# ß-GALACTOSIDASE IN NORMAL AND MUTANT HUMAN CELLS

# ß-GALACTOSIDASE IN NORMAL AND MUTANT HUMAN CELLS

## ß-GALACTOSIDASE IN CELLEN VAN GEZONDE MENSEN EN CELLEN VAN PATIENTEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. A.H.G. RINNOOY KAN
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
VRIJDAG 18 DECEMBER 1987 OM 15.45 UUR

DOOR

ADRIAAN THEODORUS HOOGEVEEN

GEBOREN TE VLAARDINGEN

1987
Offsetdrukkerij Kanters B.V.,
Alblasserdam

PROMOTIECOMMISSIE

| | |
|---|---|
| Promotor: | Prof. Dr. H. Galjaard |
| Overige leden: | Prof. Dr. J.F. Koster |
| | Prof. Dr. J.M. Tager |
| | Prof. Dr. H.F.M. Busch |

Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Faculteit der Geneeskunde, Erasmus Universiteit Rotterdam.

*Aan Marijke, Vincent en Peggy*
*Aan mijn ouders*

CONTENTS

# Chapter I

## GENERAL INTRODUCTION

### I.1. LYSOSOMES AND LYSOSOMAL ENZYMES

Lysosomes are cytoplasmic vesicles containing hydrolytic enzymes, with an acidic pH optimum. Primary lysosomes are formed at the trans side of the Golgi complex of eukaryotic cells in an area called Trans Golgi Reticulum (TGR). After fusion with endosomes or autophagosomes, they become digestive vacuoles or secondary lysosomes (for reviews see, de Duve, 1983; Mellman, 1984; Quinn, 1984). Although lysosomes are morphologically heterogeneous and undergo diverse transformations by the process of fusion with other cellular membranes, they share several functional characteristics. When a tissue homogenate, containing lysosomes, is prepared and acid hydrolases are assayed under isosmotic conditions, the activity of acid hydrolases is low. However, when the tissue is subjected to mechanical stress, or treatment with a detergent, much more activity is revealed. It was this phenomenon of latency which led to de Duve's discovery of lysosomes in 1955. The existence of lysosomes was later confirmed by the electron microscopic studies of Novikoff, (1961). The presence of an intact membrane around the lysosomal enzymes prevents the escape of macromolecular substrates and enzymes from the lysosomes.

Some 70 lysosomal enzymes have been identified which are involved in the degradation of (glyco)proteins, (phospho)lipids, glycosphingolipids, (muco)polysaccharides, DNA and RNA. After degradation the low molecular weight constituents diffuse through, or are transported across the lysosomal membrane into the cytoplasm, here they can be reutilized in metabolic processes. The substrates for the lysosomal enzymes are derived from intracellular as well as extracellular material entering the lysosomes via the mechanism

of autophagy or endocytosis.

The lysosomal enzymes themselves are glycoproteins and thus it is a question why they are not degraded by the present acid proteases. This is especially relevant since it has been demonstrated that lysosomal enzymes tested are located within the same subcellular compartment (van Dongen et al., 1984). Probably a mechanism exists which protects the lysosomal enzymes against degradation by neighbouring acid hydrolases. It is possible that a special configuration, aggregation or interaction (binding) of the lysosomal enzymes to the lysosomal membrane plays a role in this protection.

Lysosomal enzymes, along with secretory proteins and plasma membrane proteins are synthesized on ribosomes bound to the endoplasmic reticulum. The preproform of a lysosomal enzyme enters the rough endoplasmic reticulum (RER) where the "signal" sequence is removed by proteolytic cleavage and asparagine residues are glycosylated (Blobel, 1980, Erickson et al., 1984). From the RER they reach the Golgi compartment. During passage through the Golgi apparatus the secretory proteins, membrane proteins and lysosomal enzymes undergo a number of posttranslational modifications essential for their routing and destination. The oligosaccharides on secretory proteins and membrane glycoproteins and some lysosomal glycoproteins are modified to sialic-acid containing "complex" type units. However, most of the lysosomal enzymes undergo a different series of modifications. Specific "high-mannose" oligosaccharide chains are formed which can be phosphorylated at the 6-carbon position of the five outermost mannose residues. This is accomplished by two Golgi enzymes. First, N-acetylglucosaminylphosphotransferase transfers N-acetylglucosamine-1-phosphate to the mannose residues to give a phosphodiester intermediate (Reitman et al., 1981a,b; Waheed et al., 1982). Secondly N-acetylglucosamine is removed by N-acetylglucosamine-1-phosphodiester $\alpha$-N-acetylglucosaminidase (Varki and Kornfeld, 1981; Waheed et al., 1981). This modification occurs in the

cis Golgi compartment (Goldberg and Kornfeld, 1983). The mannose 6-phosphate residue acts as a recognition marker for the mannose 6-phosphate (Man 6-P) receptors to ensure transport of lysosomal proteins to the lysosomes (Kaplan et al., 1977; for reviews see Creek and Sly, 1984; Kornfeld, 1986; Von Figura and Hasilik, 1986).

Two distinct Man 6-P receptors have been identified. The first is a glycoprotein with an apparent molecular mass of 215 kDa which binds acid hydrolases independently of cations (Sahagian et al., 1981; Steiner and Rome, 1982). This receptor is present in a variety of tissues, and constitutes 0.1-0.5% of total membrane protein (Von Figura and Hasilik, 1986). The second receptor has an apparent molecular mass of 46 kDa and requires divalent cations for high affinity binding with its ligand (Hoflack and Kornfeld, 1985a,b).

Both receptors require two phosphomannosyl residues in monoester linkage on the protein for high-affinity binding (Hoflack et al., 1987). They are immunologically unrelated (Stein et al., 1987). The Man 6-P receptors are present on the plasma membrane (probably less than 10%) and on intracellular membranes especially in the Golgi apparatus (for reviews see Creek and Sly, 1984; Kornfeld, 1986; Braulke et al., 1987). The results of immunoelectron microscopical studies have been conflicting. Some workers found Man 6-P receptors only in cisternae on the cis side of the Golgi stacks, and in lysosomes (Brown and Farguhar, 1984) whereas others demonstrated receptors throughout the Golgi cisternae and not in the lysosomes (Willingham et al., 1981; Geuze et al., 1985). The genes coding for the 215 kDa and the 46 kDa Man 6-P receptors have recently been cloned (Lobel et al., 1987; Dahms et al., 1987; Pohlman et al., 1987). Knowledge of the protein structures will contribute to a better insight into the specific functions of both receptors (Stein et al., 1987).

Receptor-bound enzymes are collected in vesicles of the Trans Golgi Reticulum which form pre-lysosomes where the low

pH causes dissociation of the enzyme from the receptors. The latter recycle to the Golgi apparatus where they can be reutilized. The acidification in the (pre) lysosome is mediated by an ATP driven anion dependent proton pump. To prevent over acidification, it is suggested that the ATPase is kinetically controlled by anion transporters which modulate the chloride concentrations and in this way control the acidification of the lysosomes (for review see Mellman et al., 1986). Recently such an ATPase has been purified from chromaffin granule membranes (Moriyama and Nelson, 1987).

In addition to the Man 6-P dependent routing there must be different pathways for lysosomal enzymes and lysosomal membrane proteins to reach their correct intracellular location. Two major sets of data lead to this hypothesis. Various mouse cell lines lacking Man 6-P receptors still have lysosomal enzyme activity (Goldberg et al., 1983). Moreover in cells from patients with "I-cell" disease, which are deficient in the phosphotransferase, required for the generation of the Man 6-P recognition marker, the activity of several lysosomal enzymes is close to normal (for reviews see Creek and Sly, 1984; Erickson et al., 1984; Von Figura and Hasilik, 1986). In this latter connection it should be noted that the highest activities in "I-cell" fibroblasts are those of membrane-bound lysosomal enzymes such as ß-glucocerebrosidase. This enzyme was found to be not phosphorylated in normal cells which would point to a different routing for lysosomal membrane (associated) proteins (Murray et al., 1985; Lewis et al., 1985; Tager et al., 1986; Krentler et al., 1986).

Within the lysosomal compartment, lysosomal enzymes undergo their last posttranslational processing steps such as proteolytic and carbohydrate modifications. These specific steps may induce conformational changes necessary for activation or stabilization of the enzyme, or for protection against degradation by neighbouring lysosomal enzymes. Further interaction with substrates, activators and other

components may occur (for reviews see in: Barranger and Brady, eds., 1984; in: Dingle et al. (1984); Kornfeld, 1986; Von Figura and Hasilik, 1986).

## I.2. LYSOSOMAL STORAGE DISEASES

The physiological importance of lysosomal enzymes for the degradation of many cellular substrates is revealed by the profound effects occuring if one single lysosomal enzyme is deficient. The storage diseases are associated with (severe) physical and mental handicaps and are often lethal in early life. Out of the total number of 70 lysosomal enzymes known (Barrett and Heath, 1977) a genetic defect leading to a disease has been demonstrated for 32 of them (Galjaard and Reuser, 1984; Stanbury et al.(eds.), 1983; Wenger et al., 1986; Cooper et al., 1986; Van Diggelen et al., 1987). Nearly all lysosomal storage diseases have an autosomal-recessive pattern of inheritance but two of them, Fabry's disease ($\alpha$-galactosidase deficiency) and Hunter's disease (sulfoiduronide sulfatase deficiency) are X-linked.

Within each lysosomal storage disease a considerable clinical and biochemical heterogeneity is found (Galjaard, 1980; Stanbury et al. (eds), 1983). To investigate the cause of this heterogeneity different approaches are used.

At the genetic level complementation analysis after somatic cell hybridization using cultured fibroblasts from patients with different clinical variants of "the same" disease is a useful tool in determining whether different genes or different mutations within one gene were involved (for reviews see Ringertz and Savage, 1976; Bootsma and Galjaard, 1979; Galjaard and Reuser, 1984; Reuser, 1984 see also chapter III).

Cultured cells from two patients with a different clinical phenotype and the same enzyme deficiency are fused and the restoration of enzyme activity is measured in heterokaryons. Different approaches have been used to

analyze heterokaryons carrying the genetic information from both parental cell strains in the presence of non fused cells or homokaryons (de Weerd-Kastelein et al., 1972; Galjaard et al., 1975; Jongkind et al., 1979; Nelson and Carey, 1985). If restoration of enzyme activity is observed after fusion this points to intergenic complementation, i.e. two different gene products are involved. Possible examples of intragenic complementation after human somatic cell hybridization are extremely rare.

At the level of the enzyme protein, measurement of the catalytic activity in cells from controls, heterozygotes and patients is not only commonly used for biochemical diagnosis and genetic counseling (Galjaard, 1980, Stanbury et al. (eds), 1983), but also for understanding the cause of clinical heterogeneity (Brady and Barranger, 1983; O'Brien, 1983; Conzelmann and Sandhoff, 1983/84; Reuser et al., 1987). In this context it is important to note that many diagnostic enzyme assays are performed on easily accessible cell material such as leucocytes and skin fibroblasts whereas the clinical and pathological features are of course determined by cellular and metabolic abnormalities in a variety of organs.

Also, in diagnostic enzyme assays there may be a discrepancy between the activity as measured with an artificial substrate and with the natural substrate for the enzyme (Dreyfus et al., 1975, Navon et al., 1976).

Furthermore differences in residual enzyme activity may express differently in test tube assays then "in vivo" analyses where natural substrate is added to living fibroblasts and the metabolism of ingested substrate is followed by chromatography (Kudoh et al., 1981).

These examples and theoretical considerations described by Conzelmann and Sandhoff (1983/84) make it clear that it will often be difficult to relate residual lysosomal enzyme activity directly to the clinical expression of a lysosomal storage disease.

As has been outlined in the previous section the forma-

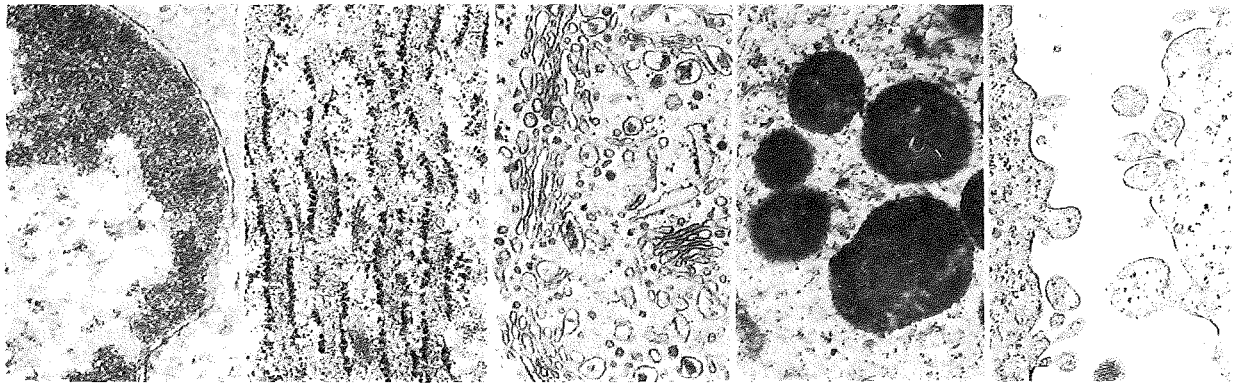
tion of mature, active lysosomal enzymes involves transcription, translation and a number of posttranslational modifications. In principle a particular lysosomal enzyme deficiency can be the result of a defect in each of these steps (Fig. 1) (see also Tager, 1985; Kornfeld, 1986).

Both when different gene products are involved and in the case of different mutations within one gene, studies on the biosynthesis and posttranslational modification of a particular (enzyme) protein in normal and mutant fibroblasts have proved to be of great help in obtaining insight in the molecular background of clinical heterogeneity. Cultivation of fibroblasts in the presence of radioactive labelled amino acid, monosaccharide or phosphate, followed by immunoprecipitation and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Hasilik and Neufeld, 1980) enabled the exact delineation of the molecular defect(s) involved in different clinical variants of several lysosomal storage disorders (for reviews see in: Barranger and Brady, (eds.) 1984)

Biochemical data on the biosynthesis of (enzyme) proteins can also be related to subcellular morphology using immunoelectron microscopy. In this respect the use of ultrathin cryosections and colloidal gold conjugates meant an important step forward in the subcellular localization of normal and mutant lysosomal enzymes and their precursor forms (Geuze et al., 1981, 1985; van Dongen et al., 1984).

The various approaches mentioned above will often provide a clue as far as the molecular impairment is concerned (see Fig. 1). For an ultimate understanding of the molecular pathogenesis and as a basis for future treatment, knowledge about the synthesis, the amino acid sequence and the tertiary structure of the normal and mutant protein(s) is of importance. Much progress is being made in the technology of amino acid sequencing and calculation of three-dimensional protein structure. However, it is often difficult or impossible to obtain sufficient cell material from patients with the genetic metabolic disease of interest.

## DIFFERENT TYPES OF GENETICALLY DETERMINED (ENZYME) PROTEIN DEFECTS



| NUCLEUS | ENDOPLASMIC RETICULUM | GOLGI | LYSOSOMES or other organelles | CELL SURFACE |
|---|---|---|---|---|
| – no or erroneous transcription | – no protein synthesis<br>– enhanced degradation of newly synthesized protein<br>– no segregation from E.R. | – impaired glycosylation<br>– impaired phosphorylation<br>– wrong compart-mentalization<br>– secretion or enhanced degradation | – transport defect<br>– no binding to other proteins<br>– enhanced degradation<br>– impaired binding to substrate<br>– impaired enzymatic activity | – absence of receptor<br>– functional inactive receptor |

Fig. 1

Also the isolation of a mutant protein may be complicated by its abnormal kinetics and even worse, there might be no protein present at all.

During the last few years the use of recombinant DNA technology has largely overcome the problems mentioned above. Thus far, the cDNA's coding for some 15 lysosomal enzymes, have been cloned. For a number of them the complete sequence of the normal gene has been reported and in a few cases the exact nature of the mutations different clinical variants has been elucidated (Myerowitz and Hogikyan, 1986; Tsuji et al., 1987; Kolata, 1987). In view of the rapid progress in this area of research, no doubt many different mutations involved in the 32 lysosomal storage diseases presently known, will be mapped within the next decade.

# Chapter II

## THEORETICAL BACKGROUND OF THE EXPERIMENTAL WORK

The experimental work presented in this thesis deals with the genetic and molecular basis of lysosomal ß-galactosidase deficiencies in man. The first section (II.1) deals with the characteristics of lysosomal ß-galactosidase and the second (II.2) with its major natural substrates, $G_{M1}$-ganglioside and keratan sulfate, and the activator proteins. A single lysosomal ß-galactosidase deficiency is the cause of the lysosomal storage diseases $G_{M1}$-gangliosidosis and Morquio B syndrome. The clinical pathological and biochemical features of the different variants of these diseases are summarized in section II.3.

A ß-galactosidase deficiency in man also occurs as part of a multiple lysosomal enzyme deficiency. In patients with galactosialidosis there is a coexistent deficiency of lysosomal neuraminidase and lysosomal ß-galactosidase, while in mucolipidosis II ("I-cell" disease) many lysosomal (enzyme) proteins, including ß-galactosidase, are deficient due to a defective Man 6-P recognition marker (see Chapter I). The major features of these two diseases are summarized in section II.4. Finally, some aspects of therapy of lysosomal storage diseases and animal models are discussed in section II.5.

### II.1. ß-GALACTOSIDASE

#### a. Acid ß-Galactosidase

Acid ß-galactosidase (E.C.3.2.1.23) is a glycoprotein which hydrolyses the terminal galactose from the natural substrates $G_{M1}$-ganglioside, asialo-$G_{M1}$-ganglioside, asialo-fetuin, keratan sulfate and lactosylceramide (for reviews see Roden, 1980; Rittman and O'Brien, 1981; Suzuki and

Suzuki, 1983). The enzyme is also able to hydrolyse galactose from artificial substrates as p-nitrophenyl-ß-D-galactopyranoside and 4-methylumbelliferyl-ß-D-galactopyranoside (Gatt and Rapport, 1966; Robins et al., 1968; Ockerman, 1968). Acid ß-galactosidase has a pH optimum of 4.2, it is heat labile and can be activated with chloride ions (Ho and O'Brien, 1971a; for review see O'Brien, 1983).

The acid ß-galactosidase activity has been demonstrated in a wide variety of human tissues and body fluids. Initially it was most extensively studied in human liver (Ho et al., 1973; Norden et al., 1974; Suzuki and Suzuki, 1974; O'Brien, 1975; Tanaka et al., 1975; Cheetham and Dance, 1976; Frost et al., 1978; Heyworth et al., 1981; Overdijk et al., 1986). Further ß-galactosidase has also been studied in human brain (Norden and O'Brien, 1973; Lisman and Hooghwinkel, 1974; Bruns and Hultberg, 1975; Tanaka and Suzuki, 1976, 1977), in cultured human skin fibroblasts (Pinsky et al., 1974; Galjaard et al., 1975; Hoeksema et al., 1979; Van Diggelen et al., 1981, 1982; d'Azzo et al., 1982; Hoogeveen et al., 1983, 1984, 1986), in placenta (Lo et al., 1979), in kidney and urine ( (Dance et al., 1969; Kress and Miller, 1978), in small intestine (Gray and Santiago, 1969; Asp, 1971) and in human leucocytes (Singer and Schafer, 1970; Kolodny and Mumford, 1976).

In addition, ß-galactosidase has been purified from other species such as calf, chicken, ox, pig, cat, dog, mouse, rabbit and monkey. The existence of multiple forms of ß-galactosidase has been reported by many workers. These forms can be separated on the basis of their different electrophoretic mobility and molecular weight (Ho et al.,1973; Norden and O'Brien, 1973). The separation and purification studies of ß-galactosidase demonstrated the existence of a monomeric, a dimeric and a multimeric form (Norden et al., 1974; Tomino and Meisler, 1975; Frost et al., 1978; Tanaka and Suzuki, 1976; Hoeksema et al., 1979; Yamamoto et al., 1982, Yamamoto and Nishimura, 1980, 1986; Rodrigues Berrocal et al., 1983). The molecular mass found

for the different ß-galactosidase forms in the liver was 65,000, 150,000 and 700,000 Da. (Norden and O'Brien, 1973; Cheetham and Dance, 1976; Frost et al., 1978). Because of their immunological relationship it was suggested that the high molecular weight form is a multimeric aggregate of the monomeric low molecular weight form (O'Brien, 1975). It has been demonstrated that the aggregation of ß-galactosidase is enhanced by the presence of its substrate (Taguchi et al., 1981), and by enzyme concentration (Cheetham and Dance, 1976; Verheijen et al., 1982).

In addition to the 65,000, 150,000 and 700,000 Da. molecular weight forms, several authors reported the presence of some low molecular weight proteins of 30,000 and 20,000 Da. in human liver (Frost et al., 1978), human placenta (Lo et al., 1979), feline liver (Holmes and O'Brien, 1979), and porcine spleen (Yamamoto et al., 1982; Yamamoto and Nishimura, 1986), the significance of which was unclear.

The first amino acid analysis for ß-galactosidase was made by Frost et al. (1978), who isolated the dimeric form of ß-galactosidase from human liver. They found a high content of neutral and acid amino acids and a low content of the basic and sulfur containing amino acids. Overdijk et al., (1986) found that the carbohydrate content of the enzyme from human liver was about 9% by weight and they reported ß-galactosidase as the first soluble lysosomal hydrolase with a high content of N-acetyllactosamine-type (complex type) of carbohydrate (60%). The rest is of the oligomannoside type. An average of three carbohydrate chains per ß-galactosidase molecule was calculated. Other human lysosomal enzymes tested have almost exclusively oligomannoside-type side chains, such as ß-glucuronidase from spleen (Natowicz et al., 1982; Howard et al., 1982), and -glucosidase from placenta (Mutsaers et al., 1987). The membrane-bound proteins which are independent of the mannose 6-phosphate receptor for their transport to the lysosome, also contain N-acetyllactosamine type carbohydrate chains

like ß-galactosidase. An example is the 80% content of N-acetyllactosamine type carbohydrate on the membrane bound lysosomal enzyme ß-glucocerebrosidase (Takasaki et al., 1984). These data are in agreement with the results of immunoelectron microscopic studies on ß-glucocerebrosidase which suggest that the membrane association of this enzyme carries it to the lysosome independent of the mannose 6-phosphate recognition system (Van Dongen et al., 1985).

The structural gene coding for ß-galactosidase was initially assigned to chromosome 22 (De Wit et al. 1977), but subsequently Bruns et al. (1978) and Shows et al. (1978) reported that the ß-galactosidase locus is on chromosome 3. Later, De Wit et al. (1979) demonstrated that two structural gene loci on chromosome 3 and 22 were required for the full expression of ß-galactosidase activity. More recently Sips et al. (1985), using monoclonal antibodies, confirmed the localization of the ß-galactosidase structural gene on chromosome 3, while a gene on chromosome 22 coded for a 32 kDa protein, necessary for the aggregation of ß-galactosidase molecules in the lysosome (Hoogeveen et al., 1983) (see also Chapter III).

b. Galactosylcerebrosidase

Another acid ß-galactosidase is the enzyme which is deficient in Krabbe's disease (Suzuki and Suzuki, 1970; Tanaka and Suzuki, 1977; for review see Suzuki and Suzuki, 1983). This enzyme catalyzes the hydrolysis of ß-linked galactose from galactosylcerebroside (Suzuki and Suzuki, 1971) and does not hydrolyse $G_{M1}$-ganglioside. Therefore it is called galactosylcerebroside-ß-galactosidase or galactosylcerebrosidase (EC 3.2.1.46).

Antibodies raised against acid ß-galactosidase do not cross-react with galactosylcerebrosidase (Norden et al., 1974), which apparently is a different protein.

c. Neutral ß-Galactosidase

Neutral ß-galactosidase has a molecular mass of 45,000-57,000 Da. and a pH optimum of 5.6-6.0. Neutral ß-galactosidase cleaves aryl-ß-D-galactoside and aryl-ß-D-glucoside linkages and is inhibited by chloride ions (Ho and O'Brien, 1971a). Studies in pig (Abrahams and Robinson, 1969), in man (Meisler, 1975; Chester et al., 1976) and in rabbit (Van Zutphen et al., 1983), strongly suggest that the neutral ß-D-galactosidase and the neutral ß-D-glucosidase are identical enzymes. To study the physiological role of neutral ß-galactosidase, the rabbit strain Y-I/J which has a deficiency for this enzyme, may serve as an interesting animal model (Van Zutphen, 1983).

Ho et al. (1973) demonstrated that the neutral ß-galactosidase does not cleave galactose from $G_{M1}$-ganglioside. Antisera raised against acid ß-galactosidase do not cross-react with neutral ß-galactosidase (Norden et al. 1974). These data indicate that the acid and neutral ß-galactosidase are genetically independent.

## II.2. NATURAL SUBSTRATES FOR ß-GALACTOSIDASE AND ACTIVATOR PROTEINS

a. Gangliosides

Glycosphingolipids are complex derivatives of ceramide which contain one or more saccharide units on the primary alcohol group of the sphingosine. The oligosaccharide moiety may be a branched chain. It is usually composed of galactose, fucose, glucose, N-acetylglucosamine and N-acetylgalactosamine. When the lipid contains a sialic acid residue, the lipid is called ganglioside (sialoglycosphingolipid).

Gangliosides were first detected by Landsteiner and Levene (1925) in kidney and later by Walz (1927) in spleen and brain. They were rediscovered by Klenk (1935) and isolated by Blix (1938) and Klenk (1942) from brain. Klenk

found the lipid mainly in the "ganglienzellen" (neuronal perikaryon cells) and named it ganglioside. In 1956 Svennerholm postulated that gangliosides are heterogeneous in their carbohydrate composition. The gangliosides have a common basic structure of galactose - N-acetylgalactosamine - galactose - glucose - N-acylsphingosine and may contain one, two or three sialic acid residues. The nomenclature still used today was based on these features (Svennerholm, 1962, 1963). In 1963 Kuhn and Wiegandt analized the structure of the four major brain gangliosides.

The ganglioside biosynthetic pathway is a stepwise process in which monosaccharide units are added to the glycolipid. The major monosialoganglioside in brain is $G_{M1}$-ganglioside ($G_{M1}$). For the biosynthesis of $G_{M1}$ five sugars are added to ceramide in a strict sequence by five different glycosyltransferases (Roseman, 1970). Gangliosides are especially abundant in nerve-ending membranes of the brain, however they are also found in extraneuronal tissues and cells such as spleen, liver, kidney, placenta, erythrocytes and also in serum. They are the characteristic lipid components of some neuronal membranes of the central nervous system, and are probably involved in the transmission of impulses along the neurons.

Gangliosides are mainly present at the outer surface of the plasma membrane, where the hydrophobic tails of the lipid molecules interact with the hydrophobic regions of the membrane. The oligosaccharide chain is exposed to the external environment of the cell. In the normal plasma membrane the lipid bilayer constitutes about 50% of the total mass since the proteins are much bigger than the lipids. Approximately one protein molecule is present for every 50 lipid molecules. Although the basic structure of the biological membrane is determined by the lipid bilayer, its specific functions are carried out mainly by the membrane proteins. Despite the knowledge of the structure, physical properties and localization of gangliosides, there are relatively few indications what their function might be. It

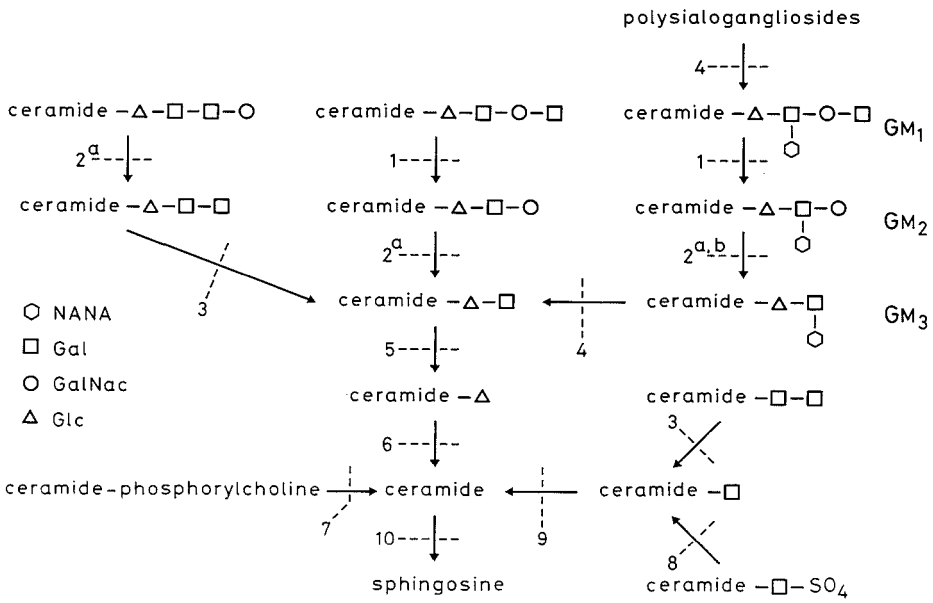
has been proposed that the variation in carbohydrate composition, exposed to the external environment make them ideal candidates to function as receptor molecules on the cell surface. For example, $G_{M1}$-ganglioside acts as a cell surface receptor for cholera toxin (Holmgren et al., 1973). However the binding of toxins cannot be the "normal" function of gangliosides. But it gives an indication that the possible function for the gangliosides is, to serve as receptors for normal signalling between cells.

They play a role in dynamic cellular processes such as the regulation of growth and proliferation (for reviews see Hakomori, 1981; Ledeen, 1983). Although the mechanism underlying these effects are unknown, several lines of evidence have emerged suggesting that gangliosides could mediate these processes by modulating protein kinase activities (Bremer et al., 1986; Kim et al., 1986; Kreutter et al., 1987). Recently it has been proposed that the inhibition of protein kinase C by sphingolipids might cause the severe neuronal degeneration and loss of cells from the cerebral and cerebellar cortex and the demyelination which has been observed in patients with sphingolipidosis and which could not be explained by lysosphingolipid accumulation alone (Hannun and Bell, 1987).

Gangliosides are catabolized by the stepwise removal of sugar molecules by hydrolases at the nonreducing end of the oligosaccharide chain (Ohman et al. 1970; Svennerholm, 1976; Brady, 1978). If one of the hydrolases is deficient, further breakdown is blocked, even though the remaining hydrolases in the pathway are present (see fig. 2). The amount and nature of lipid accumulation in the various organs will mainly depend on the normal distribution of the different sphingolipids and their rate of synthesis.

Fig. 2

CATABOLISM OF SPHINGOLIPIDS AND THE LOCATION OF THE GENETIC BLOCKS




| NO. DISEASE | ENZYMIC DEFECT | STORED MATERIAL |
|---|---|---|
| 1. $G_{M1}$-gangliosidosis | - (acid) β-galactosidase | - $G_{M1}$-ganglioside, keratan sulfate, asialo $G_{M1}$-ganglioside., galactose rich fragments of glycoproteins |
| 2 a. Sandhoff's disease | - β-hexosaminidase A,B | - $G_{M2}$-ganglioside, asialo $G_{M2}$-ganglioside, globoside |
| b. Tay Sachs disease | - β-hexosaminidase A | - $G_{M2}$-ganglioside |
| 3. Fabry disease | - α-galactosidase A | - Trihexosylceramide, digalactosylceramide |
| 4. Sialidosis | - neuraminidase | - (sialo)gangliosides, sialic acid rich fragments glycoproteins |
| 5. Lactosylceramidosis | - β-galactosidase | - lactosylceramide |
| 6. Gaucher disease | - glucocerebrosidase | - glucocerebroside |
| 7. Niemann-Pick disease | - sphingomyelinase | - sphingomyeline |
| 8 a. Metachromatic leucodystr. | - Arylsulfatase A | - lactosyl ceramide sulfate, sulfatide |
| b. Multiple sulfatase defic. | - Arylsulfatase A, B, C | - lactosyl ceramide sulfate, sulfatide, cholesteryl sulfate, sulfated glycosaminoglycans |
| 9. Krabbe's disease | - galactocerebrosidase | - galactosylcerebroside |
| 10. Farber's disease | - ceramidase | - ceramide (acylsphingosine) |

b. Carbohydrates

Lysosomal ß-galactosidase is not only involved in the intracellular degradation of gangliosides but also in the catabolism of keratan sulfate and galactose containing oligosaccharides derived from glycoproteins. As a consequence in patients with a ß-galactosidase deficiency accumulation of gangliosides, keratan sulfate and galactose containig oligosaccharides occurs in various organs; the latter two compounds are also excreted in excessive amounts in the urine (for reviews see Strecker, 1981; Warner and O'Brien, 1982; McKusick and Neufeld, 1983; O'Brien, 1983).

Of the glycosaminoglycans keratan sulfate is the only galactose containing polymer; D-galactose residues, which may be sulfated or unsulfated, alternate with N-acetylglucosamine residues.

Two types of keratan sulfate (I and II) have been distinguished (Meijer, 1970). Keratan sulfate I is a major component of the cornea and in a native state, the corneal polysaccharide is part of a proteoglycan that contains no other polysaccharide chain (Berman, 1970). Keratan sulfate I is linked to protein via the N-glycosylamine type of linkage between N-acetylglucosamine and the amide group of an asparagine residue (Baker et al., 1975).

Keratan sulfate II is mainly found in skeletal tissues. It is always associated with chondroitin sulfate as part of the same proteoglycan. Keratan sulfate II is linked to protein via O-glycosidic linkages between N-acetylgalactosamine and the hydroxyl groups of threonine and serine residues (Bray et al., 1967). In addition to the components of the repeating disaccharide unit, keratan sulfate of both types contains additional monosaccharide components such as sialic acid, galactosamine, mannose and fucose.

A number of enzymes, including ß-galactosidase, is involved in the lysosomal catabolism of keratan sulfate and a genetic deficiency of each of these enzymes results in

intracellular accumulation and increased urinary excretion of keratan sulfate (for reviews see Buddecke and Kresse, 1974; Cantz and Gehler, 1976; Roden, 1980; McKusick and Neufeld, 1983).

A large number of glycoproteins have galactose-containing oligosaccharide side chains (Strecker and Montreuil, 1979). In the case of a ß-galactosidase deficiency the catabolism of these glycoproteins will be impaired, but through the action of an endo-ß-N-acetylglucosaminidase the oligosaccharide chains are cleaved from the peptide moieties. Depending on the nature of the other (terminal) sugars, sialic acid or fucose residues will be cleaved from the oligosaccharide chains but further degradation will be blocked. In patients with a ß-galactosidase deficiency a large variety of galactose-containing oligosaccharides have been identified in liver and urine (Wolfe et al., 1974; Strecker and Montreuil, 1979; Ohkuma et al., 1981; Yamashita et al., 1981) (for review see Strecker, 1981).

c. Activator Proteins

Some water soluble hydrolases require non-enzymic, low molecular mass proteins called activator proteins, for the degradation of sphingolipids in the lysosome. Activator proteins bind to lipids to form water soluble lipid-protein complexes. They act as a kind of specific physiological detergent which facilitates the interaction between a water soluble hydrolase and its glycolipid substrate (for reviews see Sandhoff, 1984; Li and Li, 1984).

In 1964 Mehl and Jatzkewitz fractionated a partially purified arylsulfatase A preparation from porcine liver by carrier-free electrophoresis. The enzyme obtained, appeared to have lost the ability to degrade sulfatides in the absence of detergents. The enzyme activity could, however, be restored by addition of another enzymically inactive fraction which was later shown to be the first activator protein detected. This activator protein was subsequently found in

normal human tissue (Mehl and Jatzkewitz, 1968). The cerebroside sulfatase activator was purified and turned out to be a water-soluble glycoprotein with an apparent molecular weight of 21,500 (Jatzkewitz and Stinshoff, 1973). Stevens et al. (1981) reported two siblings of consanguineous parents with clinical features of juvenile metachromatic leucodystrophy who had, however, relatively high residual activities of arylsulfatase A in their fibroblasts. Addition of the sulfatide activator to the culture medium led to a normalization of the arylsulfatase A activity in the patient's cells.

Li and Li (1976) purified an activator protein which accelerates the degradation of $G_{M1}$-ganglioside (see also Wenger and Inui, 1984). Gartner et al. (1983) identified an activator protein specifically enhancing the hydrolysis of globotriaosylceramide by $\alpha$-galactosidase.

By using immunological methods and skin fibroblasts from the patients reported by Stevens et al. (1981) the identity of the three activator proteins mentioned above was demonstrated (Inui et al., 1983; Li et al., 1985). Paschke and Kresse (1982) found an increase of ß-galactosidase activity after addition of $G_{M1}$-ganglioside activator to fibroblasts from Morquio B patients.

