-114). London: Spastic International Medical Publications.

Vohr, B. R., Coll, C. G., & Oh, W. (1988). Language development of low-birth weight infants at two years. Developmental Medicine and Child Neurology, 30, 608-615.

Vohr, B. R., Coll, C. G., & Oh, W. (1989). Language and neurodevelopmental outcome of low-birthweight infants at three years. Developmental Medicine and Child Neurology, 31., 582-590.

Walker, J. (1989) The behaviour of 3-year-old children who were born preterm. Child Care, Health and Development, 15, 297-313.

Williams, H. G. (1983). Perceptual and motor development. New Jersey: Prentice Hall.

Zubrick, S. R., Macartney, H., & Stanley, F. J. (1988). Hidden handicap in school-age children who received neonatal intensive care. Developmental Medicine and Child Neurology, 30, 145-152.

APPENDICES

University of Alberta
Edmonton
Canada T6G 2G4

Faculty of Rehabilitation Medicine
Office of the Dean
316 Corbett Hall, Telephone (403) 492-2903 5991
Fax (403) 492-1626

Appendix A

EVALUATION OF LANGUAGE, PERCEPTUAL MOTOR SKILLS, AND BEHAVIOR IN 3-4 YEAR OLD CHILDREN BORN PREMATURE

Dear

We have received your name from Annette Kudje, and thank you for allowing us to contact you in regard to our current project. We are interested in learning more about how children born before their 37th week of gestation are developing. We would like to evaluate many of your childs skills, including language, balance, perception, fine motor, and gross motor, as well as your childs' behavior as you see it. If you agree to participate in this project you will be given a questionnaire to fill out pertaining to your childs' behavior and development, and you and your child will be asked to come into the University (at a time most convenient for you) for evaluations of the skills described above. The questionnaire will take about 15 minutes to complete and it can be done either before your evaluation appointment or while your child is being evaluated. The evaluation will be videotaped and will take 1-1 1/2 hours of your time. The videotapes will be used for purposes of research only. They will be maintained in an archive. An ID number will be used to identify the tapes rather than your child's name.

The results of the evaluations will be used by us to increase our understanding of the development of premature children. Should we identify any specific problems in your child, we would be glad to discuss these with you and refer you to appropriate sources for help if it is warranted, and if you so wish. We cannot provide follow up within the limits of the current study.

Your participation is entirely voluntary and you are free to withdraw your child from this project at any time without affecting any current or future care your child may need. All information gathered will be kept confidential and no publication or presentation of this material will disclose the identity of yourself or your child.

As we discussed on the phone, your appointment has been scheduled for _____. The session will take approximately 90 minutes. We will do the evaluation at the Garneau Professional Building. The address of this building is 11044-82nd Ave., and it is just one block west of campus. There is parking in an underground garage, and we will sign the parking ticket to provide you with free parking.

If you would like more information prior to that time, or have any questions at any time, please feel free to call one of us at the phone numbers on the next page. Thank you again for agreeing to participate in this project.

Sincerely,

Shelly J. Lane, PhD
Assistant Professor,
Occupational Therapy

Gary Holdgrafer, PhD
Chair and Professor
Speech Pathology and Audiology

Shelly J. Lane, PhD
492-2499
(you may call this number and leave a messege; Shelly will get back to you)

Carla Attanasio
Graduate Assistant, Occupational Therapy
431-1906

Gary Holdgrafer, PhD
492-5980

Jacquie Forwards
Graduate Assistant, Speech Pathology and Audiology
435-4786

Appendix B

University of Alberta
Edmonton
Canada T6G 2G4

Faculty of Rehabilitation Medicine
Office of the Dean
316 Corbett Hall, Telephone (403) 492-2903 / 5991
Fax (403) 492-1626

CONSENT FORM

EVALUATION OF LANGUAGE, PERCEPTUAL MOTOR SKILLS, AND BEHAVIOR IN 3-4 YEAR OLD CHILDREN BORN PREMATURE

I have read and kept for my files the cover letter explaining the project designed to evaluate many skills and behavior in my child. I understand that the objective of this project is to gain a better understanding of the development of children born prematurely.

I understand that I will be given a behavioral and developmental questionnaire to fill out and return to the researchers, and that I will be asked to attend an evaluation session at the University with my child. This evaluation will take 1 - 1 1/2 hours to complete and will look at the development of my child in the areas of language, balance, perception, and gross and fine motor skills. I understand that this session will be videotaped and that these tapes will be kept in an archive, but will be used only for research purposes. An identification number, rather than my child's name, will be used to identify the tapes.

I understand that the information gathered will be kept confidential and no publication or presentation that may come as a result of this study will disclose the identity of my child or myself.

I understand that if the researchers identify problems in my child they will discuss these with me, and will refer us to the appropriate source for follow up if I so desire. I understand that they cannot provide follow up within this project.