At present two other independent activators are known. One is unique in enhancing the hydrolyses of $G_{M2}$-ganglioside by ß-hexosaminidase A and has an apparent molecular weight of 22,000 in kidney (Conzelmann and Sandhoff, 1979) and 23,500 Da. in liver (Li et al., 1981). Several patients with a genetic deficiency of this $G_{M2}$-ganglioside activator protein have been described (for review see Sandhoff, 1984; Conzelmann and Sandhoff, 1987). The second is an activator protein which enhances the hydrolysis of glucosylceramide by glucocerebrosidase (Ho and O'Brien, 1971b; Vaccaro et al., 1985) of galactosylceramide by ß-galactocerebrosidase (Wenger et al., 1982) and of sphingomyelin by sphingomyelinase (Christomanou et al., 1986).

## II.3. SINGLE HUMAN ß-GALACTOSIDASE DEFICIENCIES

### a. $G_{M1}$-Gangliosidosis

Jatzkewitz and Sandhoff (1963) demonstrated the presence of an excessive amount of ganglioside in the brain of a patient with infantile amaurotic idiocy. They defined this ganglioside storage disorder as different from Tay-Sachs disease ($G_{M2}$-gangliosidosis). Landing et al. (1964) reported on eight patients with a lysosomal storage disease, which they called "familial neurovisceral lipidosis". They pointed out that patients described previously by various authors (Craig et al., 1959; Norman et al., 1959; Landing and Rubinstein, 1962) appeared to be examples of the same disease which they recognized as a specific clinical entity. Subsequently, other patients were identified (Gonatas and Gonatas, 1965; O'Brien et al. 1965) and on the basis of increased amounts of $G_{M1}$-ganglioside in liver and spleen the disease was called "generalized gangliosidosis" (O'Brien et al., 1965). It was also postulated that the storage of $G_{M1}$-ganglioside is caused by a defect in the enzyme system involved in the degradation of these gangliosides.

A low ß-galactosidase activity was reported in patients with a $G_{M1}$-ganglioside storage disease (Sacrez et al., 1967; Seringe et al., 1968; Dacremont and Kint, 1968). In the same year, 1968, Okada and O'Brien suggested that the ß-galactosidase deficiency is the responsible enzyme defect in this autosomal recessive disease. The enzyme activity can be assayed with its natural substrate, $G_{M1}$-ganglioside, or with artificial substrates such as p-nitrophenyl-ß-D-galactopyranoside and 4-methylumbelliferyl-ß-D-galactopyranoside. Suzuki (1968) proposed the present nomenclature of $G_{M1}$-gangliosidosis because $G_{M1}$-ganglioside is the main storage product. During the last two decades different clinical forms of $G_{M1}$-gangliosidosis have been reported (for reviews see Galjaard and Reuser, 1977; Rittman and O'Brien, 1981;

O'Brien, 1983).

A profound deficiency of ß-galactosidase activity has been demonstrated in a variety of tissues from patients with the infantile or the juvenile form of $G_{M1}$-gangliosidosis. Both with the natural substrates $G_{M1}$-ganglioside and its asialo derivative and with artificial substrate a decreased activity varying from less than 1% to 5% of control values is found in liver, brain and spleen (Norden and O'Brien, 1974; O'Brien 1983), whereas the activity is hardly detectable in leucocytes and skin fibroblasts (Galjaard and Reuser, 1977).

In the adult form of $G_{M1}$-gangliosidosis the residual activity of ß-galactosidase is of the order of 10-15% of control values.

The infantile form

Patients with the infantile form of $G_{M1}$-gangliosidosis show psychomotor retardation from birth on. Physical examination reveals gargoyle facial features, hepatosplenomegaly and skeletal deformities. The mental and motor development detoriate rapidly and ultimately the child has hardly any contact with the environment. If the patient survives the first year of life, the clinical course is that of decerebrate rigidity with blindness, deafness, spastic quadriplegia and unresponsiveness. Recurrent respiratory infections are usually responsible for an early death before the age of two years (Derry et al., 1968; van Hoof et al., 1973; Galjaard, 1980; O'Brien, 1983).

A 2-10 fold increase of $G_{M1}$-ganglioside has been demonstrated in the gray matter of brain (Gonatas and Gonatas, 1965; Suzuki et al., 1971; Berra et al., 1974). In addition an accumulation of the asialoderivative also occurs (Suzuki et al., 1969). The abnormalities of the visceral organs seem to be caused mainly by the accumulation of certain glycosaminoglycans (keratan sulfate) and glycopeptides (Suzuki et al., 1969; Callahan and Wolfe, 1970).

The pathological changes in the infantile form include neuronal lipidosis, visceral histiocytosis and cytoplasmic ballooning of the renal glomerular epithelial cells. Neurons contain numerous cytoplasmic membraneous bodies. Foamy histiocytes are found in bone marrow, liver, spleen, lymph nodes and most visceral organs.

Two patients have been reported with an atypical form of infantile $G_{M1}$-gangliosidosis (Kohlschutter et al., 1982; Charrow and Hvizd, 1986). Impaired catabolism was demonstrated by ganglioside loading of cultured skin fibroblasts and a ß-galactosidase activity of less than 1% of control values was found. Intermediate levels between controls and patient were found in leucocytes from both parents. The prominent feature is cardiac involvement suggestive for endocardial fibroelastosis but, with the recognition of developmental delay and skeletal myopathy glycogenosis type II (Pompe disease) was considered more likely. These atypical patients do not show seizures, coarse facies, macroglossia, and dysostosis multiplex. They died within the first year of life. Further genetic and molecular studies are needed for a correct classification of these atypical variants.

The juvenile form

In patients with the juvenile form of $G_{M1}$-gangliosidosis the onset of clinical features usually starts at 1 or 2 years of age and the course of the disease is milder than in the infantile form. Mental and motor development is often normal during the first year. Subsequently psychomotor deterioration becomes apparent but hepatosplenomegaly and skeletal deformities are absent, at least initially. The progression of cerebral degeneration is slower than in patients with the infantile form. There is quite some variation among the different patients described (Derry et al., 1968; Hooft et al., 1970; O'Brien et al., 1971; Gilbert et al., 1975; for review see O'Brien, 1983).

Skeletal abnormalities of a mild nature may occur and radiological changes can be of diagnostic value. Often neurological abnormalities become apparent such as locomotor ataxia, seizures, loss of coordination and speech, muscular weakness, and progressive spasticity of upper and lower extremities. Recurrent bronchopneumonia usually leads to the patient's death; the life span varies from 3 to 10 years (O'Brien, 1983).

In the juvenile form, neuronal lipidosis is found, but it is less prominent than in the infantile form. Visceral histiocytosis is usually present but not as abundant as in the infantile form. Renal glomerular cytoplasmic ballooning is similar to that in the infantile form. The accumulation of $G_{M1}$-ganglioside in the brain of several patients with the juvenile form was similar to that in the infantile form, but no storage was found in visceral organs. In the latter, accumulation of keratan sulfate has been reported (Suzuki et al., 1971; Wolfe et al., 1970; Mamelle et al., 1975).

The adult form

In the adult form of $G_{M1}$-gangliosidosis clinical features usually start at age 3 to 10 years, but several patients have been diagnosed at a much later age. Intellectual impairment has been mild but with time intellectual functions are lost. There is progressive dysarthria, gait disturbance and extrapyramidal symptoms such as dystonia. Radiologic studies reveal mild skeletal abnormalities. Progression is slow and patients survive onto late adulthood (O'Brien et al., 1976; Suzuki et al., 1977, 1979; Stevenson et al., 1978; Wenger et al., 1980, Goldman et al., 1981; Otha et al., 1985; Mutoh et al., 1986). A clear distinction between the juvenile and adult forms may be difficult. Also, biochemical and clinical studies have demonstrated that a number of patients originally described as adult $G_{M1}$-gangliosidosis, in fact are patients with galactosialidosis due to a combined deficiency of ß-galactosidase and neurami-

nidase (Suzuki et al., 1979; Wenger et al., 1980; Koster et al. 1976).

b. Morquio B syndrome (Mucopolysaccharidosis type IVB)

One of the mucopolysaccharidoses (MPS) i.e. type IV (Morquio disease) is caused by a deficiency of N-acetylgalactosamine 6-sulfate sulfatase (Matalon et al., 1974; Singh et al., 1976). Clinical variants were found with mild dysostosis multiplex, corneal clouding, short stature, keratansulfaturia but no detectable central nervous system abnormalities (O'Brien et al., 1976; Arbisser et al., 1977). In these variants a markedly reduced activity of ß-galactosidase was found while the N-acetylgalactosamine 6-sulfate sulfatase was normal. This mild form was called Morquio B syndrome (MPS IVB) (Groebe et al., 1980; Trojac et al., 1980; Van Gemund et al., 1983). The residual ß-galactosidase activity (5-10% of controls) exhibits normal thermal stability and has a normal pH optimum. However, the Km for p-nitrophenyl ß-D-galactoside is 5 times higher than normal. The idea that the ß-galactosidase deficiency is the primary defect in Morquio B syndrome was further supported by the intermediate enzyme levels found in leucocytes and fibroblasts from the parents (Groebe et al., 1980). Morquio B syndrome is thus a separate genetic and biochemical entity from the mucopolysaccharidosis IV A (Morquio A syndrome).

Paschke and Kresse (1982) as well as Van der Horst et al. (1983) found no detectable affinity of the mutant ß-galactosidase in Morquio B towards keratan sulfate and oligosaccharides isolated from the patient's urine. This explains the mucopolysaccharidosis-like phenotype of Morquio B patients. However, a very high residual activity of the mutant enzyme was found when $G_{M1}$-ganglioside was used as a substrate (Paschke and Kresse, 1982). This might explain the absence of neurological abnormalities. After hybridization of Morquio B and $G_{M1}$-gangliosidosis fibroblasts no restoration of ß-galactosidase activity was observed which in

combination with other results indicates that these two syndromes are caused by allelic mutations in the gene coding for ß-galactosidase (Van der Horst et al., 1983).

## II.4. MULTIPLE LYSOSOMAL ENZYME DEFICIENCIES

### a. Galactosialidosis

Several patients with clinical features somewhat different from those in the "classical" forms of $G_{M1}$-gangliosidosis were designated as atypical variants of this disease (Pinsky et al, 1974; Wenger et al., 1974; Loonen et al., 1974; Yamamoto et al., 1974; Andria et al., 1978). Complementation studies revealed that the mutation involved in the patients described by Pinsky et al., (1974) and Loonen et al., (1974) had to be localized in a gene different from that involved in the infantile and juvenile forms of $G_{M1}$-gangliosidosis (Galjaard et al., 1975). Later, similar somatic cell hybridization studies revealed that the patients described by Wenger et al., (1974), Suzuki et al., (1977); Andria et al., (1978) also belonged to the same complementation group (Reuser et al., 1979; Suzuki et al., 1979; Wenger et al., 1980). These atypical variants of $G_{M1}$-gangliosidosis seem to be caused by allelic mutations in a gene different from the one coding for ß-galactosidase.

The explanation was provided by Wenger et al., (1978) who found a coexistent ß-galactosidase and neuraminidase deficiency in one of their atypical variants. Later on such a combined lysosomal enzyme deficiency was found in all patients belonging to the complementation group mentioned above (Miyatake et al 1979; Kleijer et al., 1979; Hoogeveen et al., 1980, for reviews see Wenger et al., 1980; Galjaard et al., 1984).

The syndrome associated with a combined ß-galactosidase and neuraminidase has been designated as galactosialidosis by Andria et al. (1981). These authors also summarized

clinical heterogeneity among patients with this syndrome (see also Galjaard et al. 1986).

In patients with the most severe and progressive early infantile form there is kidney involvement and edema around birth, coarse facies, skeletal abnormalities, visceromegaly and ocular changes, and death occurs shortly after birth (Kleijer et al., 1979; Gravel et al., 1979).

Patients with the late infantile form show an onset of symptoms after 6-12 months of age and the main features are skeletal dysplasia, visceromegaly, macular cherry-red spot and mild mental retardation (Pinsky et al., 1974; Andria et al., 1981; Strisciuglio et al., 1984).

Still other patients have been diagnosed later in childhood or at (young) adult age and have gradually progressive skeletal abnormalities, coarse facies, angiokeratoma, neurological manifestations including myoclonus, mental retardation and loss of vision, corneal clouding and macular cherry-red spot (Loonen et al., 1974, 1984; Wenger et al., 1974; Suzuki et al., 1983, 1984). The patients mentioned in the last group are mainly of Japanese origin; some of them live up to 40-60 years of age.

All the patients with galactosialidosis have 10-15% residual activity of ß-galactosidase, while the neuraminidase activity varies from 0-5% (Hoogeveen et al., 1980; Suzuki et al., 1984; Palmeri et al., 1986).

The primary defect in galactosialidosis is the deficiency of a 32 kDa glycoprotein (D'Azzo et al., 1982) which is needed for the aggregation of ß-galactosidase monomers (Hoogeveen et al., 1983) and which is most probably an essential subunit of lysosomal neuraminidase (Verheijen et al., 1985, 1987). Part of the clinical heterogeneity in galactosialidosis has been explained by differences in the molecular processing of the 32 kD "protective protein" (Palmeri et al., 1986).

b. Mucolipidosis II ("I-cell" disease)

Mucolipidosis II is a disorder of childhood with an autosomal recessive mode of inheritance. It is apparent from birth, slowly progressive and usually has a fatal outcome in three to four years. Some patients succumb within the first year of life others reach the age of twelve years. The first patients were described by DeMars and Leroy (1967) who also designated this syndrome as "I-cell" disease because of the peculiar inclusions of cultured fibroblasts (see also Leroy et al., 1969). At birth often orthopedic abnormalities can be noted and there is generalized hypotonia, severe growth failure, coarsening of facial features, progressive psychomotor retardation and recurrent respiratory infections. Patients usually die because of bronchopneumonia or congestive heart failure (see for reviews Galjaard, 1980; Leroy, 1981; McKusick and Neufeld, 1983).

Cultured skin fibroblasts from patients with "I-cell" disease show a deficiency of a large number of lysosomal enzymes and at the same time the activity of these enzymes is markedly elevated in the medium above the cultivated mutant cells as well as in body fluids from the patients (Leroy and Spranger, 1970; Wiesmann et al., 1971).

Hickman and Neufeld (1972) were first to hypothesize that the multiple lysosomal enzyme deficiency was related to a defective recognition site common to various hydrolases. As mentioned in the previous Chapter most lysosomal enzymes, including ß-galactosidase, are dependent on the specific mannose 6-phosphate (M6P) recognition marker to reach the lysosomes (Kaplan et al., 1977; Hickman et al., 1974; Sly and Stahl 1978; Natowicz et al., 1979). Evidence of an impaired phosphorylation of the lysosomal enzymes in "I-cell" disease (Bach et al., 1979; Hasilik et al., 1980), led to the elucidation of the basic defect, a deficiency of N-acetylglucosamine-1-phosphotransferase (Hasilik et al., 1981; Reitman et al., 1981)(see Chapter I.1.). The lack of a mannose 6-phosphate marker results in the secretion of

precursor forms of those lysosomal enzymes which are dependent on this pathway for their correct subcellular compartmentalization.

In the context of this thesis it is interesting to note that in fibroblasts the main pathway of lysosomal enzymes is via the M6P receptor. When the lysosomal enzyme activities in fibroblasts from patients with "I-cell" disease are compared with the activities measured in some visceral organs, higher or normal activities are found in the latter, indicating a M6P independent pathway. However, in liver, brain and spleen the activities of ß-galactosidase and neuraminidase are reduced, while the catalytic activity of other hydrolases are within the normal range. (Leroy et al., 1972; Wenger et al., 1976; Cantz and Messer, 1979; Pittman et al., 1979; Miller et al., 1979; Minami et al.,1979; Eto et al., 1979; Pallman et al., 1980).

## II.5. THERAPY AND ANIMAL MODELS

The demonstration that lysosomal storage diseases are due to inherited lysosomal enzyme deficiencies have initiated studies on the correction of these diseases by enzyme replacement therapy. Fratantoni et al. (1968) demonstrated mutual correction of intracellular mucopolysaccharide accumulation when fibroblasts from patients with Hurler's (mucopolysaccharidosis I) and Hunter's syndrome (MPS II) were cultivated together. This correction was due to secretion and uptake of the respective normal enzymes involved in mucopolysaccharide degradation. Since then several other in vitro studies have shown that exogenous lysosomal enzymes can be taken up by (mutant) fibroblasts (for reviews see Tager et al. 1980; in: Barranger and Brady (eds), 1984). It has become clear that in various cell types ingested lysosomal enzymes are able to reach the subcellular compartments where in mutant cells storage material has accumulated (Van der Ploeg et al., 1987).

Parallel to these model studies on cultured cells, numerous attempts have been made by clinicians to improve the condition of patients with a genetic metabolic disease by enzyme replacement therapy (for reviews see in: Barranger and Brady, 1984; Krivit and Paul (eds), 1986). The first effort was the intramuscular administration of fungal $\alpha$-glucosidase to a patient with glycogenosis II (Baudhuin et al., 1964). Since then various other attempts have been made either by injection of purified enzyme, or by transplantation of organs (kidney, liver) or cells (fibroblasts, amniotic fluid cells, bone marrow).

In case of enzyme injection a constant finding is, that the enzyme is rapidly cleared from the circulation. To overcome this problem efforts have been made to stabilize the enzyme by coupling to polyvinyl pyrrolidone (Specht et al., 1979), albumin (Poznansky and Bhardwaj, 1980) or polyethylene glycol (Herschfield et al., 1987). In some instances as in Gaucher's disease there is no such problem since the storage of glucocerebrosides also occurs in the Kupffer cells (Brady et al., 1974).

Another possible problem in enzyme replacement is the formation of antibodies against the injected foreign enzyme in patients where a mutation interferes with the production of the enzyme. In a patient with Gaucher's disease where cross reactive material against (mutant) glucocerebrosidase was present, injection of normal enzyme did not lead to any immunological complication (Britton et al., 1978).

In all instances where a genetic enzyme deficiency results in brain damage by intracellular accumulation of non degraded products, passage through the blood-brain barrier is a major complication in enzyme replacement therapy both by injection of purified enzyme and by transplantation. The vast majority of lysosomal storage diseases is associated with cerebral damage and the prospects of satisfactory results of therapy either by enzyme administration or bone marrow transplantation or gene correction may not be effective.

Yet, in some patients with Fabry disease kidney transplantation has provided relief from uremia but apart from a functional replacement of a damaged organ the transplantation does not act as a source of sufficient amounts of normal enzyme to be distributed all over the body (Van den Bergh et al., 1976). The same applies to liver transplantation in some patients with Gaucher disease (Brady, 1984). Trials with fibroblast or amniotic fluid cell transplantation on patients with mucopolysaccharidoses, ß-galactosidase deficiency in Morquio B disease and "I-cell" disease have been unsuccessful, probably for the same reason of inadequate production, secretion and uptake by all mutant cells in need of normal enzyme (Gibbs et al., 1980, 1983; Tylki-Szymanska et al., 1985).

During the last 5 years treatment of lysosomal storage diseases by bone marrow transplantation has gained an increasing interest. In some cases this approach has led to improvement of the clinical condition of the patient and to a normalization of certain biochemical parameters. Studies by Ginns et al. (1984a) and Svennerholm et al., (1984) showed that certain pathologic manifestations in patients with the type 3 or Norrbottnian form of disease such as elevated plasma glucocerebroside and the number of Gaucher cells in the bone marrow are reduced after marrow transplantation. Svennerholm (1984) recommends splenectomy prior to bone marrow transplantation in order to avoid severe thrombocytopenia and to avoid delay in the degradation of glucosylceramide from vital organs such as brain, lungs and liver by the large deposite in Gaucher cells accumulated in the spleen (see also Hobbs et al., 1987). It is thought that after bone marrow transplantation just a few stem cells gain access to the nervous system, therefore one would not expect improvement of the neurological manifestations or mental deterioriation, if present. This is confirmed by the results of somewhat larger series of bone marrow transplantations in patients with different types of mucopolysaccharidoses, metachromatic leucodystrophy, the neuropathic forms of

Gaucher disease, $G_{M1}$- and $G_{M2}$-gangliosidosis and some other lysosomal storage diseases (see in: Barranger and Brady (eds), 1984; Hobbs, 1985; in: Krivit and Paul (eds) 1986). A serious practical limitation in bone marrow transplantation is the high mortality rate, mainly because of the graft versus host reaction and the minimal availability of HLA matched siblings (Hobbs et al., 1982; Ramsay et al., 1982). Finally, in most lysosomal storage disorders the genetic enzyme deficiency will exert its effect already during intrauterine development; as a consequence newborns may have intracellular storage in many cell types including those of the central nervous system which forms another complication in successful enzyme replacement therapy.

Animal models with a particular genetic enzyme deficiency may be a useful tool in testing the different problems involved in therapy before its use on humans. Since $G_{M1}$-gangliosidosis is the main topic of this thesis, data on a number of mammalian species other than man with a genetic deficiency of ß-galactosidase are summarized.

A ß-galactosidase deficiency and neuronal storage of $G_{M1}$-ganglioside is found in Siamese cats (Baker et al., 1971; Baker and Lindsey, 1974). The clinical features of these cats show similarities with the human juvenile variant of $G_{M1}$-gangliosidosis (Handa and Yamakawa, 1971; Holmes and O'Brien, 1979). Cells of the mutant animals show cross reactivity against ß-galactosidase antibodies and the enzyme was found to have different electrophoretic properties and thermostability compared with normal enzyme.

$G_{M1}$-gangliosidosis was also demonstrated in calves (Donnelly et al., 1973; Cheetham and Robinson, 1974) and again the features closely resembled those of the human juvenile variant with some residual enzyme activity and ganglioside storage largely confined to the nervous tissue.

$G_{M1}$-gangliosidosis in dogs was first described by Read et al., (1976). These authors demonstrated accumulation of $G_{M1}$-ganglioside in the brain, liver and spleen due to a generalized ß-galactosidase deficiency. Pedigree analysis

indicated an autosomal recessive pattern of inheritance. Rittman et al. (1980) purified ß-galactosidase from normal and affected dog liver and compared the normal and mutant enzyme. Immunologically, they found only 1% of the normal amount of cross reactive material against ß-galactosidase antiserum in fibroblasts; the kinetic properties of these residual enzyme molecules were normal.

Thus far, experiments on animal models have not provided impressive new knowledge about the possible fate of exogenous ß-galactosidase in patients with various forms of ß-galactosidase deficiency. More experimental work is needed in this area and another prerequisite for successful enzyme or gene replacement therapy is knowledge of the exact molecular nature of the disease to be treated. The next Chapter deals with experimental work focussed on the elucidation of the genetic and molecular nature of various human ß-galactosidase deficiencies.

# Chapter III

## RESULTS AND DISCUSSION OF THE EXPERIMENTAL WORK

### III.1. ß-GALACTOSIDASE AND ITS RELATION TO NEURAMINIDASE

Previous experimental work using somatic cell hybridization (Galjaard et al., 1975; Reuser et al., 1979) indicated that the $G_{M1}$-gangliosidoses are based on mutations in a gene different from that involved in the combined ß-galactosidase - neuraminidase deficiency in patients with galactosialidosis.

The complementation studies described in Appendix paper I were aimed at the elucidation of the genetic background of galactosialidosis and diseases with a single neuraminidase deficiency such as both clinical variants of mucolipidosis I, (sialidosis) (Cantz et al., 1977; Spranger et al., 1977; Durand et al., 1977). Also, cells from a patient with mucolipidosis II ("I-cell" disease) with a multiple lysosomal enzyme deficiency including that of neuraminidase (Thomas et al., 1976; Strecker et al., 1976) were included in the cell hybridization studies.

Cultured fibroblasts from patients were fused using polyethylene glycol yielding 70-90% of multikaryons, the majority of which containing the genetic information of both parental cell strains. At 4 days after fusion a cell homogenate was prepared and the neuraminidase activity was compared with that of both parental cell strains (each fused with itself). An additional control was the overall neuraminidase activity after 4 days of cocultivation of both parental enzyme deficient cell strains. The neuraminidase activity was measured with the fluorogenic substrate 4-methylumbelliferyl-$\alpha$-2 N-acetylneuraminic acid and with N-acetylneuraminosyl-D-lactose.

Restoration of neuraminidase activity was observed after fusion of both galactosialidosis cell strains with

either fibroblasts from patients with "I-cell" disease or mucolipidosis I (sialidosis). No complementation occurred after fusion of cells from the late infantile with the adult form of galactosialidosis. Neither was complementation found after fusion of different clinical variants of mucolipidosis I (sialidosis). The main conclusion of this work was that the combined ß-galactosidase/neuraminidase deficiency was based on a mutation in a different gene than the one involved in mucolipidosis I (sialidosis) and the one in "I-cell" disease. The different clinical variants within galactosialidosis and within mucolipidosis I (sialidosis) are probably due to allelic mutations. Later, the complementation was confirmed for other clinical and biochemical variants of galactosialidosis and mucolipidosis I, respectively, using natural glycoprotein substrates. (Swallow et al., 1981).

An interesting observation was made in the controls formed by cocultivated cells from galactosialidosis and mucolipidosis I (sialidosis). After co-cultivation the neuraminidase activity in the cell mixture became higher than the average of both parental deficient cell strains. To investigate the cause of this partial correction the two mutant cell strains were labelled in vivo with fluorescent polystyrene beads. After 3 days of cocultivation the two cell strains were separated by flow sorting using a FACS II cell sorter according to Jongkind et al. (1979). Enzyme assays on the isolated cell population showed that the increased neuraminidase activity was present in the galactosialidosis cells only. This suggested for the first time that the neuraminidase deficiency in galactosialidosis could be partly corrected by a factor secreted by other fibroblasts including neuraminidase deficient cells. Fibroblasts from "I-cell" patients, however, did not accomplish this partial correction which suggested that the unknown "corrective factor" was the phosphorylated precursor form of a lysosomal glycoprotein.

## III.2. ß-GALACTOSIDASE AND ITS CORRECTIVE FACTOR

The biochemical studies described in Appendix paper II were performed to obtain more information about the correction of galactosialidosis fibroblasts. First, Concanavalin A (Con A) fractions of human placenta and liver were prepared and used for correction studies. After addition for 2 days to the culture medium above cells from a patient with late infantile galactosialidosis, a 5-7 fold increase of neuraminidase activity was observed. The same experiment on neuraminidase deficient cells from mucolipidosis I (sialidosis) had no such effect.

It was demonstrated that $NH_4Cl$ treatment of human fibroblasts induced the secretion of precursor forms of lysosomal glycoproteins into the medium and that such molecules were more easily taken up by other cells (Hasilik and Neufeld, 1980; Gonzalez-Noriega et al., 1980). This effect is due to the presence of the mannose 6-phosphate markers on the precursor forms whereas this marker no longer occurs in mature lysosomal glycoproteins as isolated from placenta or liver. We therefore treated fibroblasts from controls, $G_{M1}$-gangliosidosis and mucolipidosis I (sialidosis) with $NH_4Cl$, collected the medium after 2-3 days and subsequently dialyzed and concentrated the proteins from the medium. Aliquots of this concentrate were then added to galactosialidosis fibroblasts and after 3-4 days both the neuraminidase and ß-galactosidase activity were nearly normalized. This remarkable effect was most likely due to the efficient uptake of the "corrective factor" because of its mannose 6-phosphate marker. When concentrated medium was added together with mannose 6-phosphate no correction occurred which can be explained by competitive inhibition (Hasilik and Neufeld, 1980; Reitman et al., 1981b). Addition of concentrated medium to other mutant cells had no effect.

The conclusion of the experiments described in Appendix paper II is that an intracellular glycoprotein must exist which is capable of correcting both the ß-galactosidase and

neuraminidase deficiency in galactosialidosis cells.

In parallel experiments, van Diggelen et al., (1981,1982) measured the turnover time of ß-galactosidase in fibroblasts from controls and different types of ß-galactosidase deficient patients. For these experiments they used a suicide substrate, ß-D-galactopyranosylmethyl-p-nitrophenyl-triazene (ß-gal-MNT) (Sinnot and Smith, 1976,1978; van Diggelen et al., 1980). The turnover time of ß-galactosidase in normal fibroblasts, and $G_{M1}$-gangliosidosis cells was found to be about 10 days. In galactosialidosis fibroblasts, however, it was reduced to less than 1 day.

When purified bovine testicular ß-galactosidase in a monomeric form was added to the culture medium above control fibroblasts or $G_{M1}$-gangliosidosis cells the enzyme was internalized and remained stable for several days. In contrast, when the same enzyme preparation was added to galactosialidosis cells it was taken up by the cells in a similar way as by control cells but once internalized it was degraded within several hours. It thus appeared that the reduced turnover time of ß-galactosidase was due to enhanced degradation (Van Diggelen et al., 1982)).

## III.3. ß-GALACTOSIDASE AND ITS PROTECTIVE PROTEIN

The experimental work described in the Appendix papers III and IV has led to the elucidation of the molecular defect responsible for the combined ß-galactosidase and neuraminidase deficiency and of the nature and function of the "corrective factor".

First, ß-galactosidase was purified from human placenta via affinity chromatography (van Diggelen et al., 1981) and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS PAGE) was performed. A major band of 64 kDa molecular mass and a minor band of 32 kDa were visible. Similar observations had been made for mouse macrophages by Skudlarek and Schwank (1979). Antiserum was raised by injecting the purified sample into a rabbit.

Using these antibodies the biosynthesis of ß-galactosidase was studied in normal and galactosialidosis fibroblasts (Appendix paper III). The cellular proteins were pulse labelled by addition of [$^3$H]-leucine to the culture medium and after a chase period varying from 3 to 20 hours immunoprecipitation was performed according to the methods of Laemmli, (1970), Bonner and Laskey, (1974) and Hasilik and Neufeld (1980a).

In normal fibroblasts ß-galactosidase appeared to be synthesized as a 85 kDa precursor which is processed to a 64 kDa mature form within hours. In galactosialidosis cells the 85 kDa precursor is synthesized in normal amounts but a 66 kDa (intermediate?) form disappears within a 20 hours chase period. A remarkable finding was the absence of two bands of 32 kDa and 20 kDa molecular mass in the galactosialidosis cells. After $NH_4Cl$ treatment the medium above control fibroblasts contained an 88 kDa ß-galactosidase precursor and a 54 kDa band which was absent in medium above galactosialidosis fibroblasts. These results suggested that the 54 kDa intracellular protein could be the precursor of the 32 kDa and 20 kDa bands (see also D'Azzo, 1982).

To study the cause of the enhanced degradation of ß-galactosidase in galactosialidosis the effect of leupeptin, a thiol protease inhibitor (Aoyagi and Umizawa, 1975) was studied. Addition of leupeptin to the culture medium during 2-5 days resulted in a 7 fold increase of ß-galactosidase activity (i.e. to the low normal range) in galactosialidosis fibroblasts. Immunoprecipitation studies after leupeptin treatment indicated an increase of both the 85 kDa and the 66 kDa band. The fact that leupeptin treatment results in an increase of the 85 kDa ß-galactosidase precursor cannot be explained satisfactorily; it may be possible that a normal distribution between the 85 kDa, 66 kDa and 64 kDa forms is disturbed when the final processing step is not performed as seems the case in galactosialidosis. In this context it is interesting to note that addition of leupeptin results in a marked increase of ß-galactosidase activity in

galactosialidosis cells which also has normal kinetic properties. This implies that a possible 66 kDa intermediate form has similar properties as mature 64 kDa ß-galactosidase (see also Appendix paper VIII).

When concentrated "corrective factor" (see Appendix paper II) is added to the medium above galactosialidosis fibroblasts a complete normalization of the immunoprecipitation pattern for ß-galactosidase is observed i.e. the 85 kDa precursor is fully processed into the mature 64 kDa enzyme.

On the basis of the results described so far it was hypothesized that the combined ß-galactosidase and neuraminidase deficiency in galactosialidosis was caused by a genetic defect of a 32 kDa protein with a 54 kDa precursor that is identical to the "corrective factor".

The work reported in Appendix paper IV led to the elucidation of the function of the 32 kDa protein which was named "protective protein" since it prevents enhanced degradation of the 66 kDa form of ß-galactosidase. Previous gel filtration experiments on normal and galactosialidosis fibroblasts (Hoeksema et al., 1979) had shown the absence of multimeric ß-galactosidase in the mutant cells whereas this is the major component in normal fibroblasts and human liver (Norden et al., 1974).

Sucrose density gradient centrifugation of lysosomal ß-galactosidase in normal and human fibroblasts indicated that under the experimental conditions mentioned in Appendix paper IV, 85% of the enzyme exists as a high molecular weight complex (600-700 kDa) at pH 6.0, whereas at pH 4.0 all ß-galactosidase exists as a high molecular weight multimer. In galactosialidosis fibroblasts the 10-15% residual ß-galactosidase activity is due to the monomeric form only. Addition of leupeptin results in an increased amount of these monomers. When, however, a concentrate of the "corrective factor" is added to galactosialidosis cells the multimeric form of ß-galactosidase appears.

Immunoprecipitation studies on pooled fractions after sucrose density gradient centrifugation showed that the high

molecular weight ß-galactosidase aggregate contains proteins of 64 kDa, 32 kDa and 20 kDa molecular mass. In addition there is an "excess" of free 32 kDa and 20 kDa molecules.

The relationship between the 54 kDa precursor and the 32 kDa and 20 kDa bands was further investigated with the following experiment. The secretion of 54 kDa molecules by [$^3$H]leucine labelled normal fibroblasts was induced by $NH_4Cl$ treatment and the medium was added for 2 days to galactosialidosis fibroblasts which do not contain any radioactive labelled molecules. The results described in Appendix paper IV showed uptake of labelled 54 kDa precursor and appearance of a strong 32 kDa band and a fainter 20 kDa band. This experiment proves the earlier suggestion that the 54 kDa protein is a precursor of the 32 kDa "protective protein" and the 20 kDa protein. Similar molecules have been identified in human liver (Frost et al., 1978), human placenta (Lo et al., 1979), feline liver (Holmes and O'Brien, 1979) and porcine spleen (Yamamoto et al., 1982, 1986). In these studies no functional significance was attributed to these molecules.

On the basis of the experiments described in Appendix papers II, III and IV, we conclude that the 32 kDa protein and perhaps the 20 kDa protein are normally required for the aggregation of ß-galactosidase monomers into a high molecular weight aggregate. In galactosialidosis a genetic defect of the 54 kDa precursor of the "protective protein" results in an inability of ß-galactosidase monomers to aggregate and as a consequence they are rapidly degraded by lysosomal proteases.

## III.4. PROCESSING OF ß-GALACTOSIDASE IN $G_{M1}$-GANGLIOSIDOSIS AND MORQUIO B SYNDROME

The experimental work reported in Appendix papers V and VI deals with immunoprecipitation studies to elucidate the molecular defect(s) in $G_{M1}$-gangliosidosis and Morquio B syndrome (see also Chapter II).

After in vivo labelling with [$^3$H]-leucine, immunoprecipitation studies using anti-ß-galactosidase antibodies were performed on skin fibroblasts from patients with the infantile or adult form of $G_{M1}$-gangliosidosis, on cells from a Morquio B patient and on medium collected 2 days after $NH_4Cl$ stimulation of mutant and normal fibroblasts. The results described in Appendix paper V show the presence of the 85 kDa precursor form of ß-galactosidase in all mutant cells tested, which is in agreement with the finding of an 88 kDa band in the medium after $NH_4Cl$ treatment. However, in steady state, cells from infantile and adult $G_{M1}$-gangliosidosis have only 4-12% of the normal radioactivity at the 64 kDa position. In pulse chase experiments no formation of mature ß-galactosidase was found despite an apparently normal synthesis of the precursor form.

In Morquio B fibroblasts a normal amount of mature 64 kDa ß-galactosidase is present which is in agreement with the normal biosynthesis of the enzyme in this mutant (Paschke and Kresse, 1982; van der Horst et al., 1983). In this syndrome a normal amount of mutant enzyme seems to be present and the mutation mainly affects the hydrolysis of galactose from keratan sulfate whereas the affinity of the mutant enzyme for $G_{M1}$-ganglioside remains unaffected (Paschke and Kresse, 1982; van der Horst et al., 1983; see also Appendix paper VIII).

In all single ß-galactosidase deficient cell strains tested normal amounts of the 32 kDa protective protein and its 54 kDa precursor were present.

Sucrose density gradient centrifugation studies indicated only a minor amount of ß-galactosidase monomer in the

infantile form of $G_{M1}$-gangliosidosis. In the adult form about 25% of the residual ß-galactosidase activity is in the multimeric form and in Morquio B this is about 50% (see also Paschke and Kresse, 1982). It should be taken into account that the activities of ß-galactosidase in all mutants are low and hence the catalytic activity of precursor ß-galactosidase may disturb a correct interpretation of the relative amounts of monomeric and multimeric form of ß-galactosidase.

The deficiency of mature ß-galactosidase in both $G_{M1}$-gangliosidosis variants cannot be explained in the same way as in galactosialidosis since the protective protein is present. Also addition of leupeptin did not result in an increase of ß-galactosidase molecules. It was therefore hypothesized that the mutant precursor form of ß-galactosidase might be degraded at an early stage of processing by proteases not sensitive to leupeptin as was reported for glycogenosis II variants (Steckel et al., 1982).

The immunological data presented here seem in contradiction with previous studies where the presence of cross reactive material was mentioned in different $G_{M1}$-gangliosidosis variants (Meissler and Ratazzi, 1974; Norden and O'Brien, 1975; Ben-Yoseph et al., 1977) and even in mutant fibroblasts now known to be derived from galactosialidosis patients (O'Brien and Norden, 1977). We can only explain these previous observations by cross reactivity with non-ß-galactosidase proteins known to be present in purified preparations used for immunization such as the 54 kDa, 32 kDa and 20 kDa proteins mentioned in paragraph 2. Also, in the previous immunotitration studies the results might be influenced by different relative amounts of ß-galactosidase monomers and multimers.

The use of pulse labelling studies (Appendix paper V) left no doubt that in both variants of $G_{M1}$-gangliosidosis only minor amounts of mature ß-galactosidase are present despite a normal synthesis of precursor form. To investigate the molecular background of this phenomenon some early processing steps in normal and mutant fibroblasts were

studied (Appendix paper VI).

The first remarkable observation was the presence of relatively large amounts of 88 kDa ß-galactosidase precursor in medium above $G_{M1}$-gangliosidosis fibroblasts without $NH_4Cl$ treatment. The amount of secreted precursor was comparable with that in medium above "I-cell" fibroblasts. This pointed to the possibility of a defective recognition marker on ß-galactosidase in $G_{M1}$-gangliosidosis. Studies with [$^{32}P$] phosphate revealed that the 88 kDa precursor secreted by infantile and adult $G_{M1}$-gangliosidosis fibroblasts is not phosphorylated in contrast to that in medium above control cells after $NH_4Cl$ treatment. The defective phosphorylation does not seem to be due to an altered glycosylation, because immunoprecipitation studies after [$^{3}H$] mannose labelling gave a similar pattern in normal and $G_{M1}$-gangliosidosis fibroblasts.

These results may be explained by a defective recognition marker on the mutant ß-galactosidase for N-acetylglucosaminylphosphotransferase. As a consequence there will be no binding to the M6P receptors, required for a correct lysosomal compartmentalization (Neufeld et al., 1981; Creek and Sly, 1984); instead ß-galactosidase precursor will be secreted. The N-acetylglucosaminylphosphotransferase itself must be normal because other lysosomal proteins such as the 54 kDa precursor of the protective protein are normally phosphorylated. Impaired phosphorylation has also been demonstrated for $\alpha$-glucosidase in some variants of glycogenosis II (Reuser et al., 1985).

The hypothesis of a defective M6P recognition marker on mutant ß-galactosidase precursor was tested by uptake experiments described in Appendix paper VI. Radiolabelled precursor ß-galactosidase secreted by $G_{M1}$-gangliosidosis fibroblasts was concentrated from the medium and added to normal fibroblasts. No uptake could be shown in contrast to labelled precursor ß-galactosidase secreted by normal fibroblasts after $NH_4Cl$ stimulation.

It thus seems that the allelic mutations involved in

all forms of $G_{M1}$-gangliosidosis tested interfere with the phosphorylation of precursor ß-galactosidase. This phenomenon might be explained by a change of the tertiary structure of the precursor protein resulting in an impaired accessibility of enzymes involved in the oligosaccharide modification. In the case of $G_{M1}$-gangliosidosis the conformation change would affect the proper action of N-acetylglucosaminylphosphotransferase (Varki et al., 1981; Lang et al., 1984, 1985; for review see Kornfeld, 1986).

Several studies have been performed to determine the common recognition marker for N-acetylglucosaminylphosphotransferase that is unique to lysosomal enzymes. It has been proposed that the N-acetylglucosaminylphosphotransferase has an active site and a second binding site which is involved in the specific recognition of lysosomal enzymes (Varki et al., 1981; Lang et al., 1985). Studies on the oligosaccharide units of several lysosomal enzymes have been performed (Hasilik et al., 1980; Goldberg and Kornfeld, 1981; Natowicz et al., 1982; Takahashi et al., 1983; Nakao et al., 1984; Mutsaers et al., 1987). It was demonstrated that phosphorylation occurs on high mannose oligosaccharide units typical for lysosomal enzymes even when these are slightly altered (Gabel and Kornfeld, 1982). Isolated high mannose oligosaccharide units, however, are poor substrates for phosphorylation (Waheed et al., 1982; Lang et al., 1984) and the combination with part of the protein is needed for the binding of the phosphotransferase. Lang et al., (1984) showed that removal of oligosaccharides make lysosomal enzymes potent inhibitors of the phosphorylation of other, intact, lysosomal enzymes. Trypsin treatment and heat denaturation of lysosomal enzymes strongly reduces the rate of phosphorylation but does not impair the binding to the phosphotransferase (Lang et al., 1984).

For a proper function of the active site and the binding site of the phosphotransferase both the conformation of the protein part as well as the carbohydrate structure are essential.

As far as ß-galactosidase is concerned it is interesting that Overdijk et al. (1986) reported that human liver ß-galactosidase is the only soluble lysosomal enzyme with a low content of the high-mannose type of carbohydrate. This enzyme therefore has relatively few sites for phosphorylation and consequently is maybe more sensitive to impairment of this process. Detailed studies on the aminoacid sequence of normal ß-galactosidase and the various mutants are likely to provide answers to many of the remaining questions.

## III.5. IN SITU LOCALIZATION OF ß-GALACTOSIDASE

Thus far most studies on ß-galactosidase were based on (immuno)biochemical analyses on cell homogenates. The possibility of immunoelectron microscopy on ultrathin frozen sections of human cultured fibroblasts using the gold labelling technique (Tokuyasu, 1973; Geuze et al., 1981) enabled us to follow the intracellular compartmentalization of ß-galactosidase in normal and mutant fibroblasts. The results of such studies are reported in Appendix paper VII.

We have used polyclonal antibodies raised against human placental ß-galactosidase, which react with the precursor form and the monomeric and multimeric form of mature ß-galactosidase as well as with the protective protein and its precursor (D'Azzo et al., 1982). In addition monoclonal antibodies were prepared which reacted only with the precursor form and the monomeric mature form of ß-galactosidase and not with the multimeric form (Sips et al., 1985). Immunoelectron microscopic studies with monoclonal antibodies revealed gold labelling of cells in the rough endoplasmic reticulum (RER), the cis and trans parts of the Golgi apparatus and weak labelling of the plasma membrane. Probably this labelling represents the 85 kDa precursor form of ß-galactosidase. When polyclonal antibodies were used, the same labelling pattern was found, but in addition there was a clear labelling of the lysosomes. The latter must repre-

sent the presence of multimeric aggregates of ß-galactosidase molecules. The absence of lysosomal labelling with the monoclonal antibody indicates that no or hardly any monomeric ß-galactosidase is present under in vivo conditions.

In fibroblasts from patients with $G_{M1}$-gangliosidosis a normal labelling pattern of the RER and Golgi apparatus was found. However, no labelling was observed in the lysosomes either with monoclonal or with polyclonal antibodies. This confirms the immunobiochemical observation of the absence of 64 kDa mature ß-galactosidase in different variants of $G_{M1}$-gangliosidosis. The reason why no lysosomal labelling was found despite the presence of the 32 kDa protective protein might be that essential determinants on the 32 kDa protein are lost under conditions appropiate for immunoelectron microscopy.

Immunoelectron microscopy was also performed on fibroblasts from patients with galactosialidosis. A similar labelling pattern was found as described for $G_{M1}$-gangliosidosis. Addition of leupeptin to galactosialidosis cells resulted in a marked increase of gold labelling in the lysosomes, both with monoclonal and polyclonal antibodies. This points to an increase of the number of monomeric ß-galactosidase molecules. When a preparation containing the precursor of the protective protein was added, increased lysosomal labelling occurred only when polyclonal antibodies were used. This implies that the restoration of the aggregation of ß-galactosidase monomers also occurs under in vivo conditions.

## III.6. IN VIVO METABOLISM OF GANGLIOSIDES

The experimental work so far described has led to the elucidation of the molecular defects responsible for $G_{M1}$-gangliosidosis and galactosialidosis. Our immunoprecipitation studies and immunoelectron microscopic observations did, however, not reveal a significant difference between

fibroblasts from patients with the infantile and the juvenile/adult form of $G_{M1}$-gangliosidosis and galactosialidosis respectively. The electron microscopical methodology used does not enable the detection of less than 10% of normal amounts of enzyme molecules and the enzyme activity measurements were based on in vitro assays with artificial substrates.

In order to approach the in vivo situation we have performed loading experiments with a radioactive labelled natural substrate for ß-galactosidase (Appendix paper VIII) $G_{M1}$-ganglioside, labelled with [$^3$H] in the ceramide portion (Schwarzmann, 1978), was kindly prepared for us by Prof. L. Svennerholm and Dr. J.E. Mansson (Dept. of Neurochemistry, University of Göteborg, St. Jörgen Hospital, Sweden). Previous experiments had indicated that $G_{M1}$-ganglioside added to the medium was ingested by cultured cells and after uptake its metabolism could be followed by chromatographic procedures (Moss et al., 1976; Suzuki et al., 1978; Fishman et al., 1976, 1978, 1983).

In experiments on normal and mutant fibroblasts 5-7% of the total amount of [$^3$H] $G_{M1}$-ganglioside added to the medium was ingested after 16 hours of incubation (Appendix paper VIII). After 8-48 hours "chase" it was shown that the ingested $G_{M1}$-ganglioside was degraded to $G_{M2}$- and $G_{M3}$-ganglioside and further to smaller metabolites. After 48 hours some of the ingested substrate still remained undegraded. In fibroblasts from a patient with infantile $G_{M1}$-gangliosidosis all $G_{M1}$-ganglioside remains undegraded and no other products are seen after thin-layer chromatography. In cells from an adult patient with $G_{M1}$-gangliosidosis clearly some of the $G_{M1}$-gangliosides are metabolized to $G_{M2}$- and $G_{M3}$-ganglioside. This is likely to be due to the residual 4-12% ß-galactosidase molecules which have been demonstrated by immunoprecipitation studies (see Appendix paper V) and which were shown to have normal kinetic properties (for review see O'Brien, 1983).

Since the ingested $G_{M1}$-gangliosides will be degraded in

the lysosomes 4-12% of the (mutant) ß-galactosidase molecules must have found their way to the lysosome independent of the mannose 6-phosphate receptor (see Appendix paper VI). The catalytic activity per molecule seems to be normal.

In the infantile form of $G_{M1}$-gangliosidosis also 4-12% of mature 64 kDa. ß-galactosidase molecules are found. Here, the catalytic activity is less than 1% probably because the mutation not only interferes with the phosphorylation but also leads to impairment of the catalytic site.

The degradation of ingested ganglioside by Morquio B fibroblasts proceeds like in control cells; this finding is in support of previous data by Paschke and Kresse (1982) that the mutation in Morquio B interferes mainly with the catalytic properties of ß-galactosidase towards keratan sulfate. The amount of mutant ß-galactosidase molecules is normal and the activity per molecule varies with the substrate.

Radioactively labelled $G_{M1}$-ganglioside ingested by galactosialidosis fibroblasts are metabolized to $G_{M2}$- and $G_{M3}$-gangliosides but then further degradation seems to be blocked. We believe that this is due to the coexistent deficiency of lysosomal neuraminidase. To test this hypothesis $G_{M1}$-ganglioside metabolism was studied in fibroblasts from a patient with mucolipidosis I and again the catabolism appeared to be blocked at $G_{M3}$-ganglioside. These results suggest a role of lysosomal neuraminidase in the degradation of $G_{M3}$-ganglioside. Up to now it was thought that the hydrolysis of neuraminic acid from gangliosides was performed by a specific ganglioside neuraminidase, located on the plasma membrane (Cantz and Messer, 1979); Zeigler and Bach, 1981, 1985). An alternative hypothesis explaining the block at $G_{M3}$-ganglioside degradation is an inhibition of the ganglioside neuraminidase by accumulated sialic acid containing storage products. Since human lysosomal neuraminidase has recently been purified (Verheijen et al., 1987)) the remaining questions on its role in ganglioside degradation will soon be resolved (Cantz, personal communication, 1987).

Finally, comparison of the in vivo degradation of $G_{M1}$-ganglioside in cells from the early infantile form and adult form of galactosialidosis did not reveal any difference. This is in agreement with the earlier observation on the residual ß-galactosidase activity of about 10% in in vitro studies in both mutant cell strains (see Appendix paper I). This residual enzyme activity must be due to monomeric ß-galactosidase since no aggregation to multimeric forms occurs because of a defective 32 kDa protective protein (see previous sections). The clinical and pathological differences between the various forms of galactosialidosis are most likely due to differences in the biosynthesis and processing of the 32 kDa protective protein and their effects on lysosomal neuraminidase activity (Palmeri et al., 1986; Verheijen, 1986).

In normal human fibroblasts nearly all ß-galactosidase in the lysosomes exists as a high molecular weight complex with neuraminidase (Verheijen et al., 1987) and the 32 kDa protective protein (Appendix paper IV). Yet, the in vivo studies of $^{3}H$ $G_{M1}$-ganglioside in galactosialidosis before and after leupeptin treatment show that monomeric ß-galactosidase also is capable of degrading $G_{M1}$-gangliosides. The exact molecular relationships between the monomeric and multimeric form of ß-galactosidase and its functions towards different substrates must await more detailed molecular analysis. This is also true for the relationship between the soluble lysosomal proteins ß-galactosidase and the 32 kDa protective protein with the membrane-bound neuraminidase.

Some indications for these relationships can, however, already be derived from the experimental work described in this thesis. Purified ß-galactosidase from bovine testis remains stable when added to $G_{M1}$-gangliosidosis fibroblasts, this must be due to an aggregation of ß-galactosidase monomers into high molecular weight multimers; this aggregation must occur in the lysosome (see also Appendix paper VII). Also the generation of the 32 kDa protective protein from its 54 kDa precursor seems to occur in the lysosome

(Appendix paper IV). The 32 kDa protein was most probably shown to be an essential subunit of lysosomal neuraminidase by Verheijen et al. (1987). Hence this protein fulfills a dual function but the number of 32 kDa protein molecules required for a normal functioning of lysosomal neuraminidase and of ß-galactosidase may be different. Also the effects of different mutations in each of the components of the ß-galactosidase-protective protein-neuraminidase complex may have very different biochemical, pathological and clinical consequences.

## SUMMARY

The importance of lysosomal enzymes in the intracellular degradation of many substrates is revealed by the profound effects of the genetic deficiency of single lysosomal enzymes in lysosomal storage diseases which are usually associated with severe physical and mental handicaps.

Some 70 lysosomal enzymes have been identified and more than 30 of them are involved in lysosomal storage diseases. During the last decade much has been learned about the biosynthesis, posttranslational modification and intracellular routing of the lysosomal enzymes. In many instances the study of cultured skin fibroblasts from patients with a deficiency of one or more lysosomal enzymes has significantly contributed to a better understanding of normal molecular and cell biological mechanisms.

The present thesis deals with experimental work on the lysosomal enzyme ß-galactosidase and its involvement in human genetic diseases such as $G_{M1}$-gangliosidosis, Morquio B syndrome and galactosialidosis.

Chapter I provides a general introduction on lysosomal enzymes and lysosomal storage diseases and summarizes the main approaches towards the study of the genetic and molecular back-ground of the clinical heterogeneity that is often observed among patients with "the same" enzyme deficiency.

Chapter II provides the theoretical background of the experimental work. The main characteristics of normal acid ß-galactosidase are described as well as the function of this enzyme in the degradation of gangliosides, keratan sulfate and galactose containing oligosaccharides derived from glycoproteins. In the same Chapter the clinical, pathological, biochemical and genetic aspects are described of those diseases in which acid ß-galactosidase is deficient. In patients with $G_{M1}$-gangliosidosis the three different clinical phenotypes are most likely based on

allelic mutations in a gene on chromosome 3 which codes for the ß-galactosidase polypeptide. Patients with the severe, progressive infantile form of $G_{M1}$-gangliosidosis and those with the milder juvenile form have hardly any detectable ß-galactosidase activity (less than 1% in fibroblasts), but in the adult form of the disease a residual activity of 10-15% of control values is present. In all clinical forms, neurological manifestations, skeletal abnormalities and intellectual impairment are present. Differences mainly concern the age of onset, the progression of the disease and the severity of the clinical features.

The mucopolysaccharidosis-like disease Morquio B syndrome is also caused by a mutation in the gene coding for ß- galactosidase. Here, the pathological abnormalities are, however, mainly restricted to the skeletal system whereas neurological manifestations are absent. This can be explained by the fact that the mutation affects the ß-galactosidase molecule in such a way that its catalytic function towards keratan sulfate is impaired whereas the hydrolysis of $G_{M1}$-gangliosides remains intact.

Chapter II also summarizes the main characteristics of two diseases where a ß-galactosidase deficiency is part of a multiple lysosomal enzyme deficiency: mucolipidosis II ("I-cell" disease) and galactosialidosis. In the former disease a defective phosphotransferase results in the lack of a mannose 6-phosphate marker on the precursor forms of many lysosomal enzymes, and as a consequence the lysosomal enzymes are secreted instead of being transported to the lysosomes. In galactosialidosis the combined deficiency of lysosomal ß-galactosidase and neuraminidase is due to the genetic defect of another glycoprotein, called the "protective protein".

Finally, the last section of Chapter II discusses the animal models available for the study of ß-galactosidase deficiencies and their use in studying the possible aproaches for therapy. The major problems encountered in the treatment of patients either by enzyme replacement or gene

therapy are mentioned.

Chapter III deals with the results and discussion of the experimental work which is basically described in eight articles published by scientific journals (Appendix papers I to VIII).

The aim of the experiments described in Appendix paper I was to study the genetic background of the single neuraminidase deficiency as present in patients with the lysosomal storage disease mucolipidosis I (sialidosis) and the combined neuraminidase and ß-galactosidase deficiency in patients with different clinical variants of the disease galactosialidosis. Cultured fibroblasts from various patients were hybridized and possible restoration of neuraminidase activity was measured in heterokaryons (containing the genome from both parental cell strains). These complementation studies indicated that galactosialidosis must be caused by a mutation in a gene different than the one involved in mucolipidosis I. Surprisingly co-cultivation experiments performed as a control, revealed some restoration of neuraminidase activity in galactosialidosis cells indicating the transfer of a "corrective factor" which was secreted even by neuraminidase deficient cells.

The nature of this "corrective factor" was further investigated (Appendix paper II). The secretion of the precursor form of the "corrective factor", was induced by the addition of $NH_4Cl$ to human fibroblasts. Application of a concentrate of this factor to the medium above galactosialidosis fibroblasts resulted in a complete restoration of the ß-galactosidase and neuraminidase activity and had no effect on the ß-galactosidase deficiency in $G_{M1}$-gangliosidosis. It was also shown that the "corrective factor" was a glycoprotein whose uptake by cultured fibroblasts could be inhibited by mannose 6-phosphate.

The experimental work described in the Appendix papers III and IV has led to the elucidation of the molecular

defect responsible for the combined ß-galactosidase and neuraminidase deficiency and of the nature and function of the "corrective factor". Immunoprecipitation studies using $^{3}$H-leucine labelled fibroblasts and antibodies against purified placental human ß-galactosidase gave insight in the biosynthesis of ß-galactosidase in normal fibroblasts and galactosialidosis cells (Appendix paper III). Normal ß-galactosidase is synthesized as a 85 kDa precursor which is processed into 64 kDa mature, fully active enzyme. The antiserum also reacted with a number of other proteins with a molecular mass of 54 kDa, 32 kDa and 20 kDa. In galactosialidosis the 85 kDa precursor ß-galactosidase was synthesized and processed normally, but the 64 kDa mature form was found to be degraded rapidly. This appeared to be due to the absence of the 32 kDa glycoprotein and its 54 kDa precursor. A relationship between the absence of this "protective protein" and an enhanced degradation of mature ß-galactosidase was supported by the fact that addition of the "corrective factor" resulted in a complete normalization of ß-galactosidase processing in galactosialidosis cells. The "corrective factor" described above thus seemed to be identical with the 32 kDa protective protein or its precursor. Furthermore, addition of leupeptin, a thiolcathepsin inhibitor prevented enhanced degradation of ß-galactosidase, but had hardly any effect on neuraminidase activity.

The experimental work in Appendix paper IV showed first of all that the 32 kDa "protective protein" is derived from the 54 kDa precursor. Sucrose-density gradient centrifugation studies indicated that in normal fibroblasts 85% of all ß-galactosidase exists as a high molecular weight multimer (600-700 kDa) together with the 32 kDa protective protein. In galactosialidosis cells only a small amount of monomeric 64 kDa ß-galactosidase is present but addition of the protective protein results in a normalization of the aggregation process. The combined results of the two sets of experiments suggest that the 32 kDa glycoprotein is required

for the aggregation of ß-galactosidase monomers. If there is a deficiency of this protective protein as in galactosialidosis, ß-galactosidase monomers cannot aggregate and are then sensitive to enhanced degradation by lysosomal proteases.

The work reported in Appendix papers V and VI involves a similar experimental approach towards the molecular processing of ß-galactosidase in patients with different clinical variants of $G_{M1}$-gangliosidosis and with Morquio B syndrome. In all mutant fibroblasts 85 kDa precursor ß-galactosidase is synthesized at a normal rate. In Morquio B also mature 64 kDa ß-galactosidase is formed which has a nearly normal affinity and activity towards ganglioside substrates but an impaired activity towards keratan sulfate and galactose-containing oligosaccharides because of its mutation. In none of the $G_{M1}$-gangliosidosis variants, however is a 64 kDa band seen and pulse-chase studies indicate that the normal processing of ß-galactosidase molecules must be impaired within hours after the synthesis of the 85 kDa precursor. Studies reported in Appendix paper VI provide the explanation for this phenomenon: the mutation of the ß-galactosidase precursor interferes with a normal phosphorylation and hence the molecules lack the mannose 6-phosphate marker required for a correct compartmentalization in the lysosome. As a consequence, the mutant precursor ß-galactosidase is secreted by the cell. The observation that in all cell lines studied so far from patients with the three different clinical phenotypes the mutation impairs phosphorylation, might be explained by a change of the three-dimensional structure of the ß-galactosidase precursor. Such a conformational change may interfere with the recognition site for N-acetylglucosaminylphosphotransferase, normally involved in the generation of the M6P marker.

All studies mentioned so far dealt with genetic, biochemical or immunological analyses on cell homogenates from cultured fibroblasts. In order to investigate whether some of the mechanisms are also operational under in vivo

conditions, immunoelectron microscopic studies were performed on frozen sections using the gold-labelling technique (Appendix paper VI). The intracellular localization of ß-galactosidase in normal and mutant fibroblasts was compared using polyclonal antibodies (reacting with both monomeric and multimeric ß-galactosidase) and monoclonal antibodies which only reacted with the monomeric form. It could be shown that also under in vivo conditions ß-galactosidase exists as a multimer in the lysosome of normal fibroblasts. In $G_{M1}$-gangliosidosis no cross reactive material could be detected in the lysosomes, but some label was found in the endoplasmic reticulum and Golgi apparatus (precursor forms) and at the cell membrane. These findings are in agreement with the biochemical data on secretion of the precursor form. In galactosialidosis cells the lysosomes contain some ß-galactosidase but only in its monomeric form. Addition of the 54 kDa precursor of the 32 kDa protective protein to these cells results in a strong labelling with polyclonal antibodies and no labelling with monoclonal antibodies which indicates that the multimerization process has been normalized in vivo.

Although more insight has been gained in the exact nature of the molecular defect(s) involved in human ß-galactosidase deficiencies the pathogenesis of the different clinical phenotypes within each of the syndromes remains difficult to explain. Test tube assays may not be sufficient for a correct understanding of in vivo mechanisms. As a first approach to study the in vivo metabolism, $G_{M1}$-ganglioside labelled in its ceramide moiety was fed to various types of mutant fibroblasts and the intracellular degradation after ingestion was studied by thin layer chromatography (Appendix paper VIII). The first observation was that there is no difference in the rate of $G_{M1}$-ganglioside degradation by monomeric and multimeric ß-galactosidase.

Also, it was shown that the residual amount of 5-15% of ß-galactosidase molecules in the adult variant of $G_{M1}$-

gangliosidosis is sufficient to degrade the ingested $G_{M1}$-ganglioside. The occurrence of pathological manifestations in patients may be related to different metabolic conditions in the various cell types, still to be studied. Further an increasing accumulation of substrate over a long period may affect the rate of hydrolysis by a decreased amount of enzyme. In cells from patients with the infantile forms of $G_{M1}$-gangliosidosis no degradation of $G_{M1}$-ganglioside was observed which is in agreement with the early and more severe manifestations in these patients.

In all galactosialidosis variants the residual amount of monomeric ß-galactosidase was found to be capable of $G_{M1}$-ganglioside degradation. The clinical manifestations in the most severe early infantile and late infantile forms are probably mainly caused by the co-existent neuraminidase deficiency. For the ß-galactosidase deficiency in the adult variant of galactosialidosis similar considerations apply as in adult $G_{M1}$-gangliosidosis. Further studies on the exact nature of the neuraminidase deficiency in the different variants of galactosialidosis and sialidosis are required. This also applies to the finding in Appendix paper VIII that in all cells with a lysosomal neuraminidase deficiency the ganglioside degradation is blocked at $G_{M3}$-ganglioside. This might point to a role of lysosomal neuraminidase in ganglioside degradation, a role which hitherto has been attributed to a distinct enzyme, ganglioside neuraminidase.

The experimental work described in this thesis has further clarified the relationship between ß-galactosidase and the protein which is required for its aggregation and thereby protecting the enzyme from rapid proteolytic degradation. The aggregation of ß-galactosidase monomers was also demonstrated under *in vivo* conditions. No difference was found in the degradation of $G_{M1}$-ganglioside by the monomeric or multimeric forms of ß-galactosidase. Our work has also revealed that the deficiency of ß-galactosidase molecules in the different clinical variants of $G_{M1}$-gangliosidosis is due to impaired phosphorylation of the

precursor form resulting in its secretion instead of a correct processing and compartmentalization in the lysosome. Future work has to elucidate the exact molecular interaction between ß-galactosidase, protective protein and neuraminidase and clarify the pathological effects of different mutations in the genes coding for each of these proteins. Knowledge of the exact biochemical background of inherited diseases is essential for optimal genetic counseling and therapy.

SAMENVATTING

Lysosomale enzymen spelen een belangrijke rol bij de afbraak van biologisch materiaal. Dit komt tot uiting bij lysosomale stapelingsziekten, waar het genetisch defect van een enkel enzym vaak leidt tot ernstige lichamelijke en geestelijke handicaps.

Van de 70 bekende lysosomale enzymen zijn er ongeveer 30 betrokken bij lysosomale stapelingsziekten.

In de afgelopen 10 jaar is veel bekend geworden over de biosynthese van lysosomale enzymen en de veranderingen die ze ondergaan na translatie en over het transport van de lysosomale enzymen naar het lysosoom. In veel gevallen hebben de studies aan fibroblasten van controle personen en patienten een grote bijdrage geleverd tot het begrip van normale moleculaire en celbiologische processen.

Het in dit proefschrift beschreven experimentele werk handelt over het lysosomale enzym ß-galactosidase en zijn rol in lysosomale stapelingsziekten zoals $G_{M1}$-gangliosidosis, Morquio B syndroom en galactosialidosis.

Hoofdstuk I geeft naast een algemene beschouwing over lysosomale enzymen en lysosomale stapelingsziekten, een samenvatting van een aantal methoden om de genetische en moleculaire achtergrond van de klinische heterogeniteit te bestuderen, die men vaak binnen eenzelfde ziekte waarneemt.

Hoofdstuk II beschrijft de theoretische achtergrond van het experimentele werk. Naast de belangrijkste karakteristieken van normale zure ß-galactosidase, wordt de functie van het enzym, de afsplitsing van galactose van $G_{M1}$ ganglioside, keratan sulfaat en oligosacchariden beschreven. In hetzelfde hoofdstuk worden de klinische, pathologische, biochemische en genetische aspecten van ziekten met een ß-galactosidase deficientie belicht. De verschillende klinische varianten van $G_{M1}$-gangliosidosis worden hoogstwaarschijnlijk veroorzaakt door een verschillend defect in het gen voor ß-galactosidase, dat zich op chromosoom 3 bevindt. Bij patienten met de ernstige, progressieve, infantiele vorm en

bij patienten met de wat mildere juveniele vorm van $G_{M1}$-gangliosidosis wordt een lage ß-galactosidase activiteit gevonden (in fibroblasten zelfs minder dan 1% ten opzichte van controle). Echter in fibroblasten van patienten met de adulte vorm van $G_{M1}$-gangliosidosis wordt een restactiviteit gemeten van 10-15%. Alle klinische vormen worden gekenmerkt door neurologische en skelet afwijkingen en mentale retardatie. De varianten verschillen meestal in de aanvang, de progressie en de ernst van de klinische verschijnselen. Bij Morquio B syndroom een ziektebeeld dat veel op mucopolysaccharidosis lijkt wordt ook een mutatie gevonden in het gen dat codeert voor ß-galactosidase. Hier zijn de pathologische afwijkingen beperkt tot het skelet, terwijl de neurologische verschijnselen afwezig zijn. Dit kan worden verklaard uit het feit dat het gemuteerde ß-galactosidase een verlaagde affiniteit heeft voor keratan sulfaat terwijl de affiniteit voor $G_{M1}$-ganglioside normaal is. In hoofdstuk II worden ook de karakteristieken van twee andere ziekten beschreven, waar naast een ß-galactosidase deficientie ook andere lysosomale enzym deficienties worden gevonden nl. mucolipidosis II ("I-cell" disease ) en galactosialidosis. In mucolipidosis II is het primaire defect een mutatie in een fosfotransferase, hierdoor kunnen precursors van lysosomale enzymen niet voorzien worden van de M6P marker. Deze M6P marker is nodig om lysosomale enzymen naar het lysosoom te transporteren. Het ontbreken van deze marker heeft als gevolg dat de meeste lysosomale enzymen door de cellen worden uitgescheiden. In galactosialidosis zijn de lysosomale enzymen ß-galactosidase en neuraminidase deficient als een gevolg van een genetisch defect in een ander glycoproteine dat "protective protein" wordt genoemd, omdat het ß-galactosidase beschermt tegen proteolytische afbraak. Tenslotte worden in hoofdstuk II de beschikbare diermodellen beschreven voor de bestudering van ß-galactosidase deficienties en om uit te zoeken wat mogelijk de juiste methode zou zijn voor eventuele therapy. De belangrijkste problemen die zich voordoen bij de behandeling

van patienten hetzij bij enzym, orgaan of gen therapy worden genoemd.

In hoofdstuk III worden de resultaten van het experimentele werk dat gebaseerd is op 8 artikelen, gepubliceerd in wetenschappelijke tijdschriften (Appendix papers I-VIII), vermeld. Het doel van het experimentele werk beschreven, in Appendix paper I, was het bestuderen van de genetische achtergrond bij klinische varianten van de lysosomale stapelingsziekte mucolipidosis I (sialidosis) en de gecombineerde neuraminidase en ß-galactosidase deficienties in klinische varianten van galactosialidosis. De gekweekte fibroblasten van de verschillende patienten werden met elkaar gefuseerd en de mogelijke verhoging van neuraminidase activiteit gemeten in de heterokaryons (deze bevatten het genoom van beide ouderlijnen). Deze fusie experimenten toonden aan dat galactosialidosis wordt veroorzaakt door een mutatie in een gen anders dan het gen dat betrokken is bij de mutatie in mucolipidosis I. Een onverwachte vinding was dat na samenkweek, bedoeld als controle experiment, de neuraminidase activiteit in fibroblasten van patienten met galactosialidosis verhoogd was. Dit wees op het transport van een corrigerende faktor uitgescheiden door zowel controle als neuraminidase deficiente cellen. De aard van deze corrigerende faktor is verder onderzocht in Appendix paper II. Door het toevoegen van $NH_4Cl$ aan het medium van fibroblasten, kon de uitscheiding van de corrigerende faktor worden verhoogd. Zodat deze in geconcentreerde vorm toegevoegd kon worden aan fibroblasten van patienten met galactosialidosis. Het resultaat was een volledige correctie van zowel ß-galactosidase als neuraminidase. Toevoeging van de faktor aan ß-galactosidase deficiente fibroblasten van patienten met $G_{M1}$-gangliosidosis had geen effect. Verder werd aangetoond dat deze faktor een glycoproteine was en dat de opname ervan geremd kon worden door toevoeging van mannose 6-fosfaat aan het medium.

Het experimentele werk beschreven in de Appendix papers III en IV heeft geleid tot de oplossing van het moleculaire

defect verantwoordelijk voor de gecombineerde deficiency van ß-galactosidase en neuraminidase in galactosialidosis en tot het begrijpen van de aard en functie van de corrective faktor. Door gebruik te maken van leucine gelabelde fibroblasten en antilichamen gericht tegen ß-galactosidase, werd meer inzicht verkregen in de biosynthese van ß-galactosidase in normale en galactosialidosis cellen. De 85 kDa precursor vorm van normale ß-galactosidase wordt "geprocessed" in een enzymatisch actieve 64 kDa mature vorm. Het antiserum reageert behalve met ß-galactosidase ook met een aantal andere eiwitten met molecuulgewichten van 54 kDa, 32kDa en 20 kDa In galactosialidosis werd aangetoond dat de 85 kDa precursor vorm normaal gemodificeerd wordt tot een 64 kDa vorm, maar dat de mature vorm in tegenstelling tot controle versneld wordt afgebroken. Dit als een gevolg van het ontbreken van een glycoproteine met een molecuul gewicht van 32 kDa. Toevoeging van de corrigerende faktor resulteerde in een normaal ß-galactosidase patroon. Uit de combinatie van de resultaten werd geconcludeerd dat het beschermende eiwit en de corrigerende faktor hetzelfde waren. Tenslotte werd nog aangetoond dat door toevoeging van leupeptine (een thiolprotease remmer) de versnelde afbraak van ß-galactosidase moleculen voorkomen kan worden. Leupeptine had echter weinig effect op de neuraminidase activiteit.

Het experimentele werk beschreven in Appendix paper IV toont de relatie tussen de 54 kDa, 32kDa en de 20 kDa eiwitten. De 54 kDa precursor wordt na toevoeging aan fibroblasten binnen de cel omgezet in een 32kDa en een 20 kDa eiwit. Experimenten met sucrose gradienten tonen aan dat in normale fibroblasten, 85% van de ß-galactosidase moleculen samen met de 32 kDa "protective protein " en het 20 kDa geaggregeerd voorkomen in een multimeer met een molecuul gewicht van 600-700 kDa In fibroblasten van patienten met galactosialidosis wordt echter een kleine hoeveelheid monomere ß-galactosidase gevonden. Toevoeging van het "protective protein" resulteert in een normaal aggregatie

patroon van ß-galactosidase en "protective protein" moleculen. Wanneer we de resultaten samenvatten wijst dit erop dat het 32 kDa eiwit noodzakelijk is voor de aggregatie van ß-galactosidase moleculen, als dit eiwit ontbreekt zoals in galactosialidosis heeft dit tot gevolg dat de ß-galactosidase monomeren niet kunnen aggregeren en hierdoor zijn zij meer gevoelig voor afbraak door lysosomale proteasen.

De bestudering van de moleculaire processing van ß-galactosidase in cellen van verschillende klinische varianten van $G_{M1}$-gangliosidosis en Morquio B syndrome wordt beschreven in de Appendix papers V en VI. De experimentele benadering is ongeveer hetzelfde als beschreven in Appendix paper IV. Er werd aangetoond dat in alle gemuteerde fibroblasten de 85 kDa precursor van ß-galactosidase normaal wordt gesynthetiseerd. In Morquio B syndroom wordt een normale hoeveelheid 64 kDa mature enzym gevonden, dat een normale affiniteit vertoont voor ganglioside substraten maar als gevolg van de mutatie een verlaagde affiniteit heeft voor keratan sulfaat en galactose bevattende oligosacchariden. In geen van de $G_{M1}$-gangliosidosis varianten die zijn getest wordt een 64 kDa mature vorm van ß-galactosidase gevonden, zelfs niet in pulse chase experimenten, waar modificaties in nieuw gevormd ß-galactosidase enkele uren na synthese kan worden bestudeerd. De normale processing van ß-galactosidase lijkt al binnen enkele uren na synthese verstoord. Studies vermelt in Appendix paper VI geven een verklaring voor dit verschijnsel. De mutatie in de ß-galactosidase precursor staat een normale fosforylering in de weg. Dit heeft als gevolg dat er geen M6P marker ontstaat, die nodig is voor het transport van ß-galactosidase naar het lysosoom. De gemuteerde precursor van ß-galactosidase wordt nu door de cel uitgescheiden. Omdat de verschillende mutaties bij de verschillende varianten niet tot een normale fosforylering leiden, zou een verklaring kunnen zijn dat een verandering in de driedimensionale structuur van het gemuteerde ß-galactosidase molecuul interfereert met het

herkenningspunt van N-acetylglucosaminylfosfotransferase, verantwoordelijk voor de vorming van de M6P marker.