I also understand that my participation is entirely voluntary and that I may withdraw my child from this project at any time without affecting any present or future treatment my child may require.

Before signing this form I have had all my questions answered to my satisfaction, but I also know that if I have any additional questions I may contact either Dr. Shelly J. Lane (492-7245) or Dr. Gary Holdgrafer (492- 5980) at any time.

_____ _____

signature date

_____

witness

APPENDIX C

Informed Consent Form for Preterm Study

THE RELATIONSHIP OF ENVIRONMENTAL/BIOLOGICAL FACTORS AND THE SENSORIMOTOR AND LANGUAGE DEVELOPMENT OF PRETERM CHILDREN

I have read the letter explaining the project. I understand that the purpose of this study is to better understand the development of children who are born early.

I understand that

1- I will complete a short questionnaire;

2- I will bring my child to the University for 40 minutes of testing. This test will look at his/her abilities in the areas of language, balance, perception, motor skills and behavior;

3- this session will be videotaped. These tapes will be marked with a number, instead of the child's name;

4- the information collected will be kept confidential. When findings are presented my child's or my name will not be given;

5- if the researcher finds problems in my child she will discuss this with me. She will suggest someone to contact if I so wish. I understand that she cannot provide follow up within this project.

6- my participation is voluntary. I may withdraw my child from this project at any time without causing any problem for my child.

Before signing this form my questions were all answered. I know that if I have any more questions I may call either Carla Attanasio (431-1906) or Joyce Magill-Evans (492-0402) at any time.

NAME (please print):_____

SIGNATURE:_____

DATE:_____

WITNESS:_____

RESEARCH:_____

APPENDIX D

THE RELATIONSHIP OF ENVIRONMENTAL/BIOLOGICAL FACTORS AND THE SENSORIMOTOR AND LANGUAGE DEVELOPMENT OF PRETERM CHILDREN.

Dear

We have received your name from Annette Kudja (research assistant). Thank you for allowing us to contact you. The purpose of this study is to learn about how children who are born early develop. We are interested in children ages three to four years.

We would like to see your child's ability to:

1. talk and understand commands (language);
2. move their arms and legs (motor skills) and balance;
3. do puzzles and find objects hidden in pictures (perception);
4. pay attention;

We would also ask you to answer some written questions about your education and if your child has been in day care.

The testing would be done by an occupational therapist at the university at a time of day that suits you. It would last 40 minutes and the children usually enjoy it. We will videotape the testing. The videotape and questionnaire will not have your child's name on it, only a number. When the findings of the study are presented at meetings or in articles, your name will not be given. The tapes will only be used for research and kept in a locked area and remain confidential.

If we find that your child has any problems, we will tell you and suggest someone you can contact if you wish. We cannot provide treatment as part of this study.

Being part of this study is completely voluntary. You may withdraw from the study at any time by telling us that you do not want to continue. This will not cause any problems for you or your child.

We will do the testing at the Garneau Professional Building. The address is 11044-82nd Ave. We will meet you on the fourth floor at the elevator. There is underground parking. We will pay for the parking.

If you have any questions, please feel free to call us at the phone numbers below. Thank you again for agreeing to participate in this project. The results of this study may help other children.

Sincerely,

Carla Soares Attanasio
Graduate Student,
Occupational Therapy
(431-1906)

Joyce Magill-Evans, PhD
Assistant Professor/Thesis Supervisor
Occupational Therapy
(492-0402)

APPENDIX E

ADDITIONAL INFORMATION

1- Mother's level of education when the child was born:

_____.

2- Mother's highest level of education: _____

3- Mother's years of education: _____

4- Has your child ever been enroled in a day care, day home or play school?

_____

If so, when and for how long?_____

APPENDIX F

Letter to the parents

Dear Mrs. _____,

Thank you very much for your cooperation in bringing ____ for evaluation last year as part of our study "The Relationship Between Biological/Environmental Factors and Sensorimotor and Language Development". In the beginning of the study, we sent you a letter explaining the objectives of the project and promised that we would inform you about any concerns which our evaluation found. We have completed our analysis of the results. According to the test we used, _____ is doing well in the areas of language, perception, balance and gross and fine motor skills. However, she was below average on some tests which evaluated the integration of sensory and motor abilities. We feel that _____ may need to be evaluated by another professional in order to better analyze her abilities as measured by other tests of these abilities. The test we used is only designed to identify children who need further evaluation. As she did well in all other areas, it is possible that her below average scores are due to a long and tiring test. If you have concerns about _____'s performance in these areas, we suggest that you contact your doctor so that he/she can refer _____ to an occupational therapist, who is able to evaluate _____'s skills in these areas. If you have any questions or concerns about our results, please contact me at 432-1134. We would like to thank you again for your participation and wish you and _____ all the best in the future.

Sincerely,

Carla Soares Attanasio
Graduate Student, Occupational Therapy