Alle genetische, biochemische en immunologische studies tot nu toe vermeld werden uitgevoerd met celhomogenaten van gekweekte fibroblasten. Om te onderzoeken of de mechanismen eerder genoemd voor ß-galactosidase ook gelden onder in vivo condities werden immunoelectronen microscopische studies uitgevoerd op bevroren coupes met gebruik van de immuno-goud markerings techniek (Appendix paper VII). De intracellulaire localizatie van ß-galactosidase in normale en gemuteerde fibroblasten werd vergeleken met behulp van polyclonale antilichamen (welke reageren met zowel de monomere als de multimere vorm van ß-galactosidase) en monoclonale antilichamen die alleen reageren met ß-galactosidase in zijn monomere vorm. Er werd aangetoond dat ook onder in vivo condities ß-galactosidase in lysosomen van normale fibroblasten als een multimeer voorkomt. In cellen van patienten met $G_{M1}$-gangliosidosis werd geen kruisreagerend materiaal gevonden in het lysosoom, wel werd label aangetoond in het endoplasmatisch reticulum, Golgi apparaat (precursor vormen) en op de plasma membraan. Deze waarnemingen vormen dus een bevestiging van de biochemische resultaten. In cellen van patienten met galactosialidosis werd een sterk verlaagde hoeveelheid ß-galactosidase moleculen gevonden in een monomere vorm. Toevoeging van het 54 kDa eiwit (de corrigerende faktor) resulteerde in een sterke labeling van het lysosoom met het polyclonale antiserum, geen labeling werd gevonden wanneer het monoclonale antiserum werd gebruikt. Dit wijst op een normaal aggregatie patroon onder in vivo omstandigheden.

Alhoewel er meer inzicht is verkregen in de aard van de moleculaire defect(en) in ß-galactosidase deficienties, blijft toch de pathogenese van de verschillende klinische phenotypen binnen elk van de syndromen moeilijk te begrijpen. Bepalingen in de reageerbuis zijn misschien niet voldoende voor het goed begrijpen van de in vivo mechanismen.

Als een eerste benadering om de functie van ß-galactosidase onder in vivo omstandigheden te bestuderen, werd $G_{M1}$-ganglioside (gelabeld in het ceramide gedeelte) toegevoegd aan fibroblasten van de verschillende patienten, en de intracellulaire afbraak werd na opname bestudeerd met behulp van dunne laag chromatography (Appendix paper VIII). De eerste waarneming was dat er geen verschil werd gevonden in de afbraak van $G_{M1}$-ganglioside door monomere of multimere ß-galactosidase. Ook werd aangetoond dat de restactiviteit van ß-galactosidase in de adulte variant van $G_{M1}$-gangliosidosis voldoende was om de opgenomen hoeveelheid $G_{M1}$-ganglioside af te breken. De pathologische verschijnselen in vivo worden waarschijnlijk veroorzaakt door de verschillende metabole condities in de verschillende cel typen. Dit zal verder onderzocht moeten worden. Een toenemende stapeling van substraat in de tijd, zou de verminderde hoeveelheid enzym kunnen remmen. In de fibroblasten van patienten met de infantiele en juveniele vorm van $G_{M1}$-gangliosidosis werd geen afbraak van $G_{M1}$-ganglioside gevonden, hetgeen in overeenstemming is met de vroege en ernstige symptomen bij deze patienten. In fibroblasten van alle galactosialidosis varianten werd aangetoond dat de restactiviteit van monomere ß-galactosidase voldoende is om $G_{M1}$-ganglioside af te breken. De ernstige klinische verschijnselen van de vroeg en laat infantiele vorm worden waarschijnlijk in hoofdzaak veroorzaakt door de eveneens aanwezige neuraminidase deficientie. Voor de adulte variant van Galactosialidosis gelden dezelfde overwegingen als voor de adulte variant van $G_{M1}$-gangliosidosis. Verdere studies zijn nodig naar de preciese aard van de neuraminidase deficientie in de verschillende vormen van galactosialidosis en salidosis. Dit geldt ook voor de waarneming vermeld in Appendix paper VIII, dat in cellen met een neuraminidase deficientie de afbraak van $G_{M3}$ ganglioside geblokkeerd wordt. Dit wijst op een rol van een lysosomale neuraminidase in de afbraak van ganglioside, een taak die wordt toegeschreven aan een ganglioside neuraminidase.

Het experimentele werk dat in dit proefschrift wordt beschreven maakt de relatie duidelijk tussen ß-galactosidase en het eiwit dat nodig is voor zijn aggregatie en bescherming daardoor tegen versnelde afbraak. Deze aggregatie werd ook onder in vivo omstandigheden aangetoond. Verder werd er geen verschil gevonden in de afbraak van $G_{M1}$-ganglioside door de monomere en multimere vorm van ß-galactosidase. Ook werd aangetoond dat de afwezigheid van ß-galactosidase moleculen in de verschillende klinische varianten van $G_{M1}$-gangliosidosis een gevolg is van een defecte fosforylering van de precursor , wat resulteert in een uitscheiding van het eiwit in plaats van een normaal transport naar het lysosoom. Verdere studies zijn nodig om een antwoord te geven op de vraag hoe de moleculaire interactie tussen ß-galactosidase, het beschermende eiwit en neuraminidase plaats vindt en om de pathologie, veroorzaakt door verschillende mutaties in de genen coderend voor elk van deze eiwitten te begrijpen. Kennis van de exacte biochemische achtergrond van erfelijke ziekten is noodzakelijk voor een goede erfelijkheidsvoorlichting en eventuele therapie.

REFERENCES

Abrahams, H.E. and Robinson, D. (1969). ß-D-Glucosidases and related enzymic activities in pig kidney. Biochem. J. 111, 749-755.

Andria, G., Del Giudice, E. and Reuser, A.J.J. (1978). A typical expression of ß-galactosidase deficiency in a child with Hurler-like features but without neurological abnormalities. Clin. Genet. 14, 16-23.

Andria, G., Strisciuglio, P., Pontarelli, G., Sly, W.S. and Dodson, W.E. (1981). Infantile neuraminidase and ß-galactosidase deficiencies (galactosidosis) with mild clinical coures. Perspectives in Inherited Metabolic Disease. 4, 379-395. (Edn Ermes s.r.l. Milan).

Aoyagi, T. and Umezawa, H. (1975). In: Proteases and biological control (Reich, E., Rifkin, D.B., and Shaw, E., eds.), pp. 429-454, Cold Spring Harbor Laboratory, Cold Spring Harbor, U.S.A.

Asp, N.G. (1971). Human small intestinal ß-galactosidases: Separation and characterization of three forms of an acid ß-galactosidase. Biochem. J. 106, 841-845.

Arbisser, A.I., Donnelly, K.A., Scott, C.I., Di Ferrante, N., Singh, J., Stevenson, R.E., Aylsworth, A.S. and Howell, R.R. (1977). Mild Morquio syndrome (MPS-IV B) with keratansulfaturia but normal 6-sulfatase activity. Am. J. Med. Genet. 1, 195-205.

Bach, G. Bargal, R. and Cantz, M. (1979). I-cell disease: deficiency of extracellular hydrolases phosphorylation. Biochem. Biophys. Res. Commun. 91, 976-981.

Baker, H.J. and Lindsey, J.R. (1974). Animal model of human disease. Am. J. Pathol. 74, 649-652.

Baker, H.J., Lindsey, J.R., McKhan, G.M. and Farrel, D.F. (1971). Neuronal $G_{M1}$ gangliosidosis in a Siamese cat with ß-galactosidase deficiency. Science. 174, 838-839.

Baker, J.R. Cifonelli, J.A. and Roden, L. (1975). The linkage of corneal keratan sulfate to protein. Connect. Tissue Res. 3, 149-156.

Barranger, J.A. and Brady, R.O. (eds.) (1984). Molecular basis of lysosomal storage disorders. Academic Press Inc.

Barrett, A.J. and Heath, M.F. (1977). Lysosomal enzymes. In: Lysosomes, a laboratory handbook (ed. Dingle, J.T.) pp. 19-146. Elsevier/North-Holland, Amsterdam.

Baudhuin, P., Hers, H.G. and Loeb, H. (1964). An electron microscopic and biochemical study of type II glycogenosis. Lab. Invest. 13, 1139-1152.

Ben-Yoseph, Y., Burton, B. and Nadler, H.L. (1977). Quantitation of the enzymatically deficient cross reacting material in $G_{M1}$ gangliosidosis. Am. J. Hum. Genet. 29, 575-580.

Berman, E.R. (1970). In "Chemistry and molecular biology of the intercellular matrix". (E.A. Balasz, ed.). Acad. Press. p. 879.

Berra, B., Dipalma, S. and Brunngraber, E.G. (1974). Altered levels of tissue gangliosidosis and glycoproteins in the infantile form of $G_{M1}$-gangliosidosis. Clin. Chim. Acta. 57, 301-306.

Blix, G. (1938). Einige Beobachtungen uber eine hexosaminhaltige Substanz in der Protagonfraktion des Gehirns. Skand. Arch. Physiol. 80, 46-51.

Blobel, G. (1980). Intracellular protein topogenesis. Proc. Natl. Acad. Sci. 77, 1496-1500.

Bonner, W.M. and Laskey, R.A. (1974). A film detection method for tritium-Labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46, 83-88.

Bootsma, D. and Galjaard, H. (1979). Heterogeneity in genetic disease studied in cultured cells. In: Models for study of Inborn Errors of Metabolism, F. Hommes (ed.) pp. 241-258. Elsevier/North-Holland Biomedical Press, Amsterdam/Oxford/New York.

Brady, R.O., Pentcher, P.G., Gol, A.E., Hibbert, S.R. and Dekabon, A.S. (1974). Replacement therapy for inherited enzyme deficiency. Use of purified glucocerebrosidase in Gaucher's disease. New Engl. J. Med. 291, 989-993.

Brady, R.O. (1978). Spingolipidosis. Ann. Rev. Biochem. 47, 687-713.

Brady, R.O. and Barranger, J.A. (1983). Glucosyl ceramide lipidosis: Gaucher's disease. In: Stanbury J.B., Wijngaarden, J.B., Frederickson, D.S., Goldstein, J.L. and Brown, M.S. (eds.). The metabolic basis of inherited disease, 4th. edn. Mc. Graw-Hill, New York. pp. 842-856.

Brady, R.O. (1984). Enzyme replacement in sphingolipidoses. In: Barranger, J.D., Brady, R.O. (eds.): Molecular basis of lysosomal storage disorders. New York, Acad. Press. pp. 426-475.

Braulke, T., Geuze, H.J., Slot, J.W., Hasilik, A. and von Figura K. (1987). On the effects of weak bases and monensin on sorting and processing of lysosomal enzymes in human cells. Eur. J. of Cell Biology. 43, 316-321.

Bray, B.A., Lickerman, R. and Meyer, K. (1967). Structure of human skeletal keratosulfate. J. Biol. Chem. 242, 3373-3380.

Bremer, E.G., Schlessinger, J. and Hakamori, S. (1986). Ganglioside-mediated modulation of cell growth. J. Biol. Chem. 261, 2434-2440.

Britton, D.E., Leinikki, P.O., Barranger, J.A. and Brady, R.O. (1978). Gaucher's disease: Lack of antibody response to intravanous glucocerebrosidase. Life Sciences 23, 2517-2520.

Brown, W.J. and Farquhar, M.G. (1984). The mannose-6-phosphate receptors for lysosomal enzymes is concentrated in cis Golgi cisternae. Cell. 36, 295-307.

Bruns, A. and Hultberg, B. (1975). Regional study of acid hydrolases and lysosomal membrane properties in the normal human brain at various ages. Mech. Age. develop. 4, 201-213.

Bruns, G.A.P., Leary, A.C., Regina, V.M. and Gerald, P.S. (1978). Lysosomal ß-D-galactosidase in manhamster somatic cell hybrids. Cytogenet. Cell. Genet. 22, 177-181.

Buddecke, E. and Kresse, H. (1974). Mammalian enzymes degrading glycosaminoglycans. In: Connective Tissues, Biochemistry and Pathophysiology, R. Fricke and F. Hartmann (eds.), p.p. 131-145, Springer-Verlag, Berlin..

Callahan, J.W. and Wolfe, L.S. (1970). Isolation and characterization of keratan sulfates from the liver of a patient with $G_{M1}$-gangliosidosis type I. Biochim. Biophys. Acta. 215, 527-543.

Cantz, M. and Gehler, J. (1976). The Mucopolysaccharidoses: inborn errors of glycosaminoglycan catabolism. Human Genet. 32, 233-255.

Cantz, M., Gehler, J. and Spranger, J. (1977). Mucolipidosis I: Increased sialic acid content and deficiency of an $\alpha$-N-acetyl neuraminidase in cultured fibroblasts. Biochem. Biophys. Res. Commun. 74, 732-738.

Cantz, M. and Messer, H. (1979). Oligosaccharide and ganglioside neuraminidase activities in mucolipidosis I (sialidosis) and mucolipidosis II (I-cell disease)

fibroblasts. Eur. J. Biochem. 79, 113-118.

Charrow, J. and Hvizd, M.G. (1986). Cardiomyopathy and skeletal myopathy in an unusual variant of $G_{M1}$ gangliosidosis. J. Pediatr. 108, 729-732.

Cheetham, P.S.J. and Robinson, D. (1974). Animal models for $G_{M1}$ gangliosidosis. Biochem. Soc. Trans. 2, 1121-1124.

Cheetham P.S.J. and Dance, N.E. (1976). The separation and characterization of the methylumbelliferyl ß-galactosidases of human liver. Biochem. J. 157, 189-195.

Chester, M.A., Hultberg, B. and Ockerman, P.A. (1976). The common identity of five glycosidases in human liver. Biochim. Biophys. Acta. 429, 517-526.

Christomanou, H., Aignesberger, A. and Linke, R.P. (1986). Immunochemical characterization of two activator proteins stimulating enzymic sphingomyelin degradation . In vitro absence of one of them in a human Gaucher disease variant. Biol. Chem. Hopper-Seyler. 367, 879-890.

Conzelmann, E. and Sandhoff, K. (1979). Purification and characterization of an activator protein for the degradation of glycolipids $G_{M2}$ and $G_{a2}$ by hexosaminidase A. Hoppe-Seyler's Z. Physiol. Chem. 360, 1837-1849.

Conzelmann, E. and Sandhoff, K. (1983/84). Partial enzyme deficiencies: Residual activities and the development of neurological disorders. Dev. Neurosci. 6, 58-71.

Conzelmann, E. and Sandhoff, K. (1987). Activator proteins for lysosomal glycolipid hydrolysis. In: Methods of biochemical analysis (ed.) Glick, D. Vol. 32 John Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore pp. 1-23.

Cooper, A., Sardharwalla, I.B. and Roberts, M.M. (1986). Human ß-mannosidase deficiency. N. Eng. J. Med. 312, 1231.

Craig, J.M., Clark, J.T. and Banker, B.Q. (1959). Metabolic neurovisceral disorder with accumulation of unidentified substance: Variant of Hurler's syndrome? Am. J. Dis. Child 98, 577.

Creek, K.E. and Sly, W.S. (1984). The role of the phosphomannosyl receptor in the transport of acid hydrolases to lysosomes. In: Lysosomes in biology and pathology, Vol. 7 (eds. Dingle, J.T., Dean, R.T. and Sly W.) pp. 63-82. Elsevier, Amsterdam, New York, Oxford.

Dahms, N.M., Lobel, P., Breitmeyer, J., Chirgwin, J.M. and

Kornfeld, S. (1987). 46 kd mannose 6-phosphate receptor: Cloning, expression, and homology to the 215 kd mannose 6-phosphate receptor. Cell. 50, 181-192.

Dacremont, G. and Kint, J.A. (1968). $G_{M1}$-ganglioside accumulation and ß-galactosidase deficiency in a case of $G_{M1}$ gangliosidosis (Landing disease). Clin. Chim. Acta. 21, 421-425.

Dance, N., Price, R.G., Robinson, D. and Stirling, J.L. (1969). ß-Galactosidase, ß-glucosidase and N-acetyl-ß-glucosaminidase in human kidney. Clin. Chim. Acta. 24, 189-197.

D'Azzo, A. (1982). Multiple lysosomal enzyme deficiency in man. Thesis, Erasmus University Rotterdam.

D'Azzo, A., Hoogeveen, A., Reuser, A.J.J., Robinson, D. and Galjaard, H. (1982). Molecular defect in combined ß-galactosidase and neuraminidase deficiency in man. Proc. Natl. Acad. Sci. USA. 79, 4535-4539.

de Duve, C., Pressman, B.C., Gianetto, R. Wattiaux, R. and Appelmans, F. (1955). Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. Biochem. J. 60, 604-617.

de Duve, C. (1983). Lysosomes revisited. Eur. J. Biochem. 137, 391-397.

DeMars, R.I. and Leroy, J.G. (1967). The remarkable cells cultured from a human with Hurler's syndrome: An approach to visual selection for in vitro genetic studies. In Vitro 2, 107-118.

Derry, D.M., Fawcett, J.S., Andermann, F. and Wolfe, L.S. (1968). Late infantile systemic lipidosis. Neurology. 18, 340-348.

de Weerd-Kastelein, E.A., Keyzer, W. and Bootsma, D. (1972). Genetic heterogeneity of xeroderma pigmentosum demonstrated by somatic cell hybridization. Nature 238, 80-83.

de Wit, J., Hoeksema, H.L., Hally, D., Hagemeijer, A., Bootsma, D. and Westerveld, A. (1977). Regional localization of a ß-galactosidase locus on human chromosome 22. Som. Cell. Genet. 3, 351-363.

de Wit, J., Hoeksema, H.L., Bootsma, D. and Westerveld, A. (1979). Assignment of structural ß-galactosidase loci to human chromosomes 3 and 22. Hum. Genet. 51, 259-267.

Dingle, J.T., Dean, R.T. and Sly, W. (eds.) (1984). Lysosomes in biology and pathology, Vol. 7. Elsevier, Amsterdam,

New York, Oxford.

Donnelly, W.J.C., Sheahan, B.J. and Kelly, M. (1973). Beta-galactosidase deficiency in $G_{M1}$ gangliosidosis of Friesian calves. Res. Vet. Sci. 15, 139-141.

Dreyfus, J.C., Poenaru, L. and Svennerholm, L. (1975). Absence of hexosaminidase A and B in a normal adult. New Engl. Med. 292, 61-63.

Durand, P., Gatti, R., Cavalieri, S., Borrone, C., Tondeur, M., Michalski, J.C. and Strecker, G. (1977). Sialidosis (mucolipidosis I). Helv. Paediat. Acta. 32, 391-400.

Erickson, A.H., Conner, G.E. and Blobel, G. (1984). Biosynthesis of lysosomal enzymes. In: Molecular basis of lysosomal storage disorders (eds. Barranger, J.A. and Brady, R.O.) pp. 235-249. Academic Press Inc.

Eto, Y., Owada, M., Katagawa, T., Kokubun, Y. and Rennert, O.W. (1979). Neurochemical abnormality in I-cell disease: chemical analysis and a possible importance of ß-galactosidase deficiency. J. Neurochem. 32, 397-405.

Fishman, P.H., Moss, J. and Vaughan, M. (1976). Uptake and metabolism of ganglioside in transformed mouse fibroblasts. Relationship of ganglioside structure to choleragen response. J. Biol. chem. 251, 4490-4494.

Fishman, P.H., Bradley, R.M., Moss, J. and Manganiello, V.C. (1978). Effect of serum on ganglioside uptake and choleragen responsiveness of transformed mouse fibroblasts. J. Lipid Res. 19, 77-81.

Fishman, P.H., Bradley, R.M., Hom, B.E. and Moss, J. (1983). Uptake and metabolism of exogenous gangliosidases by cultured cells: effect of choleragen on the turnover of $G_{M1}$. J. Lipid Res. 24, 1002-1011.

Fratantoni, J.C., Hall, C.W. and Neufeld, E.F. (1968). Hurler and Hunter's syndromes: mutual correction of the defect in cultured fibroblasts. Science. 162, 570-572.

Frost, R.G., Holmes, E.W., Norden, A.G.W. and O'Brien J.S. (1978). Characterization of purified human liver acid ß-D-galactosidases A2 and A3. Biochem. J. 175, 181-188.

Gabel, C.A. and Kornfeld, S. (1982). Lysosomal enzyme phosphorylation in mouse lymphoma cell lines with altered asparagine-linked oligosaccharides. J. Biol. Chem. 257, 10605-10612.

Galjaard, H., Hoogeveen, A., Keijzer, W., De Wit-Verbeek, H.A., Reuser, A.J.J., Mae Wan Ho and Robinson, D. (1975). Genetic heterogeneity in $G_{M1}$-gangliosidosis.

Nature 257, 60-62.

Galjaard, H. and Reuser, A.J.J. (1977). Clinical, biochemical and genetic heterogeneity in gangliosidoses. In: Inborn errors of metabolism: The cultured cell. R.A. Harkness and F. Cockburn (eds.). pp. 139-160. MPT Press. St. Leonardgate.

Galjaard, H. (1980). Genetic metabolic disease, early diagnosis and prenatal analysis. Elsevier/North Holland, Amsterdam.

Galjaard, H., D'Azzo, A., Hoogeveen, A.T. and Verheijen, F. (1984). Combined ß-galactosidase-sialidase in man: Genetic defect of a "protective protein", in molecular basis of lysosomal storage disorders, New York, Academic Press. pp. 113-131.

Galjaard, H. and Reuser, A.J.J. (1984). Genetic aspects of lysosomal storage diseases. In: Lysosomes in biology and pathalogy, Vol. 7 (eds. Dingle, J.T., Dean, R.T. and Sly, W.) pp. 315-345. Elsevier, Amsterdam, New York, Oxford.

Galjaard, H., Hoogeveen, A.T., Verheijen, F.W., Willemsen, R., Palmeri, S., Mancini, G., Svennerholm, L. and Mansson, J.E. (1986). The ß-galactosidase-sialidase complex. Studies on normal and mutant human fibroblasts. In: Enzymes of lipid metabolism II. L. Freysz, H. Dreyfus, R. Massarelli and S. Gatt (eds.). Plenum Press. New York and London. pp. 753-770.

Gartner, S., Conzelmann, E. and Sandhoff, K. (1983). Activator protein for the degradation of globotriaosyl ceramide by human $\alpha$-galactosidase. J. Biol. Chem. 258, 12378-12385.

Gatt, S. and Rapport, M.M. (1966). Isolation of ß-galactosidase and ß-glucosidase from brain. Biochim. Biophys. Acta. 113, 567-576.

Geuze, H.J., Slot, J.W., van der Ley, P.A., Scheffer, R.C.T. and Griffith, J.M. (1981). Use of colloidal gold particles in double labeling immunoelectron microscopy of ultrathin frozen tissue sections. J. Cell. Biol. 89, 653-665.

Geuze, H.J., Slot, J.W., Strous, G.J.A.M., Hasilik, A. and von Figura, K. (1985). Possible pathways for lysosomal enzyme delivery. J. Cell Biol. 101, 2253-2262.

Gibbs, D.A., Spellacy, E., Roberts, A.E. and Watts, R.W.E. (1980). The treatment of lysosomal storage diseases by fibroblast transplantation: some preliminary observations. In Birth Defects: Original Article Series.

Vol. 16, Dimes Birth Defects Foundation. pp. 457-474.

Gibbs, D.A., Spellacy, E., Tompkins, R., Watts, R.W.E. and Mowbray, J.F.A. (1983). Clinical trial of fibroblast transplantation for the treatment on mucopolisaccharidosis. J. Inher. Metab. Dis. 6, 62-81.

Gilbert, E., Varakis, J., Opitz, J. and Zu Rhein, G.M. (1975). Generalized gangliosidosis type 2. Z. Kinderheilk. 120, 151-180.

Ginns, E.I., Caplan, D.B., Rappeport, J.M. and Barranger, J.A. (1984a). Bone marrow transplantation in severe Gaucher's disease: Rappid correction of enzyme deficiency accompagnied by continued long term survival of storage cells. In: Molecular basis of lysosomal storage disorders (eds.) Barranger, J.A. and Brady, R.O. pp. 429-441.

Ginns, E.I., Choudary, P.V. Martin, B.M., Winfield, S., Stubblefield, B., Mayor, J., Merkle-Lehman, D., Murray, G.J., Bowers, L.A. and Barranger, J.A. (1984b). Isolation of cDNA clones for human ß-glucocerebrosidase using the lambda-gtll expression system. Biochem. Biophys. Res. Commun. 123, 574-580.

Goldberg, D.E. and Kornfeld, S. (1981). The phosphorylation of ß-glucuronidase oligosaccharides in mouse P388D1 cells. J. Biol. Chem. 256, 13060-13067.

Goldberg, D.E., Gabel, C.A. and Kornfeld, S. (1983). Studies of the biosynthesis of the mannose 6-phosphate receptor in receptor-positive and -deficient cell lines. J. Cell Biol. 97, 1700-1706.

Goldberg, D.E. and Kornfeld, S. (1983). Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. J. Biol. Chem. 258, 3159-3165.

Goldman, J.E., Katz, D., Rapin, I., Purpura, D.P., and Suzuki, K. (1981). Chronic $G_{M1}$ gangliosidosis presenting as dystonia. I. Clinical and pathological features. Ann. Neurol. 9, 465-475.

Gonatas, N.K. and Gonatas, J. (1965). Ultrastructural and biochemical observations on a case of systemic late infantile lipidosis and its relationship to Tay-Sachs disease and gargoylism. J. Neuropathol Exp. Neurol. 24, 318-336.

Gonzalez-Noriega, A., Grubb, J.H., Talkad, V. and Sly, W.S. (1980). Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J. Cell Biol. 85, 839-852.

Gravel, R.A., Lowden, J.A., Callahan, J.W., Wolfe, L.S. and NG Yin Ku, N.M.K. (1979). Infantile sialidosis: a phenocopy of type 1 $G_{M1}$ gangliosidosis distinguished by genetic complementation and urinary oligosaccharides. Am. J. Hum. Genet. 31, 669-679.

Gray, G.M. and Santiago, N.A. (1969). Intestinal ß-galactosidases, separation and characterization of three enzymes in normal human intestine. J. Clin. Invest. 48, 716-728.

Griffiths, G. and Simons, K. (1986). The trans Golgi network: Sorting at the exit site of the Golgi complex. Science. 234, 438-443.

Groebe, H., Krins, M., Schmidberger, H., von Figura, K., Harzer, K., Kresse, H., Paschke, E., Sewell, A. and Ullrich, K. (1980). Morquio syndrome (mucopolysaccharidosis IV-B) associated with ß-galactosidase deficiency. Report of two cases. Am. J. Hum. Genet. 32, 258-272.

Hakamori, S.I. (1981). Glycosphingolipids in cellular interaction, differentiation and oncogenesis. Ann. Rev. Biochem. 50, 733-764.

Handa, S. and Yamakawa, T. (1971). Biochemical studies in cat and human gangliosidosis. J. Neurochem. 18, 1275-1280.

Hannun, Y.A. and Bell, R.M. (1987). Lysosphingolipids Inhibit Protein Kinase C: Implications for the Sphingolipidoses, Science, Vol. 235, 670-674.

Hasilik, A., Klein, U., Waheed, A., Strecker, G. and von Figura, K. (1980). Phosphorylated oligosaccharides in lysosomal enzymes: identification of $\alpha$-N-acetylglucosamine-1-phospho-6-mannose diester groups. Proc. Natl. Acad. Sci. USA. 77, 7074-7078.

Hasilik, A. and Neufeld, E.F. (1980a). Biosynthesis of lysosomal enzymes in fibroblasts. J. Biol. Chem. 255, 4946-4950.

Hasilik, A. and Neufeld, E.F. (1980b). Biosynthesis of lysosomal enzymes in fibroblasts. J. Biol. Chem. 255, 4937-4945.

Hasilik, A., Waheed, A. and von Figura, K. (1981). Enzymatic phosphorylation of lysosomal enzymes in the presence of UDP-N-acetylglucosamine. Absence of the activity in I-cell fibroblasts. Biochem. Biophys. Res. Commun. 98, 761-767.

Hershfield, M.S., Buchley, R.H., Greenberg, M.L., Melton, A.L., Schiff, R., Hatem, C., Kurtzberg, J., Markert, M.L., Kobayashi, R.H., Kobayashi, A.L. and Abuchowski,

A. (1987). Treatment of adenosine deaminase deficiency with polyethylene glycol modified adenosine deaminase. N. Engl. J. Med. 316, 589-596.

Heyworth, C.M., Neumann, E.F. and Wynn, C.H. (1981). The stability and aggregation properties of human liver acid ß-D-galactosidase. Biochem. J. 193, 773-779.

Hickman, S. and Neufeld, E.F. (1972). A hypothesis for I-cell disease: Defective hydrolases that do not enter lysosomes. Biochem. Biophys. Res. Commun. 49, 992-999.

Hickman, S., Shapiro, L.J. and Neufeld, E.F. (1974). A recognition marker required for uptake of a lysosomal enzyme by cultured fibroblasts. Biochem. Biophys. Res. Commun. 57, 55-61.

Ho, M.W. and O'Brien, J.S. (1971a). Differential effect of chloride ions on ß-galactosidase isoenzymes: a method for separate assay. Clin. Chim. Acta 32, 443-450.

Ho, M.W. and O'Brien, J.S. (1971b). Gaucher's disease: Deficiency of 'acid' ß-glucosidase and reconstitution of enzyme activity in vitro. Proc. Natl. Acad. Sci. USA. 68, 2810-2813.

Ho, M.W., Cheetman, P. and Robinson, D. (1973). Hydrolysis of of $G_{M1}$-ganglioside by human liver ß-galactosidase. isoenzymes. Biochem. J. 136, 351-359.

Hobbs, J.R., Hugh Jones,.K., Barreti, A.J., Byron, N., Chambers, D. Henry, K., James, D.C.O., Lucas, C.F., Rogers, T.R., Benson, P.P., Tansley, L.R., Mossman, J., Young, F.P. and Patric. A.D. (1982). Reversal of clinical features of Hurler's disease and biochemical improvement after treatment by bone marrow transplantation. J. Inher. Metab. Dis. 5, Suppl. 1, 59-69.

Hobbs, J.R. (1985). Correction of 34 genetic diseases by displacement bone marrow transplantation. Plasma Ther. Transfus. Technol. 6, 221-246.

Hobbs, J.R., Shaw, P.J., Hugh Jones, K., Linsay, I., and Hancock, M. (1987). Beneficial effect of pre-transplant splenectomy on displacement bone marrow transplantatior for Gaucher's syndrome. Lancet. 16, 1111-1115.

Hoeksema, J., van Diggelen, O.P. and Galjaard, H. (1979). Intergenic complementation after fusion of fibroblasts from different patients with ß-galactosidase deficiency. Biochim. Biophys. Acta. 566, 72-79.

Hoflack, B. and Kornfeld, S. (1985a). Lysosomal enzyme binding to mouse P 388D1 macrophage membranes lacking

the 215-kDa mannose 6-phosphate receptor: evidence for the existence of a second mannose 6-phosphate receptor. Proc. Natl. Acad. Sci. USA. 82, 4428-4432.

Hoflack, B. and Kornfeld, S. (1985b). Purification and characterization of a cation-dependent mannose 6-phosphate receptor from murine P 388D1 macrophages and bovine liver. J. Biol. Chem. 260, 12008-12014.

Hoflack, B., Fujimoto, K. and Kornfeld, S. (1987). The interaction of phosphorylated oligosaccharides and lysosomal enzymes with bovine liver cation-dependent mannose 6-phosphate receptor. J. Biol. Chem. 262, 123-129.

Holmes, E.W. and O'Brien, J.S. (1979). Purification and properties of acid ß-galactosidase from feline liver. Biochem. 18, 952-958.

Holmgren, J., Lonnroth, I. and Svennerholm, L. (1973). Tissue receptor for cholera exotoxin: Postulated structure from studies with $G_{M1}$-ganglioside and related glycolipids. Infect. Immun. 5, 662-667.

Hooft, C. Vlietinck, R.F., Dacremont, G. and Kint, J.A. (1970). $G_{M1}$-gangliosidosis type 2. Eur. Neurol. 4, 1-21.

Hoogeveen, A.T., Verheijen, F.W., D'Azzo, A. and Galjaard, H. (1980). Genetic heterogeneity in human neuraminidase deficiency. Nature 285, 500-502.

Hoogeveen, A.T., Verheijen, F.W. and Galjaard, H.(1983). The relation between human lysosomal ß-galactosidase and its protective protein. J. Biol. Chem. 258, 12143-12146.

Hoogeveen, A.T., Graham-Kawashima, H., D'Azzo, A. and Galjaard, H. (1984). Processing of human ß-galactosidase in $G_{M1}$-gangliosidosis and morquio B syndrome. J. Biol. Chem. 259, 1974-1977.

Hoogeveen, A.T., Reuser, A.J.J., Kroos, M. and Galjaard, H. (1986). $G_{M1}$-gangliosidosis. Defective recognition site on ß-galactosidase precursor. J. Biol. Chem. 261, 5702-5704.

Howard, D.R., Natowicz, M. and Baenziger, J.U. (1982). Strutural studies of the endoglycosidase H-resistant oligosaccharides present on human ß-glucuronidase. J. Biol. Chem. 257, 10861-10868.

Inui, K., Emmett, M. and Wenger, D.A. (1983). Immunological evidence for deficiency in an activator protein for sulfatide sulfatase in a variant form of metachromatic leukodystrophy. Proc. Natl. Acad. Sci. USA. 80, 3074-3077.

Jatzkewitz, H. and Sandhoff, K. (1963). On a biochemically special form of infantile amaurotic idiocy. Biochim. Biophys. Acta. 70, 354-356.

Jatzkewitz, H. and Stinshoff, K. (1973). An activator of cerebroside sulfatase in human normal liver and in cases of congenital metachromatic leukodystrophy. FEBS Lett. 32, 129-131.

Jongkind, J.F., A. Verkerk, A. and Tanke, H. (1979). Isolation of human fibroblast heterokaryons with two-colour flow sorting (Facs II). Exp. Cell Res. 120, 443-448.

Kaplan, A., Achord, D.T. and Sly, W.S. (1977). Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. Proc. Natl. Acad. Sci. USA. 74, 2026-2030.

Kim, J.Y.H., Goldenring, J.R., DeLorenzo, R.J. and Yu, R.K. (1986). Gangliosides inhibit phospholipid-sensitive Ca2+ dependent kinase phosphorylation of rat myelin basic proteins. J. Neurosci. Res. 15, 159-166.

Klenk, E. (1935). Uber die Natur der Phosphatide und anderer Lipoide des Gehirns und der Leber bei der Niemann-Pickschen Krankheit. Hoppe-Seyler's Z. Physiol. Chem. 235, 24-36.

Klenk, E. (1942). Uber die Ganglioside, eine neue Grupe von zuckerhaltigen Gehirnlipoiden. Hoppe-Seyler's Physiol. Chem. 273, 76-86.

Kleijer, W.J., Hoogeveen, A., Verheijen, F.W., Niermeijer, M.F., Galjaard, H., O'Brien, J.S. and Warner, T.G. (1979). Prenatal diagnosis of sialidosis with combined ß-galactosidase and neuraminidase deficiency. Clin. Genet. 16, 60-61.

Kohlschutter, A., Sieg, K., Schulte, F.J., Hayek, H.W. and Goebel, H.H. (1982). Infantile cardiomyopathy and neuromyopathy with ß-galactosidase deficiency. Eur. J. Pediatr. 139, 75-81.

Kolata, G. (1987). New understanding of Gaucher's disease. Science 235, 1328.

Kolodny, E.H. and Mumford, R.A. (1976). Human leukocyte acid hydrolyses: characterization of eleven lysosomal enzymes and study of reaction conditions for their automated analysis. Clin. Chim. Acta. 70, 247-257.

Kornfeld, S. (1986). Trafficking of lysosomal enzymes in normal and disease states. J. Clin. Invest. 77, 1-6.

Koster, J.F., Niermeijer, M.F., Loonen, M.C.B. and Galjaard, H. (1976). ß-galactosidase deficiency in an adult: a biochemical and somatic cell genetic study. Clin. Genet. 9, 427-432.

Krentler, C., Pohlmann, R., Hasilik, A., von Figura, K. (1986). Lysosomal membrane proteins do not bind to mannose 6-phosphate specific receptors. Biol. Chem. Hoppe-Seyler 367, 141-145.

Kress, B.C. and Miller, A.L. (1978). Characterization of the acid ß-D-galactosidases from human urine. Clin. Chem. Acta. 85, 23-32.

Kreutter, D., Kim, J.Y.H., Goldenring, J.R., Rasmussen, H. Ukamadu, C., DeLorenzo, R.J. and Yu, R.K. (1987). Regulation of Protein Kinase C Activity by Gangliosides J. Biol. Chem. 262, 1633-1637.

Krivit, W. and Paul, N.W. (eds.) (1986). Bone marrow transplantation for treatment of lysosomal storage disorders. Alan, R. Liss, New York.

Kudoh, T., Sattler, M., Malmstrom, J. Bitter, M.A. and Wenger, D.A. (1981). Metabolism of fatty acid-labeled cerebroside sulfate in cultured cells from controls and metachromatic leukodystrophy patients. J. of laboratory and Clinical Medicine St. Louis. 98, 704-714.

Kuhn, R. and Wiegandt, H. (1963). Die Konstitution der Ganglio-N-tetraose und des Ganglioside G . Chem. Ber. 96,. 865-880.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Landing, B.H. and Rubinstein, J.H. (1962). Biopsy diagnosis of neurological diseases in childhood with emphasis on lipidosis. In Aronson SM, Volk BW (eds): "Cerebral Sphingolipidosis." New York: Academic Press. 1-14.

Landing, B.H., Silverman, F.N., Craig, M.N., Jacoby, M.D., Lahey, M.E. and Chadwick, D.L. (1964). Familial neurovisceral lipidosis. Am. J. Dis. Child. 108, 503-522.

Landsteiner, K. and Levene, P.A. (1925). On the heterogenetic haptene. Proc. Soc. Exptl. Biol. Med. 23, 343-344.

Lang, L., Reitman, M., Tang, J., Roberts, R.M. and Kornfeld, S. (1984). Lysosomal enzyme phosphorylation. Recognition of a protein-dependent determinant allows specific phosphorylation of oligosaccharides present on lysosomal

enzymes. J. Biol. Chem. 259, 14663-14671.

Lang, L., Takahashi, T., Tang, J. and Kornfeld, S. (1985). Lysosomal enzyme phosphorylation in human fibroblasts. Kinetic parameters offer a biochemical rationale for two distinct defects in the uridine diphospho-N-acetyl-glucosamine: Lysosomal enzyme precursor N-acetylgluco-samine-1-phosphotransferase. J. Clin. Invest. 76, 2191-2195.

Ledeen, R.W. (1983). In Handbook of Neurochemistry (Lajtha, A., ed) Vol. 3, pp. 41-90, Plenum Publishing Corp., New York.

Leroy, J.G., de Mars, R.I. and Opitz, J.M. (1969). I cell disease. In: Proc. First Conf. in Clinical Delineation of Birth Defects. Original Article Series. The National Foundation March of Dimes. Vol. 5, part 4, pp. 174-185, New York.

Leroy, J.G. and Spranger, J.W. (1970). I cell disease. New. Engl. J. Med. 283, 598-599.

Leroy, J.G., Ho, M.W., McBrinn, M.C., Zielke, K., Jacob, J. and O'Brien, J.S. (1972). I-cell disease: biochemical studies. Pediat. Res. 6, 752-757.

Leroy, J.G. (1981). Thoughts and data on the relationship between the mucolipidoses and the sialidoses. Perspect Inher. Metab. Dis. 4, 405-436.

Lewis, V., Green, M.M., Vihko, P., Helenius, A. and Mellman, I. (1985). Glycoproteins of the lysosomal membrane. J. Cell Biol. 100, 1839-1847.

Li, S.C. and Li, Y.T. (1976). An activator stimulating the enzymic hydrolysis of sphingoglycolipids. J. Biol. Chem. 251, 1159-1163.

Li, S.C., Hirabayashi, Y. and Li, Y.T. (1981). A new variant of type-AB $G_{M2}$-gangliosidosis. Biochem. Biophys. Res. Commun. 101, 479-485.

Li, Y. and Li, S. (1984). The occurrence and physiological significance of activator proteins essential for the enzymic hydrolysis of $G_{M1}$ and $G_{M2}$ gangliosides. In molecular basis of lysosomal storage disorders. (eds.) J.A. Barranger, R.O. Brady. Acad. Press. Inc. pp. 79-92.

Li, S.C., Hayato, K., Serizawa, S., Li, Y.T., Fluharty, A.L., Mayes, J.S. and Shapiro, L.J. (1985). Activator protein required for the enzymatic hydrolysis of cerebroside sulfate. J. Biol. Chem. 260, 1867-1871.

Lisman, J.J.W. and Hooghwinkel, G.J.M. (1974). Kinetic

properties of ß-galactosidase of human brain tissue. Neurobiology. 4, 167-179.

Lo, J., Mukerji, K., Awasthi, Y.C., Hanada, E., Suzuki, K. and Srivastava, S.K. (1979). Purification and properties of sphingolipid ß-galactosidases from human placenta. J. Biol. Chem. 254, 6710-6715.

Lobel, P., Dahms, N.M., Breitmeyer, J., Chirgwin, J.M. and Kornfeld, S. (1987). Cloning of the bovine 215-kDa cation-independent mannose 6-phosphate receptor. Proc. Natl. Acad. Sci. USA. 84, 2233-2237.

Loonen, M.C.B., van de Lugt, L. and Franke, C.L. (1974). Angiokeratomata corporis diffusion and lysosomal enzyme deficiency. Lancet ii, 785.

Loonen, M.C.B., Reuser, A.J.J., Visser, P. and Arts, W.F.M. (1984). Combined sialidase (neuraminidase) and ß-galactosidase deficiency. Clinical, morphological and enzymological observations in a patient. Clinical Genets. 26, 139-149.

Mamelle, J.C., Vanier, M.T., Baraton, G., Gilly, J., Carrier, H., Guichard, Y., Richard, A. and Gilly, R. (1975). Etude clinique, ultrastructurale et biochimique d'un cas de gangliosidose a $G_{M1}$ de type 2. Arch. Franc. Ped. 32, 925-940.

Matalon, R., Arbogast, B., Justice, P., Brandt, E.K. and Dorfman, A. (1974). Morquio's syndrome. Deficiency of a chondroitin sulfate N-acetyl-hexosamine sulfate sulfatase. Biochem. Biophys. Res. Commun. 61, 759-765.

McKusick, V.A. and Neufeld, E.F. (1983). The mucopolysaccharide storage diseases. In Stanbury, J.B., Wijngaarden, J.B., Frederickson, D.S., Goldstein, J.L., Brown, M.S. (eds.) "The metabolic basis of inherited disease". McGraw-Hill, New-York. pp. 751-777.

Mehl, E. and Jatzkewitz, H. (1964). Eine Cerebrosidsulfatase aus Schweineniere. Hoppe-Seyler's Z. Physiol. Chem. 339. 260-276.

Mehl, E. and Jatzkewitz, H. (1968). Cerebroside-3-sulfate as a physiological substrate of arylsulfatase A. Biochim. Biophys. Acta. 151, 619-127.

Meisler, M. and Rattazzi, M.C. (1974). Immunological studies of ß-galactosidase in normal human liver and in $G_{M1}$ gangliosidosis. Am. J. Hum. Genet. 26, 683-691.

Meisler, M.H. (1975). Inhibition of human liver ß-galactosidases and ß-glucosidase by N-bromoacetyl-ß-D-galactosylamine. Biochim. Biophys. Acta. 410, 347-353.

Mellman, I. (1984). Membrane recycling during endocytosis. In: Lysosomes in biology and pathology. (eds. Dingle, J.T., Dean, R.T. and Sly, W.). 7, 201-224.

Mellman, I., Fuchs, R. and Helenius, A. (1986). Acidification of the endocytic and exocytic pathways. Ann. Rev. Biochem. 55, 663-700.

Meyer, K. (1970). Chem. Med. Biol. Intercellular matrix, Adv. Study Inst. Vol. 1, p. 5.

Myerowitz, R. and Hogikyan, N. D. (1986). Different mutations in Ashkenazi Jewish and non-Jewish French Canadians with Tay-Sachs Disease. Science 232, 1646-1648.

Miller, A.L., Levitt, P., Ingraham, H., Converse, J. and Lewis, L. (1979). Properties of acid ß-D-galactosidase isolated from I-cell disease brain and spleen. J. Neurochem. 32, 1479-1485.

Minami, R., Kudoh, T., Oyanagi, K. and Nakao, T. (1979). Neuraminidase activity in liver and brain from patients with I-cell disease. Clin. Chim. Acta. 96, 107-111.

Miyatake, T., Yamada, T., Suzuki, M., Pallmann, B., Sandhoff, K., Ariga, T. and Atsumo, T. (1979). Sialidase deficiency in adult-type neuronal storage disease. Febs Letters. 97, 257-259.

Moriyama, Y. and Nelson, N. (1987). The purified ATPase from chromaffin granule membranes is an anion-dependent proton pump. J. Biol. Chem. 262, 9175-9180.

Moss, J., Fishman, P.H., Manganiello, V.C., Vaughan, M. and Brady, R.O. (1976). Function incorperation of ganglioside into intact cells: induction of choleragen responsiveness. Proc. Natl. Acad. Sci. USA. 73, 1034-1037.

Murray, G.J., Jonsson, L.V., Sorrel, S.H. Ginns, E.I., Tager, J.M., Schram, A.W. and Barranger, J.A. (1985). Phosphorylation of ß-glucocerebrosidase in cultured human fibroblasts. Fed. Proc. 44, 709.

Mutoh, T., Sobue, I., Nooi, M., Matsuoka, Y., Kiuchi, K. and Sugimura, K. (1986). A family with ß-galactosidase deficiency: Three adults with atypical clinical patterns. Neurology. 36, 54-59.

Mutsaers, J.H.G.M., van Halbeek, H., Vliegenthart, J.F.G., Tager, J.M., Reuser, A.J.J., Kroos, M. and Galjaard, H. (1987). Determination of the structure of the carbohydrate chains of acid $\alpha$-glucosidase from human placenta. Biochim. Biophys. Acta. 911, 244-251.

Nakao, Y., Kozutsumi, Y., Kawasaki, T., Yamashina, I., van Halbeek, H. and Vliegenthart, J.F.G. (1984). oligosaccharides on cathepsin D from porcine spleen. Arch. Biochem. Biophys. 229, 43-54.

Natowicz, M.R., Chi, M.M.Y., Lowry, O.H. and Sly, W.S. (1979). Enzymatic identification of mannose 6-phosphate on the recognition marker for receptor mediated pinocytosis of ß-glucuronidase by human fibroblasts. Proc. Natl. Acad. Sci. USA. 76, 4322-4326.

Natowicz, M., Baenziger, J.U. and Sly, W.S. (1982). Structural studies of the phosphorylated high mannose-type oligosaccharides on human ß-galactosidase. J. Biol. Chem. 25, 4412-4420.

Navon, R., Geiger, B., Ben Yoseph, Y. and Rattazzi, M.C. (1976). Low levels of ß-Hex A in healthy individuals with apparent deficiency of this enzyme. Am. J. Hum. Genet. 28, 339-349.

Nelson, P.V. and Carey, W.F. (1985). A method for enrichment of hybrid somatic cells: Complementation studies in certain lysosomal enzymopathies. J. Inher. Metab. Dis. 8, 95-99.

Neufeld, E.F. (1981). Recognition and processing of lysosomal enzymes in cultured fibroblasts. In: Lysosomes and lysosomal storage diseases (eds. Callahan, J.W. and Lowden, J.A.) pp. 115-130. Raven Press, New York.

Norden, A.G.W. and O'Brien, J.S. (1973). $G_{M1}$ ganglioside ß-galactosidase studies in human liver and brain. Arch. Biochem. Biophys. 159, 383-392.

Norden, A.G.W., Tennant, L. and O'Brien, J.S. (1974). Ganglioside $G_{M1}$ ß-galactosidase A: purification and studies of the enzyme from human liver. J. Biol. Chem. 249, 7969-7976.

Norden, A.G.W. and O'Brien, J.S. (1975). An electrophoretic variant of ß-galactosidase with altered catalytic properties in a patient with $G_{M1}$ gangliosidosis. Proc. Natl. Acad. Sci. USA. 72, 240-244.

Norman, R.M., Urich, H., Tingey, A.H. and Goodbody, R.A. (1959). Tay-Sachs disease with visceral involvement and its relationship to Niemann-Pick's disease. J. Pathol. Bacteriol. 78, 409-421.

Novikoff, A.B. (1961). Lysosomes and related particles. In: The cell, Vol. 2. Brachet, J. and Mirsky, A.E. eds. Acad. Press. New York and London, 423-488.

O'Brien, J.S., Stern, M.B., Landing, B.H., O'Brien, J.K. and

Donnel, G.N. (1965). Generalized gangliosidosis. Am. J. Dis. Child. 109, 338-346.

O'Brien, J.S., Okada, S., Ho, M.W., Fillerup, D.L., Veath, M.L. and Adams, K. (1971). Ganglioside storage diseases. Fed. Proc. 30, 956-969.

O'Brien, J.S. (1975). Molecular genetics of $G_{M1}$ ß-galactosidase. Clin. Genet. 8, 303-313.

O'Brien, J.S., Gugler, E., Giedion, A., Wiessmann, U., Herschkowitz, N., Meier, C. and Leroy, J. (1976). Spondyloepiphyseal dyplasia, corneal clouding, normal intelligence and acid ß-galactosidase deficiency. Clin. Genet. 9, 495-504.

O'Brien, J.S. (1977). Neuraminidase deficiency in the cherry-red spot, myoclonus syndrome. Biochem. Biophys. Res. Commun. 79, 1136-1141.

O'Brien, J.S. and Norden, A.G.W. (1977). Nature of the mutation in adult ß-galactosidase deficient patients. Am. J. Hum. Genet. 29, 184-190.

O'Brien, J.S. (1983). The gangliosidosis. In Stanbury,J.B., Wijngaarden, J.B., Frederickson, D.S., Goldstein, J.L., Brown, M.S. (eds.). "The metabolic basis of inherited disease". McGraw-Hill, New York, pp. 945-972.

Ockerman, P.A. (1968). Identity of ß-glucosidase, ß-xylosidase and one of the ß-galactosidase activities in human liver when assayed with 4-methylumbelliferyl-ß-D-glycosides. Studies in cases of Gauchers disease. Biochim. Biophys. Acta. 165, 59-62.

Ohkura, S., Yamashita, K. and Kobata, A. (1981). Urinary oligosaccharides of $G_{M1}$-gangliosidosis. Structures of oligosaccharides excreted in the urine of type 1 but not in the urine of type 2 patients. J. Biol. Chem. 256, 8485-8490.

Ohman, R. Rosenberg, A. and Svennerholm, L. (1970). Human brain sialidase. Biochemistry. 9, 3774-3782.

Ohta, K. Tsuji, S., Mizuno, Y., Atsumi, T., Yahagi, T., and Miyataka, T. (1985). Type 3 (adult) $G_{M1}$ gangliosidosis: Case report. Neurology 35, 1490-1494.

Okada, S. and O'Brien, J.S. (1968). Generalized gangliosidosis: Beta-galactosidase deficiency. Science. 160, 1002-1004.

Overdijk, B., Hiensch-Goormachtig, E.F.J., Beem, E.P., van Steijn, G.J., Trippelvitz, L.A.W., Lisman, J.J.W., van Halbeek, H., Mutsaers, J.H.G.M. and Vliegenthart,

J.F.G. (1986). The carbohydrate structures of ß-galactosidase from human liver. Glycoconjugate J. 3, 339-350.

Pallman, B., Sandhoff, K., Berra, B. and Miyatake, T. (1980). Sialidase in brain and fibroblasts in three patients with different types of sialidosis. In Svennerholm, L., Mandel, P., Dreyfus, H. and Urban, P.F. (eds.), "Structure and Function of Gangliosides". Plenum. Press. New York. pp. 401-414.

Palmeri, S., Hoogeveen, A.T., Verheijen, F.W. and Galjaard, H. (1986). Galactosialidosis: Molecular heterogeneity among distinct clinical phenotypes. Am. J. Hum. Genet. 38, 137-148.

Paschke, E. and Kresse, H. (1982). Morquio disease, type B: activation of $G_{M1}$-ß-galactosidase by $G_{M1}$-activator protein. Biochem. Biophys. Res. Commun. 109, 568-575.

Pinsky, L., Miller,J., Stanfield, B., Watters, G. and Wolfe, L. (1974). $G_{M1}$-gangliosidosis in skin fibroblast culture: enzymatic differences between types 1 and 2 and observations on a third variant. Amer. J. Hum. Genet. 26, 563-577.

Pittman, R.C., Williams, J.C., Miller, A.L. and Steinberg, D. (1979). Acid hydrolase deficiency in I-cell disease and pseudo-Hurler polydystrophy. Biochem. Biophys. Acta. 575, 399-409.

Pohlmann, R., Nagel, G., Schmidt, B., Stein, M., Lorkowski, G., Krentler, C., Cully, J., Meyer, H.E., Grzeschik, K. Mersmann, G., Hasilik, A., and von Figura, K. (1987) Cloning of a cDNA encoding the human cation-dependent mannose 6-phosphate-specific receptor. Proc. Natl. Acad. Sci. 84, 5575-5579.

Poznansky, M.J. and Bhardwaj, D. (1980). $\alpha$-1,4-glucosamine-albumine polymers: In vitro properties and advantages for enzyme replacement therapy. Can. J. Pharmacol. 58, 322-325.

Quinn, P. (1984). Intracellular membrane fusions. In: Lysosomes in biology and pathology. (eds. Dingle, J. and Sly, W.). vol 7, pp. 231-239

Ramsay, N.K.C., Kersey, J.H., Robinson, L.L., McGlave, P.B., Woods, W.G., Krivit, W., Kim, T.H., Goldman, A.I. and Nesbit, M.E. (1982). A randomized study of the prevention of acute graft-versus-host disease. New. Engl. J. Med. 306, 192-197.

Read, D.H., Harrington, D.D., Keenan, T.W. and Hinsman, E.J. (1976). Neuronal-visceral $G_{M1}$ gangliosidosis in a dog

with ß-galactosidase deficiency. Science. 194, 442-445.

Reitman, M.L. and Kornfeld, S. (1981a). UDP-N-acetylglucosamine: glycoprotein N-acetylglucosamine -1-phosphotransferase. J. Biol. 256. 4275-4281.

Reitman, M.L. Varki, A. and Kornfeld, S. (1981b). Fibroblasts from patients with I-cell disease and pseudo-Hurler polydystrophy are deficient in uridine 5'-diphosphate-N-acetylglucosamine: glycoprotein N-acetylglucosaminylphosphotransferase activity. J. Clin. Invest. 67, 1574-1579.

Reuser, A.J.J., Andria, G., de Wit-Verbeek, H.A., Hoogeveen, A., DelGiudice, E. and Hally, D. (1979). Biochemical, immunological and cellgenetic studies in a two-year-old patient with an atypical from of $G_{M1}$-gangliosidosis. Hum. Genet. 46, 11-19.

Reuser, A.J.J., Kroos, M., Oude Elferink, R.P.J. and Tager, J.M. (1985). Defects in synthesis, phosphorylation and maturation of acid $\alpha$-glucosidase in glycogenosis type II. J. Biol. Chem. 260, 8336-8341.

Reuser, A.J.J. (1984). Genetic heterogeneity in lysosomal storage disorders studied by somatic cell hybridization. In: Molecular basis of lysosomal storage disorders (eds. Barranger, J.A. and Brady, R.O.) pp. 235-249. Academic Press. Inc.

Reuser, A.J.J., Kroos, M., Willemsen, R., Swallow, D., Tager, J.M. and Galjaard, H. (1987). Clinical diversity in glycogenosis type II. Biosynthesis and in situ localization of acid $\alpha$-Glucosidase in mutant fibroblasts. J. Clin. Invest. 79, 1689-1699.

Ringertz, N.R. and Savage, R.E. (1976). Cell Hybrids, Academic Press, New York.

Rittmann, L.S., Tennant, L.L. and O'Brien, J.S. (1980). Dog $G_{M1}$ gangliosidosis. Characterization of the residual acid ß-galactosidase. Am. J. Hum. Genet. 32, 880-889.

Rittmann, L.S. and O'Brien, J.S. (1981). Human ß-galactosidases: Molecular and clinical aspects. Isozymes: Current Topics in Biological and Medical Research. 5, 77-113.

Robins, E., Hirsch, H.E. and Emmons, S. (1968). Glycosidases in the nervous system. Assay, some properties, and distribution of ß-glucuronidase and ß-glucosidase. J. Biol. Chem. 243, 4246-4252.

Roden, L. (1980). Structure and metabolism of connective tissue proteoglycans, in Lennarz, W.J. (ed.). The

biochemistry of glycoproteins and proteoglycans. New York, Plenum Press. p. 27.

Rodriguez-Berrocal, F.J., Perez-Gonzalez, M.N. and Cabazas, J.A. (1983). Heterogeneity of beta-galactosidase from rabbit spleen. Separation and characterization of different forms. Enzyme 30, 196-204.

Roseman, S. (1970). The synthesis of complex carbohydrates by multiglycosyltransferase systems and their potential function in intercellular adhesion. Chem. Phys. Lipids 5, 270-297.

Sacrez, R., Juif J.G., Gigonnel, J.M. and Gruner, J.E. (1967). La Maladie de Landing, ou idiote amaurotique infantile precoce avec gangliosidose generalizee de type $G_{M1}$. Pediatrie. 22, 143-162.

Sandhoff, K. (1984). Function and relevance of activator proteins for glycolipid degradation. In molecular basis of lysosomal storage disorders. (eds.) J.A. Barranger, R.O. Brady. Acad. Press. Inc. p.p. 19-49.

Sahagian, G.G., Distler, J. and Jourdian, G.W. (1981). Characterization of a membrane-associated receptor from bovine liver that binds phosphomannosyl residues of bovine testicular ß-galactosidase. Proc. Natl. Acad. Sci. USA. 78, 4289-4293.

Schwarzmann, G. (1978). A simple and novel method for tritium labeling of gangliosides and other sphingolipids. Biochim. Biophys. Acta. 529, 106-114.

Seringe, P., Plainfosse, B., Lautman, F., Lorilloux, J., Calamy, G., Berry, J.P. and Watchi, J.M. (1968). Gangliosidose generalisee du type Norman-Landing a $G_{M1}$ (Sem Hop Paris). Ann Pediatr. 44, 165-184.

Shows, T.B., Scrafford-Wolff, L., Brown, J.A. and Meisler, M. (1978). Assignment of a ß-galactosidase gene (ß-Gal a) to chromosome 3 in man. Cytogenet Cell. Genet. 22, 219-222).

Singer, H.S. and Schafer, I.A. (1970). White cell ß-galactosidase activity. N. Engl. J. Med. 282, 571.

Singh, J., Di Ferrante, N., Niebes, P. and Tavella, P. (1976). N-acetylgalactosamine-6-sulfate in man. Absence of the enzyme in Morquio disease. J. Clin. Invest. 57, 1036-1040.

Sinnot, M.L. and Smith, P.J. (1976). Active-site-directed irreversible inhibition of E.coli ß-galactosidase by the "hot" carbonium ion precursor ß-D-galactopyranosyl-methyl-p-nitrophenyltriazene. J. Chem.Soc. Chem. Commun.

223-224.

Sinnot, M.L. and Smith, P.J. (1978). Affinity labelling with a deaminatively generated carbonium ion. Biochem. J. 175, 525-538.

Sips, H.J., de Wit-Verbeek, H.A., Westerveld, A. and Galjaard, H. (1985). The chromosomal localization of human ß-galactosidase revisited: a locus for ß-galactosidase on human chromosome 3 and for its protective protein on human chromosome 22. Hum. Genet. 69, 340-344.

Skudlarek, M.D. and Swank, R.T. (1979). Biosynthesis of two lysosomal enzymes in macrophages. Evidence for a precursor of ß-galactosidase. J. Biol. Chem. 254, 9939-9942.

Sly, W.S. and Stahl, P. (1978). Receptor-mediated uptake of lysosomal enzymes. In: Transport of Macromolecules in Cellular Systems (ed. S.C. Silverstein). Dahlem Konferenzen, Berlin. 229-244.

Specht, B.U. Geiger, B., Arnon, R., Paswell, J., Keren, G., Goldman, B. and Padeh, B. (1979). Enzyme replacement in Tay-Sachs disease. Neurology. 29, 848-

Spranger, J.W., Langer, L.O. and Wiedemann, H.R. (1974). Bone dysplasias. An atlas of constitutional disorders of skeletal development. WB Saunders, Philadelphia, pp. 160-165.

Spranger, J., Gehler, J. and Cantz, M. (1977). Mucolipidosis I - a sialidosis. Amer. J. Med. Genet. 1, 21-29.

Stanbury, J.B., Wyngaarden, J.B., Frederickson, D.S., Goldstein J.L. and Brown, M.S. (eds.) (1983). The metabolic basis of inherited disease. McGraw-Hill Book Company, New York.

Steckel, F., Gieselmann, V., Waheed, A., Hasilik, A., von Figura, K., Oude Elferink, R., Kalsbeek, R. and Tager J.M. (1982). Biosynthesis of acid $\alpha$-glucosidase in late-onset forms of glycogenosis type II (Pompe's disease). Febs Letters. 150, 69-76.

Stein, M., Zijderhand-Bleekemolen, J.E., Geuze, H., Hasilik, A. and von Figura, K. (1987). Mr 46000 mannose 6-phosphate specific receptor: its role in targeting of lysosomal enzymes. EMBO Journal 6, 2677-2681.

Steiner, A.W. and Rome, L.H. (1982). Assay and purification of a solubilized membrane receptor that binds the lysosomal enzyme $\alpha$-L-iduronidase. Arch. Biochem.

Biophys. 214, 681-687.

Stevens, R.L., Fluharty, A.L., Kihara, H., Kaback, M.M., Shapioro, L.J., Marsh, B., Sandhoff, K. and Fischer, G. (1981). Cerebroside sulfatase activator deficiency induced metachromatic leukodystrophy. Am. J. Hum. Genet. 33, 900-906.

Stevenson, R.E., Taylor, H.A. and Parks, S.E. (1978). ß-galactosidase deficiency: Prolonged survival in three patients following early central nervous system deterioration. Clin. Genet. 13, 305-313.

Strecker, G., Michalski, J.C., Montreuil, J. and Farriaux, J.P. (1976). Deficit in neuraminidase associated with Mucolipidosis II (I-cell disease). Biomed. 25, 238-239.

Strecker, G. and Montreuil, J. (1979). Glycoproteins and glycoproteinoses. Biochimie, 61, 1199-1246.

Strecker, G. (1981). Oligosaccharides in lysosomal storage diseases. In lysosomes and lysosomal storage diseases. (eds.) J.W. Callahan and J.A. Lowden. Raven press. New York. pp. 95-113.

Strisciuglio, P., Creek, K.E. and Sly, W.S. (1984). Complementation, cross correction and drug correction studies of combined ß-galactosidase neuraminidase deficiency in human fibroblasts. Pediatric Res. 18, 267-171.

Suzuki, K. (1968). Cerebral $G_{M1}$-gangliosidosis: chemical pathology of visceral organs. Science. 159, 1471-1472.

Suzuki, K., Suzuki, Y. and Kamoshita, S. (1969). Chemical pathology of $G_{M1}$-gangliosidosis (generalized gangliosidosis). J. Neuropath. Exp. Neurol. 28, 25-73.

Suzuki, K. and Suzuki, Y. (1970). Globoid cell leukodystrophy (Krabbes Disease): Deficiency of galactosylcerebroside ß-galactosidase. Proc. Natl. Acad. Sci. USA. 66, 302-309.

Suzuki, K. and Suzuki, Y. (1983). Galactosylceramide lipidosis: globoid cell leukodystrophy (Krabbe's disease), In: Stanbury J.B., Wyngaarden J.B., Frederickson D.S., Goldstein J.L., Brown M.S. (eds.), "The metabolic Basis of Inherited Disease". McGraw-Hill, New York, pp. 857-880

Suzuki, Y., Crocker, A.C. and Suzuki, K. (1971). $G_{M1}$-gangliosidosis. Arch. Neurol. 24, 58-64.

Suzuki, Y. and Suzuki, K. (1971). Krabbe's globoid cell leucodystrophy: deficiency of galactocerebrosidase in

serum, leucocytes and fibroblasts. Science. 171, 73-75.

Suzuki, Y. and Suzuki, K. (1974). Glycosphingolipid ß-galactosidases IV. J. Biol. Chem. 249, 2113-2117.

Suzuki, Y., Nakamura, H., Fukuoka, K., Shimada, Y. and Uono, M. (1977). ß-galactosidase deficiency in juvenile and adult patients. Hum. Genet. 36, 219-229.

Suzuki, Y., Nakamura, N. and Fukuoka, K. (1978). $G_{M1}$-gangliosidosis: Accumulation of ganglioside $G_{M1}$ in cultured skin fibroblasts and correlation with clinical types. Hum. Genet. 43, 127-131.

Suzuki, Y., Furukawa, T., Hoogeveen, A.T., Verheijen, F. and Galjaard, H. (1979). Adult type $G_{M1}$-gangliosidosis: A complementation study on somatic cell hybrids. Brain and Development. 1, 83-86.

Suzuki, Y., Sakaraba, H., Yamanaka, T., Ko, Y.M. and Okamura, Y. (1983). Galactosialidosi (ß-galactosidase-neuraminidase deficiency): A new hereditary metabolic disease with abnormal degradation of enzyme molecules. Acta. Paediatr. Jpn. 25, 31-37.

Suzuki, Y., Sakuraba, H., Yamanaka, T., Ko, Y.M., Iimori, Y., Okamura, Y. and Hoogeveen, A.T. (1984). Galactosialidosis: A comparative study of clinical and biochemical data on 22 patients. In: The developing brain and its disorders. (eds.) Arima, M., Suzuki, Y., Yabuuchi, H. University of Tokyo Press. pp. 161-175.

Svennerholm, L. (1956). Composition of gangliosides from human brain. Nature 17, 524-525.

Svennerholm, L. (1962). The chemical structure of normal human brain and Tay-Sachs gangliosides. Biochem. Biophys Res. Commun. 9, 436-441.

Svennerholm, L. (1963). Chromatographic separation of human brain gangliosides. J. Neurochem. 10, 613-623.

Svennerholm, L. (1976). In: Perspectives in brain research. Eds. Corner, M.A. and Swaab, D.F. Elsevier/North-Holland, Amsterdam-New York, pp. 101-122.

Svennerholm, L., Mansson, J.E., Nilsson, O., and Tibblin, E. (1984). Bone marrow transplantation in the Norrbottnian form of Gaucher Disease. In: Barranger, J.A., Brady, R.O. (eds.). Molecular basis of lysosomal storage disorders. New York, Acad. Press. pp. 441-459.

Swallow, D.M., Hoogeveen, A.T., Verheijen, F.W. and Galjaard, H. (1981). Complementation analysis of human sialidase deficiency using natural substrate. Ann. Hum. Genet. 45,

105-112.

Tager, J.M., Hamers, M., Schram, A.W., Van den Bergh, R.A.J.T.M., Rietra, P.J.G.M., Loonen, P.J.M., Koster, J.F. and Slee, R. (1980). An appraisal of human trials in enzyme replacement therapy of genetic diseases. In: Enzyme therapy in genetic diseases. Birth defects: Orginal article series, Vol XVI, pp. 343-359. (ed. Desnick, R.J.) Alan, R. Liss, Inc.

Tager, J.M. (1985). Biosynthesis and deficiency of lysosomal enzymes. Trends Biochem. Sci. 10, 324-326.

Tager, J.M., Aerts, J.M.F.G., Jonsson, L.M.V., Murray, G.J., Weely, S., Strijland, A., Ginns, E.I., Reuser, A.J.J., Schram, A.W., Barranger, J.A. (1986). Molecular forms, biosynthesis and maturation of glucocerebrosidase, a membrane-associated lysosomal enzyme deficient in Gaucher disease. In: Enzymes of lipid metabolism II. Freysz, L., Dreyfus, H., Massarelli, R., Gatt, S. eds., Plenum Publ. Corp. pp. 735-745.

Taguchi, S., Kouyama, H. and Yago, N. (1981). Reversible aggregation and stability of lysosomal acid ß-galactosidase from porcine adrenal cortex. J. Biochem. 89, 483-489.

Takahashi, T., Schmidt, P.G. and Tang, J. (1983). Novel carbohydrate structures of catepsin B from porcine spleen. J. Biol. Chem. 258, 2819-2830.

Takasaki, S., Murray, G., Furbish, S., Brady, R.O., Barranger, J.A. and Kobata, A. (1984). Structure of N-Asparagine-linked oligosaccharide units of human placenta ß-Glucocerebrosidase. J. Biol. Chem. 259, 10112-10117.

Tanaka, H., Meisler, M. and Suzuki, K. (1975). Activity of human hepatic ß-galactosidase toward natural glycosphingolipid substrates. Biochim. Biophys. Acta. 398, 452-463.

Tanaka, H. and Suzuki, K. (1976). Specificities of the two genetically distinct ß-galactosidases in human sphingolipidoses. Arch. Biochem. Biophys. 175, 332-340.

Tanaka, H. and Suzuki, K. (1977). Substrate specifications of two genetically distinct human brain ß-galactosidases. Brain Res. 122, 325-335.

Thomas, G.H., Tiller, G.E., Reynolds, L.W., Miller, C.S. and Bace, J.W. (1976). Increased levels of sialic acid associated with a sialidase deficiency in I-cell disease (Mucolipidosis II) fibroblasts. Biochem. Biophys. Res. Commun, 71, 188-194.

Tokuyasu, K.T. (1973). A technique for ultracryotomy of cell suspensions and tissues. J. Cell Biol. 57, 551-565.

Tomino, S. and Meisler, M. (1975). Biochemical and immunological studies of purified mouse ß-galactosidases. J. Biochem. 250, 7752-7758.

Tsuji, S., Choudary, P.V., Martin, B.M., Stubblefield, B.K., Mayor, B.A., Barranger, J.A., Ginns, E.I. (1987). A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher's disease. N. Engl. J. Med. 316, 570-575.

Trojak, J.E., Chen-Kung, H., Roesel, R.A., Levin, L.S., Kopits, S.E., Thomas, G.H. and Toma, S. (1980). Morquio-like syndrome (MPS IV-B) associated with deficiency of a ß-galactosidase. Johns Hopkins Med. J. 146, 75-79.

Tylki-Szymanska, A., Maciejko, D., Kidawa, M. and Jablonska-Budaj, U. (1985). Amniotic tissue transplantation as a trial of treatment in some lysosomal storage diseases. J. Inher. Metab. Dis. 8, 101-104.

Vaccaro, A.M., Muscillo, M., Gallozzi, E., Salvioli, R., Tatti, M. and Suzuki, K. (1985). An endogenous activator protein in human placenta for enzymatic degradation of glucosylceramide. Biochim. Biophys. Acta. 836, 157-166.

Vaes, G. (1973). Digestive capacity of lysosomes, in: Hers, H.G., van Hoof, F. (eds) Lysosomes and Storage Diseases. New York, Academic

van den Bergh, R.A.J.T.M., Rietra, P.J.G.M., Kolk-Vegter, A.J., Bosch, E., and Tager, J.M. (1976). Therapeutic implications of renal transplantation in patients with Fabry's disease Acta Med. Scand. 200, 249-256.

van der Horst, G.T.J., Kleijer, W.J., Hoogeveen, A.T., Huijmans, J.G.M., Blom, W. and van Diggelen, O.P. (1983). Morquio B syndrome: A primary defect in ß-galactosidase. Am. J. Med. Genet. 16, 261-275.

van der Ploeg, A.T., Kroos, M., van Dongen, J.M., Visser, W.J., Bolhuis, P.A., Loonen, C.B. and Reuser, A.J.J. (1987). Breakdown of lysosomal glycogen in cultured fibroblasts from glycogenosis type II patients after uptake of acid $\alpha$-glucosidase. Neurol. Sciences. 79, 327-336.

van Diggelen, D.P., Galjaard, H., Sinnot, M.L. and Smith, P.J. (1980). Specific inactivation of lysosomal glycosidases in living fibroblasts by the corresponding glycosylmethyl-p-nitrophenyltriazenes. Biochem. J. 188, 337-343.

van Diggelen, O.P., Schram, A.W., Sinnott, M.L., Smith, P.J. Robinson, D. and Galjaard, H. (1981). Turnover of ß-galactosidase in fibroblasts from patients with genetically different types of ß-galactosidase deficiency. Biochem. J. 200, 143-151.

van Diggelen, O.P., Hoogeveen, A.T., Smith, P.J., Reuser, A.J.J. and Galjaard, H. (1982). Enhanced proteolytic degradation of normal ß-galactosidase in the lysosomal storage disease with combined ß-galactosidase and neuraminidase deficiency. Biochim. Biophys. Acta. 703, 69-76.

van Diggelen, O.P., Schindler, D., Kleijer, W.J., Heijmans, J.M.G., Galjaard, H., Linden, H.U., Peter-Katalinie, J., Egge, H., Dabrowski, U. and Cantz, M. (1987). Lysosomal α-N-acetylgalactosaminidase deficiency: A new inherited metabolic disease. Lancet No. 8562, Vol.II, 804.

van Dongen, J.M., Barneveld, R.A., Geuze, H.J. and Galjaard, H. (1984). Immunocytochemistry of lysosomal hydrolases and their precursor forms in normal and mutant human cells. Histochemical J. 16, 941-954.

van Dongen, J.M. and Willemsen, R., Ginns, E.I., Tager, J.M., Barranger, J.A., Reuser, A.J.J. (1985). The subcellular localization of soluble and membrane-bound lysosomal enzymes in I-cell fibroblasts: a comparative immunocytochemical study. Eur. J. Cell Biol. 39, 179-189.

Van Gemund, J.J., Giesberts, M.A.H., Eerdmans, R.F., Blom, W. and Kleijer, W.J. (1983). Morquio-B disease. Spondyloepiphyseal dysplasia associated with acid ß-galactosidase deficiency. Report of three cases in one family. Hum. Genet. 64, 50-54.

van Hoof, F. (1973). $G_{M1}$-gangliosidosis. In: Lysosomal Storage Diseases. H.G. Hers. and F. van Hoof (eds.), pp. 305-321. Academic Press, New York.

van Zutphen, L.F.M., den Bieman, M.G.C.W. and Fox, R.R. (1983). Separation of ß-D-galactosidases in rabbit tissues: Genetics of neutral ß-D-galactosidase. Biochem. Genet. 21, 177-189.

Varki, A.P., Reitman, M.L. and Kornfeld, S. (1981). Identification of a variant of mucolipidosis III (pseudo-Hurler polydystrophy): a catalytically active N-acetylglucosaminylphosphotransferase that fails to phosphorylate lysosomal enzymes. Proc. Natl. Acad. Sci. USA. 78, 7773-7777.

Varki, A. and Kornfeld, S. (1981). Purification and

characterization of rat liver α-N-acetylglucosaminyl phosphodiesterase. J. Biol. Chem. 256, 9937-9943.

Verheijen, F.W., Brossmer, R. and Galjaard, H. (1982). Purification of acid ß-galactosidase and acid neuraminidase from bovine testis: evidence for an enzyme complex. Biochem. Biophys. Res. Commun. 108, 868-875.

Verheijen, F., Palmeri, S., Hoogeveen, A.T. and Galjaard, H. (1985). Human placental neuraminidase. Eur. J. Biochem. 149, 315-321.

Verheijen, F.W. (1986). Lysosomal neuraminidase in human genetic disease. Thesis, Erasmus University Rotterdam.

Verheijen, F.W., Palmeri, S. and Galjaard, H. (1987). Purfication and partial characterization of lysosomal neuraminidase from human placenta. Eur. J. Biochem. 162, 63-67.

Von Figura, K. and Hasilik, A. (1986). Lysosomal enzymes and their receptors. Ann. Rev. Biochem. 55, 167-193.

Waheed, A., Hasilik, A. and von Figura, K. (1981). Processing of the phosphorylated recognition marker in lysosomal enzymes. J. Biol. Chem. 256, 5717-5721.

Waheed, A., Hasilik, A. and von Figura, K. (1982). UDP-N-acetylglucosamine: lysosomal enzyme precursor N-acetylglucosamine-1-phosphotransferase. J. Biol. Chem. 257, 12322-12331.

Walz, E. (1927). Uber das Vorkommen von Kerasin in normaler Ochsenmilz. Hoppe-Seyler's Z. Physiol. Chem. 251, 7517-7520.

Warner, T.G. and O'Brien, J.S. (1982). Structure analysis of the major oligosaccharides accumulating in canine $G_{M1}$ gangliosidosis liver. J. Biol. Chem. 257, 224-232.

Wenger, D.A., Goodman, S.I. and Myers, G.G. (1974). ß-galactosidase deficiency in young adults. Lancet ii, 1319-1320.

Wenger, D., Sattler, M., Clark, C. and Wharton, C. (1976). I-cell disease: activities of lysosomal enzymes towards natural and synthetic substrates. Life Sci. 19, 413-420.

Wenger, D.A., Tarby, T.J. and Wharton, C. (1978). Macular cherry-red spots and myoclonus with dementia: coexistent neuraminidase and ß-galactosidase deficiencies. Biochem. Biophys. Res. Commun. 82, 589-595.

Wenger, D.A., Satter, M., Mueller, T., Myers, G.G., Schneiman, R.S. and Nixon, G.W. (1980). Adult $G_{M1}$ gangliosidosis:

Clinical and biochemical studies on two patients and comparison to other patients called varant or adult $G_{M1}$ gangliosidosis. Clin. Genet. 17, 323-334.

Wenger, D.A., Sattler, M. and Roth, S. (1982). A protein activator of galactosylceramide ß-galactosidase. Biochim. Biophys. Acta. 712, 639-649.

Wenger, D.A. and Inui, K. (1984). Studies on the sphingolipid activator protein for the enzymatic hydrolysis of $G_{M1}$ ganglioside and sulfatide. In molecular basis of lysosomal storage disorders (eds.) J.A. Barranger, R.O. Brady. Acad. Press. Inc. pp.61-78.

Wenger, D.A., Sujansky, E., Fennessey, P.V. and Thompson, J.N. (1986). Human ß-mannosidase deficiency. N. Eng. J. Med. 315, 1201-1205.

Wiesmann, U., Vassella, F. and Herschkowitz, N.N. (1971). I-cell disease: leakage of lysosomal enzymes into extracellular fluids. N. Engl. J. Med. 285, 1090-1091.

Willingham, M.C., Pastan, I.H., Sahagian, G.G., Jourdian, G.W. and Neufeld, E.F. (1981). Morphologic study of the internalization of a lysosomal enzyme by the mannose 6-phophate receptor in cultured Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA. 78, 6967-6971.

Wolfe, L.S., Callahan, J., Faweett, J.S., Andermann, F. and Server, C.R. (1970). $G_{M1}$-gangliosidosis without chondrodystrophy and visceromegaly. Neurology. 20, 23-44.

Wolfe, L.S., Senior, R.G. and Ng Ying Kin, N.M.K. (1974). The structures of oligosaccharides accumulating in the liver out $G_{M1}$-gangliosidosis, type I. J. Biol. Chem. 249, 1828-1838.

Yamamoto, Y., Adachi, S., Kamamura, S., Takashi, M., Ohtori, T., Shinji, Y. and Nishikawa, M. (1974). Localized ß-galactosidase deficiency. Occurrence in cerebellar ataxia with myoclonus, epilepsy and macular cherry-red spot. A new variant of $G_{M1}$-gangliosidosis. Arch. Intern. Med. 134, 627-634.

Yamamoto, Y. and Nishimura, K. (1980). pH-Dependent association dissociation of $G_{M1}$-ß-galactosidase purified from porcine spleen. J. Biochem. 88, 705-713.

Yamamoto, Y., Fujie, M. and Nishimura, K. (1982). The interrelation between high- and low-molecular-weight forms of $G_{M1}$-ß-galactosidase purified from porcine spleen. J. Biochem. 92, 13-21.

Yamamoto, Y. and Nishimura, K. (1986). Aggregation-

dissociation and stability of acid ß-galactosidase purified from porcine spleen. Int. Biochem. 4, 327-335.

Yamashita, K., Ohkura, T., Okada, S., Yabuuchi, H. and Kobata, A. (1981). Urinary oligosaccharides of $G_{M1}$-gangliosidosis. Different excretion patterns of oligosaccharides in the urine of type 1 and type 2 subgroups. J. Biol. Chem. 256, 4789-4798.

Zeigler, M. and Bach, G. (1981). Cellular localization of neuraminidases in cultured human fibroblasts. Biochem. J. 198, 505-508.

Zeigler, M. and Bach, G. (1985). Ganglioside distribution in mucolipidosis type IV cultured fibroblasts. Arch. Biochem. Biophys. 241, 602-607.

DANKWOORD

Graag wil ik iedereen bedanken die op enigerlei wijze heeft bijgedragen aan het tot stand komen van dit proefschrift. In het bijzonder ben ik mijn promotor Prof. Dr. H. Galjaard erkentelijk voor de stimulerende begeleiding die hij mij gaf bij mijn wetenschappelijke vorming en voor de mogelijkheden die ik kreeg om in het buitenland kennis op te doen.

Ik dank de overige leden van de promotie commissie Prof. Dr. J.F. Koster, Prof. Dr. J.M. Tager en Prof. Dr. H.F.M. Busch voor het kritisch doorlezen van dit proefschrift en voor hun waardevolle suggesties omtrent de vorm en inhoud ervan.

Van de lysosomenclub wil ik mijn (ex)collega's bedanken voor hun hulp, interesse en de stimulerende discussies. In het bijzonder dank ik Arnold Reuser die voor mij meer is dan een collega. Jouw bereidwilligheid om op elk moment een ieder te helpen is heel bijzonder. Frans en Sandra veel van het werk in dit proefschrift is met jullie hulp tot stand gekomen. Rob en Han altijd opgewekt en direct klaar om de helpende hand te bieden. Bedankt Marjan, Elly, Joke en Ans het is fijn dat er mensen zijn die naast het werk ook aan de sociale contacten denken. Bert de discussies tijdens de reis naar huis waren te kort. Lies, Ben, Dicky, Niels, Nynke, Nicole, Hans, Pauline, Marianne ik hoop dat de contacten in de toekomst beter worden. Jetty, Eveline, Ruud, Marja, Margien en Herman ik mis jullie.

De 24e (Otto, Wim, Henk, Diana, Ruud, Eva en Martinus) ik weet jullie altijd te vinden. Martinus jou wil ik bedanken voor de onvermoeibare wijze waarop jij mij attendeerde op artikelen die van nut konden zijn bij mijn onderzoek.

Het fotografische werk was bij Joop Fengler en Tar van Os in zeer goede handen. Het tekenwerk werd uitstekend verzorgd door Pim Visser. Nelly bedankt voor het goede type- en printwerk en voor de belangstelling die jij en je collega's

toonden. Diana jij bent er altijd om me even tussendoor te helpen. Piet Hartwijk en Jan de Josselin de Jong zorgde voor de technische kant. Piet bedankt voor de openhartige gesprekken die wij zowel binnen als buiten onze werkkring voerden. Rein ook bij jou kom ik niet alleen om te bestellen. Jopie, Elly en mevr. Godijn dank voor de goede zorg, als jullie er niet zijn merkt iedereen dit direct. Prof. Dr. H. Jongkind en Ton Verkerk als goede buren zijn jullie er wanneer nodig.

I am very grateful to Prof. Dr. D. Robinson (University of London) for his contribution to the work described in this thesis and I appreciated very much his stimulating discussions. I thank Prof. Dr. R. Brossmer (University of Heidelberg ), Prof. Dr. L. Svennerholm and Dr. J.E. Mansson (University of Goteburg) for their hospitality during my stay in their institutes and for providing me the substrates necessary for experimental work mentioned in this thesis. I am also very grateful to Prof. Dr. Y. Suzuki (University of Tokyo) who invited me to work in his institute and I thank him and his collegues for their hospitality during my stay.

Further I like to thank the guests (Dr. Hiroko Kawashima, Dr. Silvia Palmeri, Dr. Grazia Mancini, Dr. Giancarlo Parenti) who have worked with me during the last years and contributed to the experimental work mentioned in this thesis.

## CURRICULUM VITAE

De schrijver van dit proefschrift werd op 23 februari 1947 te Vlaardingen geboren. Van 1965 tot 1970 was hij als analist werkzaam bij de (toenmalige) Fabriek van Chemische Producten Vondelingenplaat. Tussentijds werd de militaire dienstplicht vervuld bij de verbindingsdienst van de Koninklijke Marine. Na de middelbare school werd een Hogere Beroeps Opleiding gevolgd aan het Van 't Hoff Instituut te Rotterdam.

In 1970 trad hij in dienst van het instituut Histologie en Celbiologie I van de Medische Faculteit te Rotterdam. In 1977 werd hij bevorderd tot scheikundige. Naar aanleiding van zijn onderzoek op het gebied van erfelijke stofwisselingsziekten werd hij uitgenodigd door verschillende buitenlandse instituten.

In 1985 volgde een aanstelling als wetenschappelijk onderzoeker bij de afdeling Celbiologie en Genetica. Vanaf die periode werd ook onderwijs in histologie gegeven aan 1e jaars studenten geneeskunde. In 1986 werd zijn werk bekroond met de "Steven Hoogendijk Prijs".

Het in dit proefschrift beschreven onderzoek beslaat de werkzaamheden verricht in de periode 1980-1986 onder leiding van Prof. Dr. H. Galjaard op de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam.

# P A P E R  I

Nature

(1980) 285, 500-502

Reprinted from Nature, Vol. 285, No. 5765, pp. 500-502, June 12 1980


# Genetic heterogeneity in human neuraminidase deficiency

A. T. Hoogeveen, F. W. Verheijen, A. d'Azzo & H. Galjaard

Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands

**There is a deficiency of human α-*N*-acetylneuraminidase in several inherited diseases. In patients with mucolipidosis I (refs 1,2) and in adults with a variant form without bony abnormalities and mental retardation[3,4], both also classified as sialidoses[4], it is the only deficient enzyme. In mucolipidosis II ('I-cell' disease) neuraminidase is one of many deficient lysosomal hydrolases[5-7] and a third manifestation combines deficiency of neuraminidase and β-galactosidase[8,9]. We have investigated the genetic background of these various neuraminidase deficiencies by somatic cell hybridization and co-cultivation. The principal conclusions from work on mutant fibroblasts, reported here, are that at least three gene mutations are involved and that the combined β-galactosidase/neuraminidase deficiency is likely to be due to defective post-translational modification of these enzymes.**

Table 1 summarizes the neuraminic acid content and the activities of β-galactosidase and neuraminidase in the different mutant fibroblasts and 4 days after fusion of each cell type with itself (parental fusion). The activity of neuraminidase was measured with *N*-acetylneuraminosyl-D-lactose and with 4-methylumbelliferyl-α-2-*N*-acetylneuraminic acid (provided by Dr J. S. O'Brien), and similar results were obtained with both substrates. Because of its simplicity and high sensitivity, we have used principally the fluorometric assay, the results of which are reported here. As Table 1 shows, the neuraminidase activity in fibroblasts from a severely mentally retarded 11-yr-old girl with the classical form of mucolipidosis I (ML I), also classified as sialidosis 2 (ref. 4), was 1% of control values. Cells from a patient with a variant form described by Durand *et*

**Table 2** Cell hybridization and co-cultivation of neuraminidase-deficient human fibroblasts

| Combination of cell strains | Neuraminidase activity ($\times 10^{-9}$ mol per h per mg protein) Hybridization | Co-cultivation |
|---|---|---|
| I-cell and class. ML I | 6.2 | 0.5 |
| I-cell and variant ML I | 9.6 | 1.2 |
| I-cell and adult β-gal⁻/neur⁻ | 3.5 | 0.6 |
| I-cell and infantile β-gal⁻/neur⁻ | 5.8 | 0.2 |
| Class. ML I and variant ML I | 1.6 | 1.2 |
| Adult β-gal⁻/neur⁻ and infant. β-gal⁻/neur⁻ | 0.8 | 1.0 |
| Class. ML I and adult β-gal⁻/neur⁻ | 9.4 | 3.7 |
| Class. ML I and infant. β-gal⁻/neur⁻ | 5.0 | 3.0 |
| Variant ML I and adult β-gal⁻/neur⁻ | 7.0 | 4.0 |
| Variant ML I and infant. β-gal⁻/neur⁻ | 6.8 | 4.9 |

Values are the means of three to six independent experiments. Hybridization was carried out with polyethylene glycol as before[15] adapted for human fibroblasts in monolayer. $10^6$ cells of both cell strains were mixed 4 days before fusion and cultivated in Ham's F10 medium with 10% fetal calf serum. The medium was changed after 3 days and 1 day later cells were rinsed twice with medium without serum, and after removal of the medium, hybridization was carried out as follows: 1 ml of 42% polyethylene glycol (PEG) molecular weight 1,000 (Koch-Light) in Ham's F10 with 15% DMSO was added. The mixture was rocked for 2 min, 1 ml 25% PEG in Ham's F10 was added, the mixture was rocked again and 8 ml Ham's F10 was added twice. After rocking, the medium was removed, the cells rinsed with Ham's F10 and then Ham's F10 with 10% fetal calf serum was added. After 4 days of cultivation in the same medium, the heterokaryon population was collected and analysed for neuraminidase activity using 4-methylumbelliferyl as substrate. Microscopy of stained preparations after cell fusion revealed that 70–90% of the cells contained more than one nucleus, and autoradiography after incorporation of $^3$H-thymidine showed that nearly all multinucleate cells were heterokaryons.

**Table 1** Activities of β-galactosidase and neuraminidase, and neuraminic acid content in mutant human fibroblasts and effect of cell fusion

| Cell type | Total neuraminic acid ($10^{-9}$ mol per mg) | β-Galactosidase | Neuraminidase Unfused | Neuraminidase After parental fusion |
|---|---|---|---|---|
| I-Cell* | 117 | 4 | 0.3 | 0.2 |
| Classical ML I* | 71 | 730 | 1.3 | 0.7 |
| Variant ML I* | 40 | 850 | 3.8 | 3.9 |
| Adult β-gal⁻/neur⁻ | 60 | 45 | 1.3 | 1.5 |
| Infantile β-gal⁻/neur⁻ | 59 | 48 | 0.6 | 0.7 |
| Heterozygous mother | 16 | 574 | 31 | — |
| Heterozygous father | 17 | 627 | 34 | — |
| Control fibroblasts | | | | |
| Mean | 22 | 630 | 82 | — |
| Range | 15–31 ($n=17$) | 350–1,050 ($n=36$) | 43–129 ($n=17$) | — |

Activities are expressed as nmol per h per mg protein and the values given are the means of three to six independent experiments on each mutant strain and on a large number of control fibroblasts. In all instances fibroblasts were cultured in Ham's F10 medium with 10% fetal calf serum and they were collected by trypsinization, rinsed in saline and centrifuged. Cells were disrupted by addition of bidistilled water to the pellet, and after shaking, the homogenate was used directly for biochemical analysis. Neuraminidase activity was determined with methylumbelliferyl substrate (provided by Drs T. Warner and J. S. O'Brien, see ref. 20 for synthesis). Cell homogenate (1 μl) was incubated with 2 μl 2 mM substrate in 0.25 M Na-acetate buffer, pH 4.3, for 1–2 h at 37 °C; the fluorescence of the liberated methylumbelliferone was measured after addition of 500 μl 0.5 M sodium carbonate buffer, pH 10.7, at 448 nm. The activity of β-galactosidase was also measured with methylumbelliferyl substrate as before[9]. Total neuraminic acid content was measured after hydrolysis of the cells for 1 h at 80°C in 0.1 N $H_2SO_4$ according to Warren[21].

*Cultured fibroblasts from a patient with mucolipidosis I were provided by Dr H. D. Bakker; those from a patient with a variant form (De PF in ref. 3) by Dr P. Durand, and those from a patient with I-cell disease by Dr A. Boué.

*al.*[3], and sometimes classified as sialidosis 1 (ref. 4), had a residual activity of 4–5%. This higher residual activity is probably responsible for the lower neuraminic acid content in these fibroblasts.

Fibroblasts from a mentally retarded adult male with myoclonus and ataxia[10] and from a 2-yr-old boy with Hurler-like features but no neurological abnormalities[11] had a profound neuraminidase deficiency in addition to a 10% residual activity of β-galactosidase (Table 1). These two patients had previously been classified as variant forms of $G_{M1}$-gangliosidosis[12] or β-galactosidase deficiency[13] on the basis of complementation after fusion with fibroblasts from patients with a primary defect of β-galactosidase. Fibroblasts from both parents of the patient with infantile β-galactosidase/neuraminidase deficiency (β-gal$^-$/neur$^-$) had normal activity of β-galactosidase but neuraminidase activity was about half the mean control value (Table 1). This makes it unlikely that β-galactosidase deficiency is the primary defect in this condition. One of our patients with β-gal$^-$/neur$^-$ has recently been classified as sialidosis II (ref. 4). Because the primary defect in this condition has not yet been resolved and the syndromes included in the category sialidosis 2 are so different clinically, biochemically and even genetically, such classification seems somewhat premature.

We have studied the genetic background of the various neuraminidase deficiencies by complementation analysis after somatic cell hybridization. The results of enzyme assays 4 days after fusion of each cell strain with itself (parental fusion in Table 1) indicate that the polyethylene glycol (PEG) method of hybridization[14] does not affect neuraminidase activity. The results of fusions of different mutant cell strains are summarized in the second column of Table 2. Compared with parental fusions, there was 4–15 times as much neuraminidase activity after fusion of I-cells with each of the other mutant fibroblasts. The activities after co-cultivation (third column of Table 2) were not higher than the average values for each pair of parental cells. The complementation observed after fusion of I-cells with each of the other mutant fibroblasts is most probably due to a correction of the post-translational defect in I-cell disease[7]. The rapid generation of neuraminidase activity and of other lysosomal hydrolases after fusion of I-cells with various other mutant fibroblasts[15] would agree with a normalization of the processing and/or activation of preformed glycosidases.

The fusions of classical MLI × variant MLI did not result in complementation and these conditions probably represent different mutations within one gene. The same is true for the adult type and infantile type of β-gal$^-$/neur$^-$ deficiency. Fusions of each of the MLI strains with each of the β-gal$^-$/neur$^-$ deficient fibroblasts, however, resulted in a clear increase in neuraminidase activity (3–9 times the values after parental fusion). This indicates that two different gene mutations are involved in the neuraminidase deficiency of sialidosis 1 and the combined β-gal$^-$/neur$^-$. The restoration of neuraminidase activity after fusion of MLI cells with β-gal$^-$/neur$^-$ fibroblasts might result because one cell type, most probably MLI, is deficient in the structural part of the enzyme and the other is defective in a modifying enzyme or a regulatory factor. Another explanation could be that neuraminidase is made up of different subunits which must be normal for the expression of its activity.

Neuraminidase activity also increased after co-cultivation of the different types of MLI cells with each of the β-gal$^-$/neur$^-$ fibroblasts (compare last column Table 2 with average values of both parental strains in Table 1). This partial restoration of neuraminidase activity was investigated further by labelling the mutant fibroblasts with fluorescent polystyrene beads, followed by co-cultivation for 3 days, separation of the two cell populations by two-colour flow sorting (FACS II) according to Jongkind *et al.*[16] and assay of neuraminidase. Table 3 shows a marked increase in neuraminidase activity in the combined β-gal$^-$/neur$^-$ fibroblasts whereas the MLI cells remain deficient. We could find no neuraminidase activity in the culture medium above the mutant cells and the labelling did not affect the enzyme activity. These experiments suggest the transfer of an unknown factor from MLI cells to fibroblasts with a combined β-gal$^-$/neur$^-$ deficiency, a factor which can increase the neuraminidase activity in the latter cells four- to sevenfold. Normal human fibroblasts also secrete this 'correction factor'. We found no evidence of an activator that could act *in vitro*, for mixing of homogenates of the two kinds of cells did not affect enzyme activity. The correction observed after hybridization (Table 2) and after co-cultivation (Table 3) may represent a normalization of the post-translational processing of neuraminidase and β-galactosidase. It remains to be seen how neuraminidase is related to these processes, for neuraminidase deficiency can also occur without β-galactosidase deficiency. *N*-acetylneuraminic acid is, however, a common component of glycoproteins, certain glycosaminoglycans and gangliosides[17,18], and different neuraminidases have a role in the degradation of these compounds[8,19]. A better understanding of the nature of the neuraminidase deficiency in the various mutant cells might resolve the interrelationship with β-galactosidase deficiency. Further characterization of the correction factor is in progress; its heat lability, affinity for concanavalin A and the fact that I-cells cannot provide it (co-cultivation studies in Table 2) suggest that it is a glycoprotein.

**Table 3** Co-cultivation of different neuraminidase-deficient cell strains and enzyme assays after two-colour flow sorting (FACS II)

| Cell type | Neuraminidase activity ($\times 10^{-9}$ mol per h per mg protein) Before co-cultivation | After 3 d co-cultivation |
|---|---|---|
| Classical MLI | 0.7 | 0.7 |
| Infantile β-gal$^-$/neur$^-$ | 0.8 | 5.3 |
| Mixed cell population | 0.7 | 2.9 |

Red polystyrene beads were added to the culture medium above classical MLI cells, left for 2 days, and cells were collected by trypsinization, rinsed and centrifuged (1,000 r.p.m. for 5–10 min). The same was done with green beads for β-gal$^-$/neur$^-$ fibroblasts. About $2 \times 10^6$ cells of each labelled type were seeded and co-cultivated in confluency for 3 days. The two labelled cell populations were then separated and collected with a FACS II cell sorter according to Jongkind *et al.*[16] and neuraminidase activity was measured as described in Table 1. The values given are the mean of three independent experiments which gave very similar values.

Received 16 November 1979; accepted 24 March 1980.

1. Cantz, M., Gehler, J. & Spranger, J. *Biochem. biophys. Res. Commun.* **74**, 732–738 (1977).
2. Spranger, J., Gehler, J. & Cantz, M. *Am. J. med. Genet.* **1**, 21–29 (1977).
3. Durand, P. *et al. Helv. paediat. Acta* **32**, 391–400 (1977).
4. Lowden, J. A. & O'Brien, J. S. *Am. J. hum. Genet.* **31**, 1–18 (1979).
5. Thomas, G. H. *et al. Biochem. biophys. Res. Commun.* **71**, 188–195 (1976).
6. Strecker, G., Michalski, J. C., Montreuil, J. & Farriaux, J. P. *Biomedicine*, Paris **25**, 210–238 (1976).
7. Hasilik, A., Rome, L. H. & Neufeld, E. F. *Fedn. Proc.* **38**, 467 (1979).
8. Wenger, D. A., Tarby, T. J. & Wharton, C. *Biochem. biophys. Res. Commun.* **82**, 589–595 (1978).
9. Okada, S. *et al. Eur. J. Pediat.* **130**, 239–249 (1979).
10. Loonen, M. C. B., Lugt, van der, L. & Franke, C. L. *Lancet* **ii**, 785 (1974).
11. Andria, G., Del Guidice, E. & Reuser, A. J. J. *Clin. Genet.* **14**, 16–23 (1978).
12. Galjaard, H. *et al. Nature* **257**, 60–62 (1975).
13. Reuser, A. J. J. *et al. Hum. Genet.* **46**, 11–19 (1979).
14. Hales, A. *Somat. Cell Genet.* **3**, 227–230 (1977).
15. d'Azzo, A., Halley, D. J. J., Hoogeveen, A. & Galjaard, H. *Am. J. hum. Genet.* (in the press).
16. Jongkind, J. F., Verkerk, A. & Tanke, H. *Expl Cell Res.* **120**, 444–448 (1979).
17. Rosenberg, A. & Schengrund, C. L. *Biological Roles of Sialic Acid* (Plenum, New York, 1976).
18. Ledeen, R. W. *J. supramolec. Struct.* **8**, 1–17 (1978).
19. Cantz, M. & Messer, H. *Eur. J. Biochem.* **97**, 113–118 (1979).
20. Warner, T. G. & O'Brien, J. S. *Biochemistry* **18**, 2783–2787 (1979).
21. Warren, L. *J. biol. Chem.* **234**, 1971–1975 (1959).

Printed in Great Britain by Macmillan Production Ltd., Basingstoke, Hampshire

# P A P E R  II

Biochem. Biophys. Res. Comm.

(1981) 103, 292-300

Vol. 103, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 16, 1981

# CORRECTION OF COMBINED β-GALACTOSIDASE/NEURAMINIDASE DEFICIENCY IN HUMAN FIBROBLASTS

André Hoogeveen, Alessandra d'Azzo, Reinhard Brossmer* and Hans Galjaard

Dept. of Cell Biology and Genetics, Medical Faculty, Erasmus University Rotterdam, the Netherlands

*Institut für Biochemie II, Universität Heidelberg, W. Germany

Received September 8, 1981

## SUMMARY

The combined deficiency of β-galactosidase and neuraminidase in human fibroblasts can be corrected to nearly normal values. This can be accomplished by addition of concentrated culture medium obtained after $NH_4Cl$ stimulation of different types of human fibroblasts, including those with an isolated β-galactosidase or neuraminidase deficiency. The corrective factor is a macromolecular glycoprotein, which is labile at 60°C. Its uptake by human fibroblasts is competitively inhibited by mannose-6-phosphate and its corrective action within β-gal$^-$/neur$^-$ fibroblasts continues during a "chase" of 72 hours.

## INTRODUCTION

Various patients with skeletal abnormalities, skin lesions, retinal cherry-red spot, myoclonus and dementia have been found to have a combined deficiency of β-galactosidase and neuraminidase (1-4). The nature of this combined lysosomal enzyme deficiency has not yet been resolved but complementation studies after fusion of different types of human mutant fibroblasts indicate that the responsible gene mutation is different from that involved in the isolated β-galactosidase deficiency in $G_{M1}$-gangliosidosis (5, 6) and that causing isolated neuraminidase deficiency in mucolipidosis I (4, 7).

When fibroblasts with a combined deficiency of β-galactosidase and neuraminidase (β-gal$^-$/neur$^-$) were co-cultivated with other human fibroblasts, including those with an isolated neuraminidase deficiency (neur$^-$), a slight increase of neuraminidase activity in the β-gal$^-$/neur$^-$ cells was observed (4).

Preliminary experiments suggested that this partial correction was due to the uptake of a glycoprotein that is secreted by other cells into the culture medium.

The present studies were carried out to obtain more information about the nature of the correction of this combined lysosomal enzyme deficiency.

## MATERIALS AND METHODS

### Cell cultivation and analysis

Human skin fibroblasts were cultivated in Ham's F10 medium supplemented with 10% fetal calf serum and antibiotics. Fibroblasts from patients with infantile and adult $G_{M1}$-gangliosidosis were kindly provided by Dr. H. Goldman (Montreal) and Dr. Y. Suzuki (Tokyo) respectively. Cells with an isolated neuraminidase deficiency were derived from a patient with mucolipidosis I (Dr. Bakker, Amsterdam) and two cell strains with a combined deficiency of β-galactosidase and neuraminidase were derived from a 2-year-old boy described by Andria et al. (8) and from an affected 21-week-old fetus described by Kleijer et al. (3). The cells were free of mycoplasm as judged by the method of Chen (9). Enzyme analyses were performed after trypsinization, rinsing in saline, centrifugation (10 min. 100 g) and lysis in double distilled water. The activities of β-galactosidase and neuraminidase were assayed with 4-methylumbelliferyl-β-D-galactopyranoside (Koch-Light) and 4-methylumbelliferyl-α-D-N-acetylneuraminic acid (prepared in Institut für Biochemie II (Med. Fak.), Universität Heidelberg).

To stimulate the secretion of "high-uptake" forms of glycoproteins, fibroblasts were grown to confluency in a 200 $cm^2$ flask, whereafter the growth medium was replaced by 40 ml of serum free medium to which $NH_4Cl$ was added in a final concentration of 10 mM. After 2 or 3 days the medium was collected, dialyzed against Dulbecco's phosphate buffered saline and concentrated on an Amicon PM 10 filter to a final volume of 1 ml (concentrate). In case cells with normal β-galactosidase activity were used, the β-galactosidase secreted into the medium after $NH_4Cl$ treatment was removed by affinity column chromatography using a p-aminophenylthiogalactoside-CH-sepharose affinity matrix (10).

### Correction

To study the corrective effect an aliquot of 40 µl concentrated medium after $NH_4Cl$ treatment was added to 1 ml of Ham's F10 medium supplemented with fetal calf serum and left for a few hours up to three days on different types of mutant fibroblasts.

Correction was also studied by addition of a Concanavalin A preparations of human placenta and liver. Concanavalin A - sepharose 4B (Pharmacia, Uppsala) was used according to a procedure described earlier (11). After elution at 20°C with 0.75 M α-

Table I

EFFECT OF CONCANAVALIN A PREPARATIONS FROM HUMAN PLACENTA AND LIVER ON THE β-GALACTOSIDASE AND NEURAMINIDASE ACTIVITY IN NEURAMINIDASE DEFICIENT FIBROBLASTS*

| | ENDOGENOUS ACTIVITY | | AFTER ADDITION OF CON.A PREPARATIONS | |
|---|---|---|---|---|
| | β-gal. | neur. | β-gal. | neur. |
| β-GAL$^-$/NEUR$^-$ (8) | 59.3 | 1.3 | (1) 58.7 | 3.6 |
| | | | (2) 67.7 | 5.9 |
| MUCOLIPIDOSIS I | 640 | 0.4 | (2) 650 | 0.3 |

*Activities have been measured with 4-methylumbelliferyl substrate and are expressed as $10^{-9}$.moles.mg$^{-1}$.hr$^{-1}$. (1) represents Con.A preparation from human liver and (2) from placenta.

methylglucoside and dialysis against 10 mM Na-phosphate buffer pH.7 the preparation was concentrated on an Amicon PM 10 filter till a final protein concentration of 15 mg/ml. An aliquot of 50 µl of this preparation was added to 1 ml medium.

Inhibition of β-galactosidase activity

Irreversible inhibition of β-galactosidase activity in cultured human fibroblasts was performed by growing cells for 2 hours in medium containing 0.2 mM β-D-galactopyranosylmethyl-p-nitrophenyltriazene (β-galMNT) kindly provided by Dr. M. Sinnott (School of Chemistry, University of Bristol) according to procedures described earlier (12, 13). After refreshing the medium, the reappearance of β-galactosidase activity was studied during a period of 3 days.

RESULTS

Concanavalin A - sepharose 4B (Con.A) preparations of human placenta and human liver were added for two days to the medium above β-gal$^-$/neur$^-$ fibroblasts and to cells with an isolated neuraminidase deficiency (neur$^-$). The results in Table 1 show a 3 to 5 times increase of the neuraminidase activity in β-gal$^-$/

Table II

EFFECT OF CONCENTRATED "CORRECTIVE FACTOR" ON THE ACTIVITIES OF β-GALACTOSIDASE AND NEURAMINIDASE IN DIFFERENT TYPES OF HUMAN MUTANT FIBROBLASTS*

| MUTANT CELL STRAIN | β-GALACTOSIDASE | | NEURAMINIDASE | |
|---|---|---|---|---|
| | endo-genous | after addition "corrective factor" | endo-genous | after addition "corrective factor" |
| $\beta$-GAL$^-$/NEUR$^-$ (8) | 64 | 474 | 2.2 | 32 |
| $\beta$-GAL$^-$/NEUR$^-$ (3) | 80 | 380 | 1.0 | 21 |
| INFANTILE $G_{M1}$-GANGLIOSIDOSIS | 5.5 | 4.5 | 90 | 82 |
| ADULT VARIANT $G_{M1}$-GANGLIOSIDOSIS (15) | 65 | 55 | 170 | 172 |
| MUCOLIPIDOSIS I | 610 | 540 | 0.5 | 1.0 |
| CONTROL FIBROBLASTS | 350-1050 $\bar{x}$ = 630 | | 43-172 $\bar{x}$ = 82 | |

*Activities are measured with 4-methylumbelliferyl substrate and are expressed as $10^{-9}$moles.mg$^{-1}$.hr$^{-1}$. The concentrate of "corrective factor" in these experiments is prepared from medium above $G_{M1}$-gangliosidosis fibroblasts (infantile type) after $NH_4Cl$ stimulation (see Materials and methods).

neur$^-$ fibroblasts. This increase cannot be due to uptake of neuraminidase, because the activity of neur$^-$ fibroblasts did not increase. Heat inactivation (15' at 100°C) of the Con.A fraction abolishes the partial restoration of the neuraminidase activity of $\beta$-gal$^-$/neur$^-$ fibroblasts. These observations suggest that the correction factor is a glycoprotein.

To achieve a more efficient uptake of the correction factor another source of glycoproteins was found in media of various types of human fibroblasts that were treated with $NH_4Cl$ to stimulate the secretion of precursor forms of glycoproteins (14). The medium containing these "high-uptake" forms of glycoproteins was then concentrated and dialyzed on an Amicon pM 10 filter, to remove most of the molecules with a molecular weight lower than 20 x $10^3$. The concentrated medium obtained in this way was

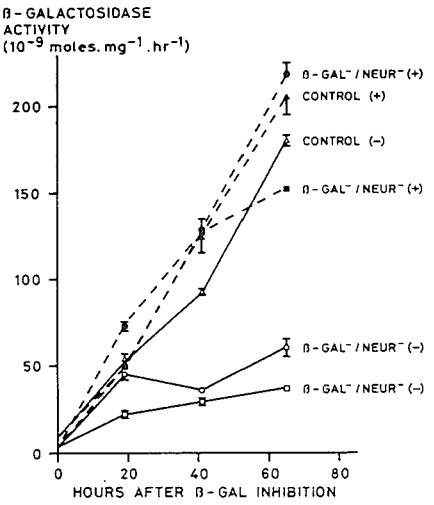



Fig. 1 CORRECTION FOR β-GALACTOSIDASE ACTIVITY IN β-GAL⁻/NEUR⁻ FIBROBLASTS.

After irreversible inhibition of β-galactosidase with a triazene analogue the reappearance of β-galactosidase was studied in two different β-gal⁻/neur⁻ cell strains with and without addition of "corrective factor" prepared from medium above $G_{M1}$-gangliosidosis fibroblasts after $NH_4Cl$ stimulation.

□ = β-gal⁻/neur⁻ fibroblasts (8)

○ = β-gal⁻/neur⁻ fibroblasts (3)

△ = normal fibroblasts.

Closed symbols represent the activity of β-galactosidase in the same cell strains but after addition of "corrective factor".

added to the culture medium above various types of human mutant fibroblasts and left for 2-3 days. Table II summarizes the effect of concentrate on the β-galactosidase and neuraminidase activity in the different cell types. There was no effect on fibroblasts with an isolated β-galactosidase deficiency (β-gal⁻) nor on neur⁻ cells. In two different strains of β-gal⁻/neur⁻ fibroblasts, however, the activity of both β-galactosidase and neuraminidase increases markedly and approaches low control values. In the experiment illustrated in Table II concentrated medium containing "corrective factor" was prepared from $NH_4Cl$ stimulated medium of $G_{M1}$-gangliosidosis fibroblasts (β-gal⁻), but similar results were obtained with concentrates from normal and mucolipidosis I media.

Addition of mannose-6-phosphate (1 mM) interferes with the reappearance of β-galactosidase and neuraminidase activities in β-gal$^-$/neur$^-$ cells after addition of "corrective factor". Heat treatment (15 min. at 60°C) of the corrective factor results in a complete loss of its effect, but at 50°C correction is still retained (data not shown).

The reappearance of newly synthesized β-galactosidase molecules after correction of β-gal$^-$/neur$^-$ cells could be studied with the use of an irreversible inhibitor of β-galactosidase (10, 12, 13). Addition of this inhibitor to the medium above β-gal$^-$/neur$^-$ fibroblasts results in a 94% inhibition of the residual β-galactosidase activity. Fig. 1 shows the reappearance of β-galactosidase activity during 65 hours after removal of the inhibitor. In β-gal$^-$/neur$^-$ cells the β-galactosidase activity reaches its original value within 24 hours and subsequently levels off. When a concentrate of "corrective factor" was added, however, the activity of β-galactosidase continues to progress as in control fibroblasts. The reappearance of β-galactosidase activity in control fibroblasts is not affected by addition of "corrective factor".

In subsequent experiments "corrective factor" was added to the medium above β-gal$^-$/neur$^-$ cells during 16 hours followed by a chase of 72 hours (Fig. 2). The intracellular increase of both neuraminidase and β-galactosidase activity was found to continue during 3 days after removal of the "corrective factor" from the medium. In contrast, medium from β-gal$^-$/neur$^-$ fibroblasts, prepared in the same way, had no effect on the intracellular activities of β-galactosidase or neuraminidase in the mutant cells.

## DISCUSSION

The complementation for β-galactosidase after fusion of different types of human β-galactosidase deficient cell strains (5) was surprising in view of the fact that normal human β-galactosidase consists of one polypeptide of about 70,000 m.w. which also occurs in an aggregate of molecular weight 700,000 (16). Later, Wenger et al. (1) found a coexistent deficiency of neuraminidase in one of their atypical patients with β-galactosidase deficiency. Fibroblasts from several other patients were sub-

sequently found to have this combined β-galactosidase/neuraminidase deficiency (2, 4). Analysis of interspecies and intraspecies hybrids, Hoeksema et al. (17, 18) showed that the isolated β-galactosidase deficiency in classical forms of $G_{M1}$-gangliosidosis is due to a structural mutation in the gene on chromosome 3 coding for the β-galactosidase polypeptide chain. In variants, later identified as β-gal$^-$/neur$^-$, the residual β-galactosidase activity consists of the monomeric form of β-galactosidase and aggregation to higher molecular weight forms seems to be impaired.

Using an irreversible inhibitor of β-galactosidase, van Diggelen et al. (10) observed that the turnover time of β-galactosidase in normal human fibroblasts and in $G_{M1}$-gangliosidosis cells is about 10 days whereas that in β-gal$^-$/neur$^-$ fibroblasts is reduced to less than 1 day. This was found to be due to enhanced degradation of β-galactosidase in these mutant cells (10).

In co-cultivation studies the neuraminidase activity of β-gal$^-$/neur$^-$ cells increased 5-7 fold as a result of transfer of a "corrective factor" secreted by other fibroblasts including those with an isolated neuraminidase deficiency (4). In the present study a similar effect was found after addition of a Con.A preparation of human liver or human placenta to medium above β-gal$^-$/neur$^-$ cells. In both instances only a partial restoration of neuraminidase activity (up to 10-15% of control values) and none of β-galactosidase occurs. When concentrates of culture media from $NH_4Cl$ stimulated fibroblasts are used, however, a complete restoration of the activity of both neuraminidase and β-galactosidase in β-gal$^-$/neur$^-$ cells is achieved. The rate of reappearance of β-galactosidase in β-gal$^-$/neur$^-$ fibroblasts in the presence of corrective factor (Fig. 1) is similar to that in control fibroblasts.

The corrective factor or its precursor form is secreted by various types of human fibroblasts including those from $G_{M1}$-gangliosidosis (β-gal$^-$) and mucolipidosis I (neur$^-$). No correction is obtained with concentrate prepared from medium after $NH_4Cl$ stimulation of β-gal$^-$/neur$^-$ fibroblasts. This points to the specific nature of the corrective factor. The experimental results presented indicate that the corrective factor is a macromolecular glycoprotein, excluded by an Amicon PM 10 filter, which

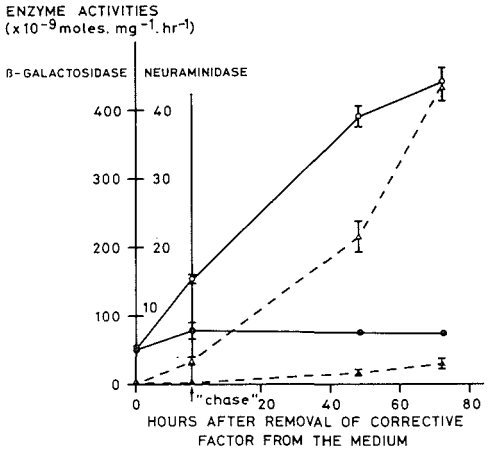



Fig. 2 ACTIVITIES OF β-GALACTOSIDASE AND NEURAMINIDASE IN β-GAL⁻/NEUR⁻ FIBROBLASTS AFTER ADDITION AND "CHASE" OF CORRECTIVE FACTOR.

During a period of 16 hours corrective factor was added to the medium above β-gal⁻/neur⁻ fibroblasts. Subsequently a "chase" was carried out during 72 hours and the intracellular activities of β-galactosidase (full line) and neuraminidase (dotted line) were measured.

○ and △ represent activities after addition of corrective factor prepared from medium above $G_{M1}$-gangliosidosis cells after $NH_4Cl$ stimulation.

● and ▲ represent activities after addition of medium above β-gal⁻/neur⁻ cells, treated in the same way.

is stable at 50°C but loses its activity at 60°C. I-cell secreted glycoproteins which are not phosphorylated, are not taken up by other fibroblasts (19). Our observation that I-cell medium cannot correct β-gal⁻/neur⁻ fibroblasts (4) is in agreement with the phosphoglycoprotein nature of the "corrective factor" described in the present paper. This is also supported by the fact that mannose-6-phosphate inhibits the uptake of the "corrective factor" (14, 20, 21, 22).

The finding that the β-galactosidase and neuraminidase activities in β-gal⁻/neur⁻ cells continue to increase after removal of the corrective factor from the medium (Fig. 2) might point to a (lysosomal) enzymic nature of the corrective factor.

Acknowledgements

We thank Dr. F.W. Verheijen for his contribution to parts of the experimental work and Dr. A.J.J. Reuser for stimulating discussions.

REFERENCES

1. Wenger, D.A., Farby, T.J., and Wharton, C. (1978) Biochem. Biophys. Res. Commun. 82, 589-595.
2. Lowden, J.A. and O'Brien, J.S. (1979) Am. J. Hum. Gen. 31, 1-18.
3. Kleijer, W.J., Hoogeveen, A., Verheijen, F.W., Niermeijer, M.F., Galjaard, H., O'Brien, J.S., and Warner, T.G. (1979) Clin. Genet. 16, 60-61.
4. Hoogeveen, A.T., Verheijen, F.W., d'Azzo, A., and Galjaard, H. (1980) Nature 285, 500-502.
5. Galjaard, H., Hoogeveen, A., Keijzer, W., De Wit-Verbeek, H.A., Reuser, A.J.J., Ho, M.W., and Robinson, D. (1975) Nature 257, 60-62.
6. Suzuki, Y., Furukawa, T., Hoogeveen, A., Verheijen, F., and Galjaard, H. (1979) Brain and Development 1, 83-86.
7. Swallow, D.M., Hoogeveen, A.T., Verheijen, F.W., and Galjaard, H. (1981) Ann. Hum. Genet. 45, 105-112.
8. Andria, G., Del Giudice, E., and Reuser, A.J.J. (1978) Clin. Genet. 14, 16-23.
9. Chen, T.R. (1977) Exp. Cell Res. 104, 255-262.
10. Van Diggelen, O.P., Schram, A.W., Sinnott, M.L., Smith, P.J., Robinson, D., and Galjaard, H. (1981) Biochem. J. (in press).
11. Miller, A.L., Frost, R.G., and O'Brien, J.S. (1976) Anal. Biochem. 74, 537-545.
12. Sinnott, M.L. and Smith, P.J. (1978) Biochem. J. 175, 525-538.
13. Van Diggelen, O.P., Galjaard, H., Sinnott, M.L., and Smith, P.J. (1980) Biochem. J. 188, 337-343.
14. Hasilik, A., and Neufeld, E.F. (1980) J. Biol. Chem. 255, 4937-4945.
15. Suzuki, Y., Nakamura, H., Fukuoka, K., Shimada, J., and Uono, M. (1977) Hum. Genet. 36, 219-229.
16. Norden, A.G.W., Tennant, L., and O'Brien, J.S. (1974) J. Biol. Chem. 249, 7969-7976.
17. Hoeksema, H.L., Van Diggelen, O.P., and Galjaard, H. (1979) Biochim. Biophys. Acta 566, 72-79.
18. Hoeksema, H.L., De Wit, J., and Westerveld, A. (1980) Hum. Genet. 53, 241-247.
19. Hickman, S., and Neufeld, E.F. (1972) Biochem. Biophys. Res. Commun. 49, 992-999.
20. Sly, W.S. and Stahl, P. (1978) In: Transport of Macromolecules in Cellular Systems (ed. Silverstein, S.C.) Dahlem Konferenzen, Berlin, 229-244.
21. Hasilik, A., Waheed, A., and Von Figura, K. (1981) Biochem. Biophys. Res. Commun. 98, 761-767.
22. Reitman, M.L., Varki, A., and Kornfeld, S. (1981) J. Clin. Invest. 67, 1574-1579.

P A P E R  III

Proc. Natl. Acad. Sci.

(1982) 79, 4535-4539

*Proc. Natl. Acad. Sci. USA*
Vol. 79, pp. 4535–4539, August 1982
Biochemistry

# Molecular defect in combined β-galactosidase and neuraminidase deficiency in man

(precursor β-galactosidase/leupeptin/corrective factor)

A. D'AZZO, A. HOOGEVEEN, A. J. J. REUSER, D. ROBINSON*, AND H. GALJAARD

Department of Cell Biology and Genetics, Medical Faculty, Erasmus University, Rotterdam, the Netherlands

*Communicated by Victor A. McKusick, April 5, 1982*

ABSTRACT In normal human fibroblasts, an enzymically active 85,000-dalton precursor form of β-galactosidase is processed, via a number of intermediates, into a mature 64,000-dalton form. In addition there is an enzymically inactive 32,000-dalton component and its 54,000-dalton precursor. In fibroblasts from patients with a combined deficiency of β-galactosidase and neuraminidase these last two components are absent and hardly any mature β-galactosidase can be demonstrated. Nevertheless, in the mutant fibroblasts, precursor β-galactosidase is synthesized and processed normally. The excessive intralysosomal degradation that is responsible for the deficiency of mature β-galactosidase can be partially corrected by addition of the protease inhibitor leupeptin, which results in the accumulation of 85,000-dalton precursor β-galactosidase and of a partially processed 66,000-dalton form. When mutant cells were grown in the presence of a "corrective factor" purified from the medium of $NH_4Cl$-stimulated cell cultures, both β-galactosidase and neuraminidase activities were restored to low control levels. The immunoprecipitation pattern was completely normal after addition of the corrective factor, and mature 64,000-dalton β-galactosidase accumulated in the mutant fibroblasts. We propose that the combined β-galactosidase/neuraminidase deficiency is caused by a defective 32,000-dalton glycoprotein which is normally required to protect β-galactosidase and neuraminidase against excessive intralysosomal degradation and to give these enzymes their full hydrolytic activity.

Acid β-galactosidase in human liver (1) and cultured fibroblasts (2) consists of a 70,000-dalton (Dal) monomeric form and an aggregate of the same polypeptide (600,000–800,000 Dal). Since the observation by Okada and O'Brien (3) that the autosomal recessive disease $G_{M1}$-gangliosidosis is due to a β-galactosidase deficiency, various patients with infantile, juvenile, and adult forms of this disease have been reported (4–7). Analyses of interspecies hybrids indicated that the β-galactosidase deficiency in the various forms of $G_{M1}$-gangliosidosis is based on a mutation of a structural gene on chromosome 3 coding for the enzyme polypeptide (8).

A number of patients have been described with a coexistent deficiency of β-galactosidase and neuraminidase (6, 7, 9, 10). Previous studies on fibroblasts from these types of patients had shown that the 10–15% residual β-galactosidase had normal kinetic properties (11) and that the aggregation of monomeric β-galactosidase was impaired (2). No evidence for a mutation of the structural locus was found (8).

Neuraminidase deficiency, however, also can occur without any abnormality of β-galactosidase, as in various types of patients with sialidosis (mucolipidosis I) (12–15). Complementation studies after somatic cell hybridization (10) indicated that three different gene mutations are responsible for the enzyme deficiencies in $G_{M1}$-gangliosidosis [β-galactosidase deficiency (β-gal$^-$)], sialidosis [neuraminidase deficiency (neur$^-$)], and combined β-galactosidase and neuraminidase deficiency (β-gal$^-$/neur$^-$).

The turnover time of β-galactosidase has been measured in different cell strains (16, 17). In normal fibroblasts and in $G_{M1}$-gangliosidosis cells, the enzyme has a half-life of approximately 10 days, whereas in β-gal$^-$/neur$^-$ fibroblasts it is <24 hr. Subsequent studies revealed that this reduction is due to enhanced degradation of β-galactosidase; the enzyme is synthesized at a normal rate (18). It was also shown that both the β-galactosidase and neuraminidase activities in β-gal$^-$/neur$^-$ fibroblasts could be restored by a "corrective factor" of a glycoprotein nature that is produced by normal fibroblasts and other mutant cells, including $G_{M1}$-gangliosidosis fibroblasts (19). Because the latter contain no measurable amount of β-galactosidase, a convenient source of this factor is the medium obtained when these mutant cells are cultured in the presence of ammonium chloride which diverts newly synthesized precursor forms of glycoproteins into the medium, thus enhancing their secretion (20–22).

In this paper we examine the molecular nature of the genetic defect responsible for β-gal$^-$/neur$^-$. Using immunoprecipitation studies on radiolabeled β-galactosidase and related components, we demonstrate the sequence of events involved in the processing of mature β-galactosidase from its precursor forms in control and mutant fibroblasts. We also show the molecular background of leupeptin inhibition of the excessive degradation of β-galactosidase in β-gal$^-$/neur$^-$ cells and that of full correction by addition of the putative factor.

## MATERIALS AND METHODS

**Cell Culture.** Human skin fibroblasts were maintained in Ham's F10 medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum and antibiotics. Fibroblasts from patients with the infantile or adult form of $G_{M1}$-gangliosidosis were kindly provided by H. Goldman (Montreal) and Y. Suzuki (Tokyo), respectively. Cells with isolated neur$^-$ were derived from a patient with classical mucolipidosis I (sialidosis) (from H. D. Bakker, Amsterdam). Two cell strains with β-gal$^-$/neur$^-$ were obtained from patients described earlier (23, 24).

**Preparation and Assay of $NH_4Cl$-Induced Secretions.** Normal culture medium above confluent cultures was replaced by serum-free medium supplemented with $NH_4Cl$ at a final concentration of 10 mM. Two days later the medium was collected

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: β-gal$^-$, β-galactosidase deficiency or deficient; β-gal$^-$/neur$^-$, β-galactosidase and neuraminidase deficiency or deficient; neur$^-$, neuraminidase deficiency or deficient; Dal, dalton.

* Permanent address: Dept. Biochemistry, Queen Elizabeth College, University of London.

and centrifuged (90 × g, 5 min) and enzymic activities were measured in 20-μl aliquots. The cells were harvested by trypsinization, rinsed in saline, and disrupted in distilled water. Enzymic analyses were carried out by using the appropriate 4-methylumbelliferyl substrates (Koch–Light) as described by Galjaard (25). Activities measured in 1 ml of culture medium are derived from approximately $10^6$ fibroblasts corresponding to 0.3 mg of cellular protein.

**Enzyme Purification.** Fibroblasts were grown to confluency in 200-$cm^2$ flasks and subcultured for 2 days in 40 ml of serum-free medium containing 10 mM $NH_4Cl$. The medium was collected and ultrafiltered to approximately 5 ml on an Amicon PM10 filter; then its pH was adjusted to 5.2 with 20 mM sodium acetate/0.1 M NaCl. The concentrate was applied to a 2.5-ml *p*-aminophenylthiogalactoside-CH-Sepharose affinity matrix (17). The column was washed with 20 mM sodium acetate, pH 5.2/1 M NaCl and eluted with 20 mM sodium acetate, pH 5.2/0.5 M NaCl containing 0.1 M γ-galactonolactone. The eluate was dialyzed against Dulbecco's phosphate-buffered saline and concentrated on an Amicon PM10 filter to a final volume of about 1 ml. The whole procedure was performed at 4°C.

Gel filtration studies of β-galactosidase were carried out on Sephracryl S-200 as described by Hoeksema *et al.* (2).

**Uptake Studies.** Cells were seeded at near confluency in 24-well plates (Costar) and cultured for 3–4 days. The purified β-galactosidase ($2.5 \times 10^{-5}$ unit) was added to the medium (1 unit hydrolyzes 1 μmol of 4-methylumbelliferyl-β-D-galactopyranoside per min). After 16-hr incubation in the presence of exogenous enzyme, the cells were cultured in fresh medium for 2 days prior to analysis. Where appropriate mannose 6-phosphate was used at a final concentration of 1 mM.

**Immunoprecipitation of Labeled β-Galactosidase.** This was carried out by using purified specific immunoglobulins immobilized to Sepharose-4B beads. The rabbit antiserum used for their production was raised against a highly purified human placental β-galactosidase prepared by affinity chromatography. It was virtually homogeneous and showed one 64,000-Dal band on polyacrylamide gel electrophoresis, corresponding to the known size of mature human β-galactosidase with traces of a smaller molecule (30,000 Dal). The amount of coupled IgG that precipitated 95% of the β-galactosidase in the cells was judged from a previously determined enzyme activity/IgG ratio.

Normal and mutant fibroblasts were grown in 75-$cm^2$ flasks for 2 weeks, to a cell density equivalent to 1.5 mg of protein per flask. At 3–4 days before labeling, Ham's F10 medium was replaced by Dulbecco's modification of Eagle's medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum and antibiotics. Prior to labeling, the cultures were rinsed and incubated for 1 hr with 6 ml of the medium free of leucine to induce the depletion of the intracellular leucine pool. The labeling medium (6 ml) was this same medium supplemented with 0.2 ml of dialyzed fetal calf serum and 0.2 ml of L-[4,5-$^3$H]leucine (0.2 mCi; 135 Ci/mmol; Amersham Radiochemical Center; 1 Ci = $3.7 \times 10^{10}$ becquerels). After incubation in the presence of the label, the cells were harvested and processed according to the method of Hasilik and Neufeld (20).

**Polyacrylamide Gel Electrophoresis and Fluorography.** Electrophoresis in the presence of $NaDodSO_4$ was performed on 12% slab gels according to Laemmli (26), except that the ratio of acrylamide to methylene-bisacrylamide was changed to 30:0.312. The gels were prepared for fluorography as described (27). [$^{14}$C]methyl-labeled protein molecular weight standards (from Amersham Radiochemical Center) were: phosphorylase B, 92,000; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000.

**Protease Inhibition.** In these experiments leupeptin (Sigma) was included in the medium above normal and mutant fibroblasts at a final concentration of 0.02 mM. The incubation period varied between 2 and 5 days.

**Correction Studies.** The corrective factor from $G_{M1}$-gangliosidosis fibroblasts was prepared as described (19). β-gal$^-$/neur$^-$ fibroblasts were incubated with concentrated corrective factor for 2 days in the presence of [$^3$H]leucine and the radiolabeled products were then examined by $NaDodSO_4$ electrophoresis.

## RESULTS

Because the β-galactosidase deficiency in β-gal$^-$/neur$^-$ cells is due to enhanced degradation (7, 17), the effect of leupeptin, a thiol protease inhibitor, was studied. Addition of 0.02 mM leupeptin to the culture medium for 5 days resulted in an increase in β-galactosidase activity from 40 and 47 nmol/hr per mg of protein in two different β-gal$^-$/neur$^-$ cell strains (from patients described in refs. 23 and 24) to 305 and 315 nmol/hr per mg. This is a restoration to low control levels (activity range in normal fibroblasts is 350–1,050). The effect of leupeptin on neuraminidase activity was less clear. Only in one cell strain (23) was there a significant increase and that only reached 10% of normal levels.

In medium from cultures of normal and different types of mutant fibroblasts, hardly any β-galactosidase activity could be detected (0.3–0.4 nmol/hr per ml). Addition of $NH_4Cl$ increased the extracellular β-galactosidase activity to 10–14 nmol/hr per ml for both β-gal$^-$/neur$^-$ fibroblasts and control cells. Such increase did not occur for $G_{M1}$-gangliosidosis fibroblasts. Neuraminidase activity could not be detected in the medium from any of the cells tested. The ability of β-gal$^-$/neur$^-$ cells to synthesize β-galactosidase precursor therefore is not impaired.

This extracellular β-galactosidase was purified from both normal and β-gal$^-$/neur$^-$ culture media by affinity chromatography. Comparison of the two preparations showed them to be identical with regard to a number of criteria.

Gel filtration studies indicated an apparent size of 80,000–90,000 Dal, consistent with this being the precursor form of the enzyme. Both samples had a pH optimum of 3.9 and a $K_m$ of 1.4 mM, compared to values of 4.3 and 0.25 mM for the mature intracellular enzyme.

Both preparations were taken up from medium from $G_{M1}$-gangliosidosis fibroblasts, and this uptake could be strongly inhibited by 1 mM mannose 6-phosphate (Table 1). A similar amount of β-galactosidase was ingested after a 16-hr incubation period when either preparation was used, and the levels in the recipient cells remained unchanged when cultured for a further 48 hr in fresh medium. However, when purified β-galactosidase precursor from control fibroblasts was administered to β-gal$^-$/neur$^-$ fibroblasts, no increase in enzyme activity could be detected after a 16-hr incubation. The excessive degradation of β-galactosidase in β-gal$^-$/neur$^-$ cells therefore is not due to obvious mutant properties of the β-galactosidase precursor.

In order to study possible molecular differences in the enzyme components, immunoprecipitation was carried out on radiolabeled normal and mutant cells and on media collected from the different cultures after $NH_4Cl$ treatment. The constituent polypeptides were then examined by gel electrophoresis under reducing and denaturing conditions.

**Control Fibroblasts.** After incubation for 24 hr in the presence of [$^3$H]leucine, control fibroblasts showed four radiolabeled immunoprecipitable components (Fig. 1). Two of these corresponded to the components of the placental preparations that showed a prominent 64,000-Dal form and traces of a 32,000-Dal form in Coomassie blue-stained gels. The 85,000-Dal precursor form that was also seen and the 64,000-Dal mature β-

Table 1. Uptake of β-galactosidase precursor purified from control medium and β-gal⁻/neur⁻ medium by human mutant fibroblasts

| Cell strain | Incubation | β-Galactosidase activity, nmol/hr/mg protein† | | |
|---|---|---|---|---|
| | | No addition | With β-galactosidase from control medium | With β-galactosidase from β-gal⁻/neur⁻ medium |
| β-gal⁻* | 16 hr | 3.7 | 13.0 | 15.1 |
| | 16 hr followed by 48-hr chase | 3.3 | 12.6 | 14.5 |
| | 16 hr in presence of 1 mM Man-6-*P* | 3.0 | 3.9 | 3.8 |
| β-gal⁻/neur⁻ | 16 hr | 40.5 | 42.0 | 39.5 |

* From $G_{M1}$-gangliosidosis.
† Each value is the mean of two or three separate experiments.

galactosidase were enzymically active. The 51,000- and 32,000-Dal components were inactive, as can also be deduced from their presence in $G_{M1}$-gangliosidosis fibroblasts which have less than 1% enzyme activity (unpublished data). After $NH_4Cl$ stimulation the immunoprecipitable forms in the medium had apparent sizes of 88,000 and 54,000 Dal but the cells lacked the 64,000- and 32,000-Dal components. This suggests that the medium forms are precursors of the mature 64,000-Dal β-galactosidase and of the enzymically inactive 32,000-Dal component.

The sequence of events and the relationship among these different constituents were clarified in pulse–chase experiments. After a 3-hr pulse, almost all immunoprecipitable radioactivity was present in the 85,000-Dal form of β-galactosidase (Fig. 1). After a 5-hr chase this band disappeared and transient intermediates gave rise to the 64,000-Dal mature form that was fully established over a period of 3 days. The 32,000-Dal component, which was not seen after the 3-hr pulse, became evident after the shortest period of chase with a concomitant disappearance of a 54,000-Dal component. A 51,000-Dal component was visible during the whole period of the pulse–chase experiment.

**β-gal⁻/neur⁻ fibroblasts.** Corresponding immunoprecipitation experiments with radiolabeled β-gal⁻/neur⁻ fibroblasts showed clear differences from control cells. Intracellularly, the mature 64,000-Dal β-galactosidase was barely visible and the 32,000-Dal component was absent (Fig. 2). As with the controls, 85,000- and 51,000-Dal bands were visible. After $NH_4Cl$ stimulation the intracellular pattern remained unchanged. Extracellularly, unlike the control, the 54,000-Dal band was absent and the only detectable constituent was 88,000 Dal.

In pulse–chase experiments on β-gal⁻/neur⁻ cells the labeling pattern after 3 hr was similar to that in control cells. After

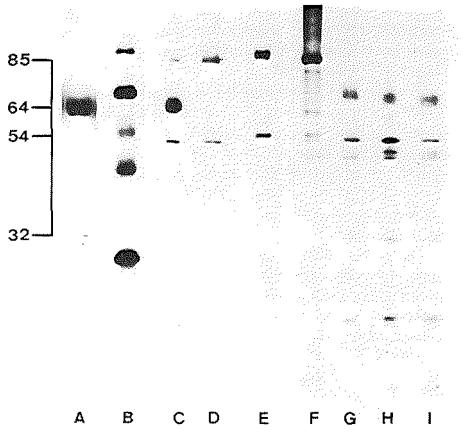



FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of β-galactosidase from normal fibroblasts and culture medium immunoprecipitated with antiserum after [³H]leucine labeling. Lanes: A, placental β-galactosidase (40 μg) stained with Coomassie blue; B, radiolabeled marker proteins; C, control fibroblasts labeled for 24 hr; D, control fibroblasts labeled for 24 hr in the presence of $NH_4Cl$; E, medium from control cells labeled for 24 hr in the presence of $NH_4Cl$. Lanes F–I show pulse labeling of β-galactosidase from control fibroblasts: F, 3-hr labeling; G, 3-hrs labeling followed by 5-hr chase in the presence of unlabeled leucine; H, 3-hr labeling and 20-hr chase; I, 3-hr labeling and 70-hr chase. Numbers show sizes in kDal.

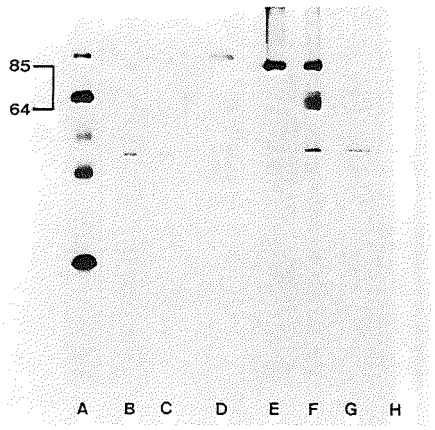



FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of β-galactosidase from β-gal⁻/neur⁻ fibroblasts and culture medium immunoprecipitated with antiserum after [³H]leucine labeling. Lanes: A, radiolabeled marker proteins; B, β-gal⁻/neur⁻ cells labeled for 24 hr; C, β-gal⁻/neur⁻ cells labeled for 24 hr in the presence of $NH_4Cl$; D, medium from β-gal⁻/neur⁻ cells labeled for 24 hr in the presence of $NH_4Cl$. Lanes E–H show pulse labeling of β-galactosidase from β-gal⁻/neur⁻ fibroblasts: E, 3-hr labeling; F, 3-hr labeling followed by 5-hr chase; G, 3-hr labeling and 20-hr chase; H, 3-hr labeling and 70-hr chase.

a 5-hr chase, when 85,000-Dal precursor β-galactosidase was still present, a 66,000-Dal form appeared (Fig. 2). Subsequently, both these bands disappeared but, unlike with the controls, mature 64,000-Dal β-galactosidase did not accumulate in significant quantity. Another feature was the absence of the 32,000-Dal component in β-gal$^-$/neur$^-$ cells during the whole period of chase, whereas the 51,000-Dal band was visible, as in controls.

Treatment with leupeptin led to an accumulation of both 85,000-Dal precursor β-galactosidase and of a partially processed 66,000-Dal form in β-gal$^-$/neur$^-$ cells and in control fibroblasts (Fig. 3).

Addition of the corrective factor (19) had an effect quite different from that of leupeptin: a complete normalization of the β-galactosidase labeling pattern occurred (Fig. 3). There was no accumulation of precursor form but instead, as in control cells, this was rapidly converted to the mature 64,000-Dal β-galactosidase which seemed to be resistant to excessive intralysosomal degradation.

## DISCUSSION

Previous studies (2, 7, 17) indicated that the 10–15% residual β-galactosidase activity in β-gal$^-$/neur$^-$ fibroblasts has normal properties but that there is an excessive intralysosomal degradation of β-galactosidase, shortening its half-life to about 1/10th (17, 18). The present paper shows that, in normal human fibroblasts, mature 64,000-Dal β-galactosidase is produced via an 85,000-Dal precursor form. Similar observations have been made for β-galactosidase in mouse macrophages (28). We have shown that in β-gal$^-$/neur$^-$ fibroblasts the 85,000-Dal form is synthesized normally and that all properties tested are similar to those of precursor β-galactosidase in control cells. The excessive degradation of β-galactosidase in β-gal$^-$/neur$^-$ fibroblasts therefore cannot be due to an abnormality of the precursor form.

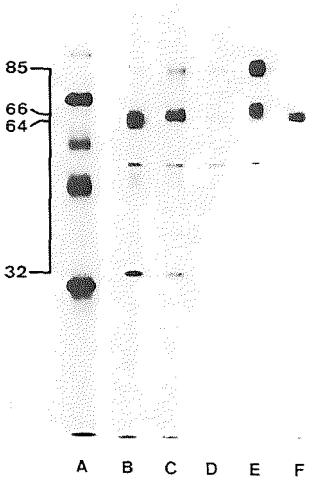



FIG. 3. Effects of leupeptin and corrective factor on the $NaDodSO_4$/polyacrylamide gel electrophoresis patterns of immunoprecipitated radiolabeled β-galactosidase from control fibroblasts and β-gal$^-$/neur$^-$ cells. Lanes: A, radiolabeled marker proteins; B, control fibroblasts; C, control fibroblasts plus leupeptin; D, β-gal$^-$/neur$^-$ cells; E, β-gal$^-$/neur$^-$ cells plus leupeptin; F, β-gal$^-$/neur$^-$ fibroblasts plus corrective factor. The cells were grown in the presence of leupeptin or factor for 2 days.

In immunoprecipitation studies the main mutant characteristics of β-gal$^-$/neur$^-$ cells are a decreased amount of mature β-galactosidase and the absence of a nonenzymic 32,000-Dal component. Pulse–chase experiments in the mutant cells demonstrated conversion of the 85,000-Dal precursor to a 66,000-Dal form. Both forms disappear within 20 hr without the appearance of the mature 64,000-Dal β-galactosidase, which became apparent in control cells.

Addition of leupeptin to both normal and β-gal$^-$/neur$^-$ fibroblasts results in an increase of the 85,000-Dal precursor and of the 66,000-Dal form. This suggests that during normal processing the 85,000-Dal precursor may be degraded to some extent by intralysosomal proteases. The accumulation of the 66,000-Dal form possibly is due to leupeptin inhibition of the last maturation step to the 64,000-Dal form [like β-hexosaminidase (29)]. In experiments not reported here we found that the effect of the corrective factor was not inhibited by leupeptin. It is therefore unlikely that this factor is involved in this final maturation step. Instead, the corrective factor seems to protect β-galactosidase monomer against excessive degradation and, at the same time, enables its aggregation into the high molecular weight forms that were absent from β-gal$^-$/neur$^-$ fibroblasts (2).

Our immunoprecipitation studies demonstrate complete lack of a 32,000-Dal component that is present in control cells (Figs. 1 and 2) and in other mutant cells (unpublished data). Similarly, in the culture medium of β-gal$^-$/neur$^-$ cells a 54,000-Dal component is absent. Together with the results of pulse–chase experiments, this indicates that the latter larger form is a precursor of the 32,000-Dal component. The exact relationship between intracellular 51,000- and 54,000-Dal forms is not yet understood. Neither of them has β-galactosidase activity but apparently they are copurified with the precursor and mature forms of β-galactosidase.

We propose that the 32,000-Dal component is the genetically defective factor causing the combined β-galactosidase/neuraminidase deficiency and is identical to the corrective factor described by Hoogeveen *et al.* (19). It is able to convert the β-galactosidase labeling pattern completely to normal (Fig. 3). Unlike leupeptin, it allows complete processing of the precursor form to a 64,000-Dal mature β-galactosidase which can accumulate normally.

The 32,000-Dal glycoprotein lacking in "I-cell" disease (19) apparently plays a role in a final intralysosomal step leading to protection of β-galactosidase against excessive proteolytic degradation. It also stabilizes neuraminidase but, unlike the case with β-galactosidase, the presence of the 32,000-Dal glycoprotein seems to be essential to neuraminidase's catalytic activity. After our report that leupeptin results in an increased activity of both β-galactosidase and neuraminidase in one cell strain (7), Suzuki *et al.* (30) did not find an effect of protease inhibition on neuraminidase in cells from their β-gal$^-$/neur$^-$ patients. These observations and our analyses of the corrective factor in different types of β-gal$^-$/neur$^-$ fibroblasts (unpublished data) point to molecular heterogeneity within this group of patients.

It may well be that the 32,000-Dal glycoprotein is normally required to unite β-galactosidase monomers and neuraminidase in a complex attached to the lysosomal membrane. Further studies on the nature of this mutation which affects the protection of lysosomal enzymes against the aggression of neighboring "colleague enzymes" are likely to give more insight in the normal cell biology of lysosomal enzymes.

We are grateful to Dr. R. Barneveld for providing us with the antiserum, and to Dr. F. Verheijen and Dr. A. Konings for stimulating discussions. We thank Mr. T. van Os for the preparation of the illustrations and Ms. M. J. C. H. van Woensel for the typing of the manuscript.

1. Norden, A. G. W., Tennant, L. L. & O'Brien, J. S. (1974) *J. Biol. Chem.* **249**, 7969–7976.
2. Hoeksema, H. L., Van Diggelen, O. P. & Galjaard, H. (1979) *Biochim. Biophys. Acta* **566**, 72–79.
3. Okada, S. & O'Brien, J. S. (1968) *Science* **160**, 1002–1004.
4. Suzuki, Y., Nakamura, H., Fukuoka, K., Shimada, Y. & Uono, M. (1977) *Hum. Genet.* **36**, 219–229.
5. O'Brien, J. S. (1978) in *The Metabolic Basis of Inherited Disease*, eds. Stanbury, J. B., Wyngaarden, J. B. & Frederickson, D. S. (McGraw-Hill, New York), pp. 841–865.
6. Lowden, J. A. & O'Brien, J. S. (1979) *Am. J. Hum. Genet.* **31**, 1–18.
7. Galjaard, H., Hoogeveen, A., Verheijen, F., Van Diggelen, O. P., Konings, A., D'Azzo, A. & Reuser, A. J. J. (1981) *Perspectives in Inherited Metabolic Disease* (Edi Ermes, Milan), Vol. 4, pp. 317–333.
8. Hoeksema, H. L., De Wit, J. & Westerveld, A. (1980) *Hum. Genet.* **53**, 241–247.
9. Wenger, D. A., Tarby, T. J. & Wharton, C. (1978) *Biochem. Biophys. Res. Commun.* **82**, 589–595.
10. Hoogeveen, A. T., Verheijen, F. W., D'Azzo, A. & Galjaard, H. (1980) *Nature (London)* **285**, 500–502.
11. Galjaard, H. & Reuser, A. J. J. (1977) in *The Cultured Cell and Inherited Metabolic Disease*, eds. Harkness R. A. & Cockburn, F. (MTP Press, Lancaster), pp. 139–160.
12. Cantz, M., Gehler, J. & Spranger, J. W. (1977) *Biochem. Biophys. Res. Commun.* **74**, 732–738.
13. Thomas, G. H., Tiller, G. E., Reynolds, L. W., Miller, C. S. & Bace, J. W. (1976) *Biochem. Biophys. Res. Commun.* **71**, 188–194.
14. Thomas, G. H., Tipton, R. E., Ch'ien, L. T., Reynolds, L. W. & Miller, C. (1978) *Clin. Genet.* **13**, 369–379.
15. Durand, P., Gatti, R., Cavalieri, S., Borrone, C., Tondeur, M., Michalski, J. C. & Strecker, G. (1977) *Helv. Paediatr. Acta* **32**, 391–400.
16. Van Diggelen, O. P., Galjaard, H., Sinnott, M. L. & Smith, P. J. (1980) *Biochem. J.* **188**, 337–343.
17. Van Diggelen, O. P., Schram, A. W., Sinnott, M. L., Smith, P. J., Robinson, D. & Galjaard, H. (1981) *Biochem. J.* **200**, 143–151.
18. Van Diggelen, O. P., Hoogeveen, A. T., Smith, P. J., Reuser, A. J. J. & Galjaard, H. (1982) *Biochim. Biophys. Acta* **703**, 69–76.
19. Hoogeveen, A., D'Azzo, A., Brossmer, R. & Galjaard, H. (1981) *Biochem. Biophys. Res. Commun.* **103**, 292–300.
20. Hasilik, A. & Neufeld, E. F. (1980) *J. Biol. Chem.* **255**, 4937–4945.
21. Gonzalez-Noriega, A., Grubb, J. H., Talkad, V. & Sly, W. S. (1980) *J. Cell Biol.* **85**, 839–852.
22. Sly, W. S., Natowicz, M., Gonzalez-Noriega, A., Grubb, J. H. & Fischer, H. D. (1981) in *Lysosomes and Lysosomal Storage Diseases*, eds. Callahan, J. W. & Lowden, G. A. (Raven, New York), pp. 131–146.
23. Andria, G., Del Giudice, A. & Reuser, A. J. J. (1978) *Clin. Genet.* **14**, 16–23.
24. Kleijer, W. J., Hoogeveen, A., Verheijen, F. W., Niermeijer, M. F., Galjaard, H., O'Brien, J. S. & Warner, T. G. (1979) *Clin. Genet.* **16**, 60–61.
25. Galjaard, H. (1980) in *Genetic Metabolic Diseases* (Elsevier, North-Holland), pp. 817, 825.
26. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
27. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88.
28. Skudlarek, M. D. & Swank, R. T. (1979) *J. Biol. Chem.* **254**, 9939–9942.
29. Frisch, A. & Neufeld, E. F. (1981) *J. Biol. Chem.* **256**, 8242–8246.
30. Suzuki, Y., Sakuraba, H., Hayashi, K., Suzuki, K. & Imahori, K. (1981) *J. Biochem.* **90**, 271–273.

# P A P E R  IV

J. Biol. Chem.

(1983) 258, 12143-12146

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 20, Issue of October 25, pp. 12143–12146, 1983
*Printed in U.S.A.*

# The Relation between Human Lysosomal β-Galactosidase and Its Protective Protein*

(Received for publication, May 2, 1983)

**André T. Hoogeveen, Frans W. Verheijen, and Hans Galjaard‡**

*From the Department of Cell Biology and Genetics, Erasmus University, Rotterdam, the Netherlands*

**Cultured skin fibroblasts from patients with the lysosomal storage disease galactosialidosis lack a 54-kDa protein which is a precursor of 32-kDa and 20-kDa proteins, which immunoprecipitate with human anti-β-galactosidase antiserum. The lack of a 32-kDa "protective protein" results in a combined deficiency of β-galactosidase and sialidase. The mechanism of protection of lysosomal β-galactosidase against proteolytic degradation is elucidated by sucrose density gradient centrifugation and immunoprecipitation studies. In normal fibroblasts at the low intralysosomal pH, more than 85% of β-galactosidase exists as a high molecular weight (600–700 kDa) multimer and about 10% as a monomer of 64-kDa. In mutant cells from galactosialidosis patients, the residual enzyme activity, about 10%, is present as a monomer and no multimer exists. After addition of the 54-kDa precursor form of the protective protein, the density pattern of β-galactosidase in galactosialidosis cells is normalized. Immunoprecipitation studies after sucrose density gradient centrifugation on homogenate and on purified β-galactosidase from normal fibroblasts show that the protective protein is associated only with the multimeric form of β-galactosidase. We propose that intralysosomal protection against proteolysis of β-galactosidase and sialidase is accomplished by aggregation into a high molecular weight complex consisting of multimeric β-galactosidase, sialidase, and protective protein. The genetic deficiency of the latter, as in galactosialidosis, results in a rapid degradation of monomeric β-galactosidase and a loss of sialidase activity.**

The lysosomal enzyme β-D-galactosidase (EC 3.2.1.23) catalyzes the hydrolysis of the β-D-galactosyl moiety from $G_{M1}$-ganglioside,[1] asialo-$G_{M1}$-ganglioside, galactose containing glycosaminoglycans, and a variety of synthetic substrates (1, 2). Genetic deficiencies in man involving β-galactosidase have been found in patients with different types of lysosomal storage disease; biochemical and genetic studies of cultured skin fibroblasts from such patients have contributed to our understanding of both the normal and mutant enzyme (for reviews, see Refs. 2–4).

In normal human tissues and cultured cells, active lysosomal β-galactosidase exists as a monomeric form with a molecular mass of 64-kDa and a high molecular mass aggregate of the same polypeptide of approximately 700-kDa (5, 6). In purified preparations from human placenta (7) and porcine spleen (8), additional proteins with a molecular mass of 31-kDa and 21-kDa have been designated as contaminants. Recently, d'Azzo *et al.* (9) reported the absence of these latter proteins in cells from patients with galactosialidosis, an autosomal recessive inherited lysosomal storage disease which is associated with a coexistent deficiency of β-galactosidase and sialidase (10, 11). The same investigators also showed that the lack of these 32-kDa and 20-kDa proteins leads to enhanced intralysosomal degradation of β-galactosidase. It was postulated that in normal cells a 32-kDa protective protein is required to protect β-galactosidase against the action of intralysosomal proteases (9).

The purpose of the present study was to investigate the relationship between the protective protein(s) and the absence of the high molecular weight form of β-galactosidase which was previously found in cells from galactosialidosis patients (6). Using sucrose density gradient centrifugation followed by immunoprecipitation studies with anti-β-galactosidase antiserum, we show that the 32-kDa and 20-kDa protective proteins are derived from a 54-kDa precursor protein and that these proteins are essential for the aggregation of monomeric β-galactosidase. When β-galactosidase is not aggregated into a high molecular weight form, it is rapidly degraded by lysosomal cathepsins. The functional significance of this observation is discussed.

## EXPERIMENTAL PROCEDURES

*Preparation of Cell Materials*—Human skin fibroblasts from a normal individual and from a patient with galactosialidosis described by Kleijer *et al.* (12) were obtained from the Rotterdam Cell Repository (Dr. M. F. Niermeijer). The cells were cultivated in Ham's F10 medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum and antibiotics (Gibco). Early passages of the cell strains were grown in 75 $cm^2$ Falcon flasks to a density of 1 to 2 mg of protein/flask. The cells from one flask were harvested by trypsinization, rinsed in saline, and centrifuged (10 min at 90 × g) as reported earlier (13). The cell pellet was lysed by suspending in 100 μl of 20 mM Na phosphate, pH 6.0, containing 100 mM NaCl and 1% (w/v) Zwitterionic detergent 3–12 (Calbiochem) (Buffer A). This sample is centrifuged at 10,000 rpm during 10 min at 4 °C. The supernatant was used for further studies.

*Sucrose Density Gradient Centrifugation*—Sucrose density gradient centrifugation was performed by the method of Martin and Ames (14). Linear gradients of 20 to 40% (w/v) sucrose in Buffer A were prepared in a total volume of 5 ml. Samples of 100 μl of supernatant were layered on to the top of the gradient and centrifugation was carried out at 40,000 rpm for 15 h at 4 °C in a Beckman L5-65 ultracentrifuge with an SW 50-1 rotor. After centrifugation, 175-μl fractions were collected and β-galactosidase assays with 1 mM 4-methylumbelliferyl-β-D-galactopyranoside as a substrate were performed on 10-μl samples as described earlier (13).

*Immunoprecipitation of Radioactive β-Galactosidase*—This immunoprecipitation was performed according to procedures described earlier (9, 15). At 3–4 days before labeling, the Ham's F10 medium was replaced by Dulbecco's modification of Eagle's medium (Flow Laboratories) supplemented with 10% fetal calf serum and antibiotics. One hour prior to labeling, this medium was replaced by Dulbecco's medium free of leucine. Subsequently, replacement by 6 ml of

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom reprint requests should be addressed at, Department of Cell Biology and Genetics, Erasmus University, P. O. Box 1738, 3000 DR Rotterdam, the Netherlands.

[1] The trivial name used is: $G_{M1}$, *N*-acetylneuraminylgangliotetraglycosylceramide.

this same medium supplemented with 0.2 ml of dialyzed fetal calf serum and 0.2 ml of L-[4,5-$^3$H]leucine is performed (0.2 mCi, 135 Ci/mmol, Amersham Radiochemical Centre). The cells were grown for 48 h in the presence of this labeled medium. After incubation, the cells were harvested and centrifuged as described earlier (15).

The cell pellet was either lysed in Buffer A and directly layered on the sucrose gradient, or β-galactosidase in the lysate was first purified using affinity chromatography (16) before sucrose density gradient centrifugation. For this latter purpose, the cell pellet from one or two Falcon flasks was lysed in 500 μl of 20 mM Na acetate, pH 5.2, containing 0.1 M NaCl, 1% (w/v) Zwitterionic detergent and 0.2% (w/v) bovine serum albumin (Buffer B). This sample was applied to a 60-μl *p*-aminophenylthiogalactoside-CH-Sepharose affinity matrix (16). The column was washed with 600 μl of Buffer B supplemented with 0.5 M NaCl, and eluated in buffer B supplemented with 0.1 M γ-galactonolactone. The eluate was dialyzed against buffer A supplemented with 0.2% (w/v) bovine serum albumin, according to Penefski (17).

Pooled fractions were mixed with excess of conventional anti-β-galactosidase antiserum raised against human placental enzyme (9) and incubated for 1 h at 20 °C followed by 15 h at 4 °C. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 12% slab gels according to Laemmli (18) with modifications described by Hasilik and Neufeld (15). Fluorography was carried out according to Bonner and Laskey (25) and the [$^{14}$C]methyl-labeled protein molecular weight standards (phosphorylase *b*, 92,000; bovine serum albumin, 65,000; IgG, 55,000; ovalbumin, 46,000; carbonic anhydrase, 30,000 (Amersham)) were used. Visualization of the labeled proteins was possible after 1–2 weeks of exposure to X-Omat Kodak film.

Correction of the combined β-galactosidase and neuraminidase deficiency in galactosialidosis fibroblasts was studied in two ways. Firstly, the intralysosomal proteolytic degradation of the lysosomal enzymes was inhibited by addition to the medium of leupeptin (Sigma) at a final concentration of 0.02 mM for 4 days prior to sucrose density gradient centrifugation. Secondly, the "corrective factor" (19) and 54-kDa identified glycoprotein (9) was prepared by addition of 10 mM $NH_4Cl$ to the medium above $G_{M1}$-gangliosidosis fibroblasts during 2 days followed by concentration on an Amicon PM10 filter to a final volume of 1 ml as described before (9, 19). An aliquot of 40 μl of this concentrate was added per 1 ml of the Ham's F10 medium above galactosialidosis fibroblasts during 4 days and then sucrose density gradient centrifugation was performed to study the β-galactosidase pattern in the mutant cells.

Finally, radioactively labeled corrective factor was prepared in the same way but after addition of both [$^3$H]leucine (see above) and $NH_4Cl$ during 2 days prior to preparation of the concentrate. The radiolabeled concentrate was dialyzed against Ham's F10 medium added to galactosialidosis fibroblasts for 4 days and subsequently immunoprecipitation studies with anti-β-galactosidase antiserum were carried out to identify the "corrective protein" and its fate after being taken up by the mutant fibroblasts.

## RESULTS AND DISCUSSION

*Protective Protein for β-Galactosidase*—In previous studies (9), it was shown that galactosialidosis fibroblasts lack a 32-kDa protein which precipitated with an antiserum against human β-galactosidase but which did not show any hydrolytic activity. In all other human cells tested, including those with a β-galactosidase deficiency, this 32-kDa protein is present. On the basis of pulse-chase experiments and the absence of a 54-kDa glycoprotein in ammonium chloride-stimulated medium above galactosialidosis fibroblasts, d'Azzo *et al.* (9) postulated that the 54-kDa protein is the precursor of the 32-kDa protective protein." In immunoprecipitation studies summarized in Fig. 1, experimental evidence is presented for this hypothesis. Ammonium chloride-stimulated and [$^3$H]leucine-labeled medium above control fibroblasts contains the 85-kDa precursor of β-galactosidase and a 54-kDa glycoprotein (Fig. 1*b*). When this medium is given to galactosialidosis cells, a 32-kDa and 20-kDa protein band becomes apparent (Fig. 1*d*). This is not the case when $NH_4Cl$-stimulated and radiolabeled medium derived from galactosialidosis cells (Fig. 1*c*), which lacks the 54-kDa protein, is added (Fig. 1*e*). These

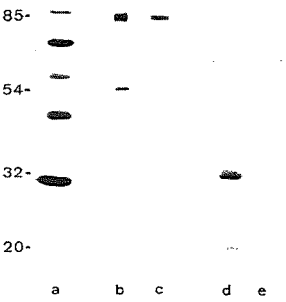



FIG. 1. **Polyacrylamide gel electrophoresis of immunoprecipitated β-galactosidase.** *a*, Standards. *b*, $NH_4Cl$-stimulated medium above control fibroblasts. *c*, $NH_4Cl$-stimulated medium above galactosialidosis (β-gal$^-$/neur$^-$) cells. *d*, Galactosialidosis fibroblasts after addition of medium mentioned under *b*. *e*, Galactosialidosis fibroblasts after addition of medium mentioned under *c*.

results show that the 54-kDa protein is the precursor form of the 32-kDa and 20-kDa proteins; the stronger 32-kDa protein is thought to be required to protect β-galactosidase against intralysosomal proteolytic degradation (9).

*Sucrose Density Gradient Centrifugation*—The relationship between the 32-kDa protective protein and β-galactosidase was further investigated with sucrose density gradient centrifugation. In a lysate in buffer A of control fibroblasts, two peaks are demonstrated at pH 6.0 (Fig. 2*a*). About 15% of the total activity is present as a monomer and 85% as a high molecular weight multimer. Previous studies using gel filtration had already shown that β-galactosidase exists in two forms *i.e.* a monomer of about 64kDa and a high molecular weight aggregate of about 600 to 700kDa (5–8). In contrast to previous observations, we find that most of the enzyme exists as a multimer which might be due to the experimental conditions used in this study. When the cell sample was homogenized by sonication or freeze-thawing, nearly all enzyme was found to occur as a monomer. The ratio monomer:multimer is also pH-dependent because, under our conditions avoiding sonication or freeze-thawing and using Zwittergent as a detergent, all β-galactosidase activity exists as a multimer at pH 4.0. In the *in vivo* situation within the lysosome where the pH is in the order of 5.0 (20), most of the β-galactosidase presumably exists as a high molecular weight aggregate.

As is shown in Fig. 2*b*, a lysate from a patient with galactosialidosis only shows the monomer peak after sucrose density gradient centrifugation. This finding is in agreement with earlier gel filtration studies, which indicated a marked decrease of high molecular weight β-galactosidase in galactosialidosis cells (6). This figure also shows that when these mutant cells are treated with leupeptin, which inhibits intralysosomal cathepsins, the β-galactosidase activity rises but the density pattern remains unchanged (Fig. 2*b*). The increased activity seems thus to be due to an increase in monomeric β-galactosidase. When cells with other types of β-galactosidase deficiency such as those derived from patients with $G_{M1}$-gangliosidosis are treated with leupeptin, there is no significant rise of β-galactosidase activity (data not shown).

Addition to galactosialidosis fibroblasts of $NH_4$-stimulated medium from control fibroblasts known to contain the 54-kDa precursor of the 32-kDa protective protein results in a normalization of the density pattern of β-galactosidase in the

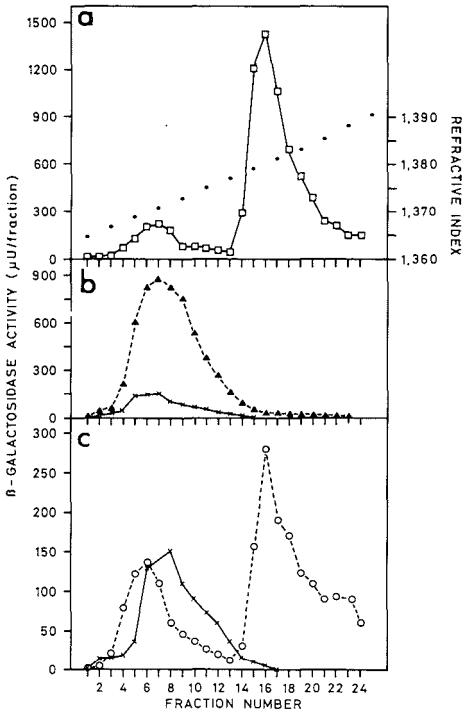



FIG. 2. **Sucrose density gradient centrifugation of lysosomal β-galactosidase in normal and mutant human fibroblasts.** *a*, Density pattern of β-galactosidase in normal fibroblasts (□——□); and ··· indicate the respective indices of the linear sucrose gradient from 20 to 40% in 20 mM Na phosphate, pH 6.0, containing 100 mM NaCl and 1% (w/v) Zwitterionic detergent. *b*, Pattern in galactosialidosis cells (×——×) and of the same cells after inhibition of lysosomal cathepsins by leupeptin (▲- - -▲). *c*, Pattern in galactosialidosis cells before (×——×) and after addition of $NH_4Cl$-stimulated medium containing the 54-kDa precursor of the protective protein (○- - -○).

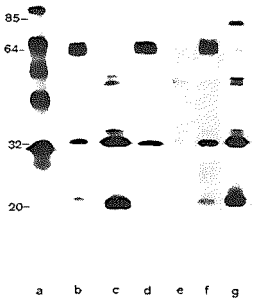



FIG. 3. **Polyacrylamide gel electrophoresis of immunoprecipitated β-galactosidase in pooled fractions after sucrose density gradient centrifugation.** *a*, Standards. *b*, Immunoprecipitate of the total lysate of control fibroblasts. *c*, Cell lysate after sucrose density gradient centrifugation; pooled fractions 5–11. *d*, The same; pooled fractions 14–20. *e*, Purified β-galactosidase from control fibroblasts; pooled fractions 5–11. *f*, The same; pooled fractions 14–20. *g*, Immunoprecipitate of the unretained material of the affinity column used for the purification of β-galactosidase.

mutant cells (Fig. 2*c*). After 4 days administration of this "corrective factor", about two-thirds of the enzyme appears as a high molecular weight multimer. These results suggest that the 54-kDa precursor and the 32-kDa protein derived from it is required for the aggregation of monomeric β-galactosidase into a high molecular weight form. This observation explains previous results by Hoeksema *et al.* (6), who found a normalization of the gel filtration pattern of β-galactosidase after somatic cell hybridization of β-galactosidase-deficient $G_{M1}$-gangliosidosis fibroblasts and galactosialidosis cells. The former cells will synthesize the 32-kDa and 20-kDa proteins whereas the nucleus of the galactosialidosis cells will code for normal β-galactosidase polypeptide chains.

*Immunoprecipitation*—A lysate of [$^3$H]leucine-labeled control fibroblasts was exposed to sucrose density gradient centrifugation. Fractions 1–4, 5–11, and 14–20, corresponding to the pattern illustrated in Fig. 2, were pooled and treated with conventional antiserum against human β-galactosidase. After immunoprecipitation, polyacrylamide gel electrophoresis was carried out under denaturing conditions and the results are shown in Fig. 3. In the fractions 1–4, no β-galactosidase and minor quantities of the 32-kDa and 20-kDa proteins are present. The fractions 5–11 corresponding with the monomer (Fig. 2) contain the expected amount of 64-kDa β-galactosidase accompanied by large quantities of the 32-kDa and 20-kDa proteins (Fig. 3, *lane c*). The fractions 14–20 corresponding with the multimeric form contain the majority of the 64-kDa β-galactosidase and about equal amounts of the 32-kDa protein and only traces of the 20-kDa protein (Fig. 3, *lane d*).

Similar immunoprecipitation studies were performed after purification of β-galactosidase from control fibroblasts using affinity column chromatography. When the eluate of the column is exposed to sucrose density gradient centrifugation, a similar pattern of β-galactosidase activity with two peaks (Fig. 2) is obtained. The results of immunoprecipitation studies on the pooled fractions 5–11 and 14–20 are shown in the right-hand part of Fig. 3. Hardly any bands are visible in the fractions 1–4. In fractions 5–11, again a faint 64-kDa β-galactosidase band is seen, but contrary to the studies on total cell lysate, hardly any 32-kDa and no 20-kDa proteins are present in this purified preparation (Fig. 3, *lane e*). In the fractions 14–20, most of the 64-kDa β-galactosidase and the 32-kDa protein and minor quantities of the 20-kDa protein are present (Fig. 3 *lane f*). When immunoprecipitation studies were performed on the unretained material of the affinity column used for purification of β-galactosidase, most of the 32-kDa and 20-kDa proteins were found there (Fig. 3, *lane g*).

These immunoprecipitation studies suggest that the 32-kDa component found in the low density fraction of cell lysate is not bound to the β-galactosidase monomer and the latter is easily separated from it using affinity chromatography. On the other hand, affinity chromatography of the β-galactosidase in the high density fraction copurifies apparently equal amounts of the 32-kDa protein. This strongly suggests that these two components are intimately bound in the high molecular weight aggregate.

## CONCLUSION

We have shown that in normal human fibroblasts a 54-kDa precursor protein is processed into a 32-kDa glycoprotein, which in turn is required for the aggregation of monomeric

(64-kDa) β-galactosidase into a high molecular mass multimer of 600 to 700-kDa. There seems to be an excess amount of 32-kDa protein in proportion to the amount of β-galactosidase monomer. This has also been observed for the microsomal accessory protein egasyn which binds β-glucuronidase to the endoplasmic reticulum in mouse (21).

We have found for human fibroblasts that at the low intralysosomal pH, most of the β-galactosidase exists as a 600 to 700-kDa multimer and that the 32-kDa protective protein is firmly bound to this high molecular mass aggregate. Previous studies of human placenta (7) and porcine spleen (8) have also demonstrated the presence of low molecular weight proteins together with the high molecular weight fraction of β-galactosidase but these have wrongly been considered as contaminants. Verheijen *et al.* (22) recently showed that sialidase and the high molecular weight form of β-galactosidase exist as a complex. It is likely that this complex is membrane-bound. Cheetham and Dance (23) suggested earlier that the high molecular weight form of β-galactosidase consists of low molecular weight enzyme bound to small fragments of the lysosomal membrane.

d'Azzo *et al.* (9) have recently shown that the autosomal recessive lysosomal storage disease galactosialidosis is associated with the absence of the 54-kDa precursor protein of the 32-kDa and 20-kDa glycoproteins mentioned above. They explained the combined deficiency of β-galactosidase and sialidase as being due to enhanced degradation of β-galactosidase whereas the protective protein would also be required to activate sialidase.

In the present study, we have shown that the protection of β-galactosidase is realized by aggregation of monomers into high molecular weight multimers. This is supported by the observation that the stability of β-galactosidase increases with conditions favoring the formation of multimers (24). When the aggregation of monomers cannot take place, as in galactosialidosis, β-galactosidase remains in its monomeric form which apparently is very sensitive to intralysosomal proteolytic degradation. It seems likely that sialidase needs to be incorporated into a membrane-bound high molecular weight complex with β-galactosidase in order to become active (22). If no such complex can be formed, as in galactosialidosis, sialidase remains inactive (9, 22).

Further studies are now under way to investigate the role of monomeric β-galactosidase and of the high molecular weight complexes of multimeric β-galactosidase, protective protein(s), and sialidase in the hydrolysis of various substrates.

*Acknowledgments*—We thank Dr. Arnold Reuser and Dr. Herman Sips for stimulating discussions and Prof. D. Robinson (Department of Biochemistry, University of London) for critically reviewing the manuscript.

REFERENCES

1. Barrett, A. J., and Heath, M. F. (1977) in *Lysosomes* (Dingle, J. T., ed) 2nd Ed., pp. 19–127, Elsevier/North-Holland, Amsterdam and New York
2. O'Brien (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Frederickson, D. S., Goldstein, J. L., and Brown, M. S., eds) 5th Ed., pp. 945–972, McGraw-Hill, New York
3. Sandhoff, K., and Christomanou, H. (1979) *Hum. Genet.* **50,** 107–143
4. Galjaard, H., Hoogeveen, A. T., Verheijen, F., van Diggelen, O. P., Konings, A., d'Azzo, A., and Reuser, A. J. J. (1981) in *Perspectives in Inherited Metabolic Diseases*, Vol. 4, pp. 317–333, Edi. Ermes, Milano
5. Norden, A. G. W., Tennant, L. L., and O'Brien, J. S. (1974) *J. Biol. Chem.* **249,** 7969–7976
6. Hoeksema, J., van Diggelen, O. P., and Galjaard, H. (1979) *Biochim. Biophys. Acta* **455,** 72–79
7. Lo, J., Mukerji, K., Awasthi, Y. C., Honada, E., Suzuki, K., and Srivastava, S. K. (1979) *J. Biol. Chem.* **254,** 6710–6715
8. Yamamoto, Y., Fujie, M., and Nishimura, K. (1982) *J. Biochem.* **92,** 13–21
9. d'Azzo, A., Hoogeveen, A. T., Reuser, A. J. J., Robinson D., and Galjaard, H. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 4535–4539
10. Wenger, D. A., Tarby, T. J., and Wharton, C. (1978) *Biochem. Biophys. Res. Commun.* **82,** 589–595
11. Hoogeveen, A. T., Verheijen, F. W., d'Azzo, A., and Galjaard, H. (1980) *Nature (Lond.)* **285,** 500–502
12. Kleijer, W. J., Hoogeveen, A. T., Verheijen, F. W., Niermeijer, M. F., Galjaard, H., O'Brien, J. S., and Wanner, T. G. (1979) *Clin. Genet.* **16,** 60–61
13. Galjaard, H. (1980) *Genetic Metabolic Diseases; Early Diagnosis and Prenatal Analysis*, Elsevier/North-Holland, Amsterdam and New York
14. Martin, R. G., and Ames, B. N. (1961) *J. Biol. Chem.* **236,** 1372–1379
15. Hasilik, A., and Neufeld, E. F. (1980) *J. Biol. Chem.* **255,** 4937–4945
16. Van Diggelen, O. P., Galjaard, H., Sinnott, M. L., and Smith, P. J. (1980) *Biochem. J.* **188,** 337–343
17. Penefski, H. S. (1977) *J. Biol. Chem.* **252,** 2891–2899
18. Laemmli, U. K. (1970) *Nature (Lond.)* **227,** 680–685
19. Hoogeveen, A. T., d'Azzo, A., Brossmer, R., and Galjaard, H. (1981) *Biochem. Biophys. Res. Commun.* **103,** 292–300
20. Hollemans, M., Reyngoud, D. J., and Tager, J. M. (1979) *Biochim. Biophys. Acta* **551,** 55–56
21. Lusis, A. J., Tomino, S., and Paigen, K. (1976) *J. Biol. Chem.* **251,** 7753–7760
22. Verheijen, F., Brossmer, R., and Galjaard, H. (1982) *Biochem. Biophys. Res. Commun.* **108,** 868–875
23. Cheetham, P. S. J., and Dance, N. E. (1976) *Biochem. J.* **157,** 189–195
24. Heyworth, C. M., Neumann, E. F., and Wynn, C. H. (1981) *Biochem. J.* **193,** 773–779
25. Bonner, W. M., and Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88

# P A P E R V

J. Biol. Chem.
(1984) 259, 1974-1977

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 259, No. 3, Issue of February 10, 1974–1977, 1984
Printed in U.S.A.


# Processing of Human β-Galactosidase in $G_{M1}$-Gangliosidosis and Morquio B Syndrome*

(Received for publication, August 10, 1983)

**André T. Hoogeveen, Hiroko Graham-Kawashima‡, Alessandra d'Azzo, and Hans Galjaard§**
*From the Department of Cell Biology and Genetics, Erasmus University, Rotterdam, the Netherlands*

**The nature of the molecular defect resulting in the β-galactosidase deficiency in different forms of $G_{M1}$-gangliosidosis and mucopolysaccharidosis IV B (Morquio B syndrome) was investigated. Normal and mutant cultured skin fibroblasts were labeled *in vivo* with [$^3$H]leucine and immunoprecipitation studies with human anti-β-galactosidase antiserum were performed, followed by polyacrylamide gel electrophoresis and fluorography. In Morquio B syndrome, the mutation does not interfere with the normal processing and intralysosomal aggregation of β-galactosidase. In cells from infantile and adult $G_{M1}$-gangliosidosis, 85-kDa precursor β-galactosidase was found to be synthesized normally but more than 90% of the enzyme was subsequently degraded at one of the early steps in post-translational processing. The residual 5–10% β-galactosidase activity in adult $G_{M1}$-gangliosidosis is 64-kDa mature lysosomal enzyme with normal catalytic properties but with a reduced ability of the monomeric form to aggregate into high molecular weight multimers. Knowledge of the exact nature of the molecular defect underlying β-galactosidase deficiency in man may lead to a better understanding of the clinical and pathological heterogeneity among patients with different types of $G_{M1}$-gangliosidosis and Morquio B syndrome.**

A genetic deficiency of lysosomal β-galactosidase (EC 3.2.1.23) in man is responsible for the lysosomal storage diseases $G_{M1}$-gangliosidosis (1, 2)[1] and mucopolysaccharidosis IV B or Morquio B syndrome (3, 4). On the basis of different clinical and pathological manifestations, infantile, juvenile, and adult forms of $G_{M1}$-gangliosidosis are distinguished (for reviews, see Refs. 2 and 5). Somatic cell hybridization studies have shown that the different forms of $G_{M1}$-gangliosidosis and Morquio B syndrome are based on different mutations in the gene on chromosome 3, which codes for the β-galactosidase polypeptide (6–8).

The β-galactosidase activity in cultured fibroblasts from patients with infantile or juvenile $G_{M1}$-gangliosidosis is less than 1% of control values whereas the residual enzyme activity varies from 5 to 10% in cells from patients with adult $G_{M1}$-gangliosidosis or Morquio B syndrome (2–5, 9, 10). Immunological studies using antisera against purified human β-galactosidase showed the presence of normal amounts of cross-reactive material in liver or fibroblasts from patients with infantile or adult $G_{M1}$-gangliosidosis (11, 12).

Studies after *in vivo* labeling of normal fibroblasts with [$^3$H]leucine followed by immunoprecipitation with anti-β-galactosidase antiserum and polyacrylamide gel electrophoresis have shown that β-galactosidase is synthesized as a 85-kDa precursor molecule which is processed via intermediate forms into 64-kDa mature enzyme (13). This finding was in agreement with previous studies on mouse macrophage β-galactosidase (14). Within the lysosome, this molecule aggregates into a high molecular mass form (600- to 700-kDa) in the presence of a 32-kDa "protective protein," which prevents rapid proteolytic degradation of β-galactosidase (13, 15).

The purpose of the present work was to obtain a better insight into the nature of the molecular defect in the infantile and adult forms of $G_{M1}$-gangliosidosis by analyzing the processing of mutant β-galactosidase using immunoprecipitation. In addition, the aggregation pattern of the residual β-galactosidase in mutant fibroblasts was studied using sucrose density gradient centrifugation.

## EXPERIMENTAL PROCEDURES

*Materials*—Skin fibroblasts from patients with the infantile form of $G_{M1}$-gangliosidosis were kindly provided by Dr. L. Pinsky (Montreal), Drs. P. Aula and P. Ämmälä (Helsinki), Dr. T. Tønnesen (Glostrup), and Drs. M. C. B. Loonen and W. F. Arts (Rotterdam). Cells from patients with the adult form of $G_{M1}$-gangliosidosis were obtained from Dr. D. Wenger (Denver) (case II-4, Ref. 10) and Dr. Y. Suzuki (Tokyo) (cases 5 and 6, Ref. 9), and Dr. K. von Figura (Münster) provided fibroblasts from a patient with Morquio B syndrome (case 2, Ref. 4). All fibroblasts were cultured in Ham's F-10 medium supplemented with 10% fetal calf serum and antibiotics.

*Preparation and Enzyme Assays*—For intracellular enzyme assays, cells were grown in 75-cm$^2$ Falcon flasks to a density of 1 to 2 mg of protein/flask. The cells were harvested by trypsinization, rinsed in saline, and centrifuged (10 min at 90 × *g*), the cell pellet was lysed in distilled water, and β-galactosidase and β-*N*-acetylhexosaminidase activities were measured with the appropriate 4-methylumbelliferyl substrates (Koch-Light) as reported earlier (5). The effect of protease inhibition on the activity of β-galactosidase was studied by addition of leupeptin (Sigma) to the culture medium in a final concentration of 0.02 mM. To measure the activity of lysosomal enzymes secreted into the culture medium, fibroblasts were seeded in 24-well (16-mm diameter) tissue culture plates (2 × 10$^5$ cells/well), and 2 days later, when the cells had reached confluency, the medium was replaced by 500 μl of fresh medium with 5% inactivated (60 °C for 3 h) fetal calf serum and $NH_4Cl$ at a final concentration of 10 mM. After 2 days, the medium was collected and centrifuged (5 min at 90 × *g*) and enzymatic activities were measured in 10-μl aliquots.

*Immunoprecipitation and Polyacrylamide Gel Electrophoresis*—Cells were labeled with 0.2 mCi of L-[4,5-$^3$H]leucine (135 Ci/mmol, Amersham Radiochemical Centre) for periods varying from 2 h to 3 days and processed for immunoprecipitation using anti-β-galactosidase antiserum raised against human placental enzyme as described (13, 16). The immunoprecipitates were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Working as a fellow from the Department of Pediatrics, Kanazawa University, Kanazawa, Japan.

§ To whom reprint requests should be addressed at the Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, the Netherlands.

[1] $G_{M1}$ refers to *N*-acetylneuraminylgangliotetraglycosylceramide.

described, and the radioactive bands were visualized in fluorography (13, 16). Quantitative measurements of incorporated radioactivity in distinct protein bands were performed by liquid scintillation counting of dissected pieces of acrylamide gel; the total incorporaton of [$^3$H] leucine in cellular proteins was determined in 10-μl aliquots of cell [illegible]genate.

Sucrose density gradient centrifugation was performed according to Martin and Ames (17). After harvesting and centriguation as described above, the fibroblast pellet from one Falcon flask was lysed by suspension in 100 μl of 20 mM sodium phosphate, pH 6.0, containing 100 mM NaCl and 1% (w/v) zwitterionic detergent 3-12 (Calbiochem). Linear gradients of 20 to 40% (w/v) sucrose in this same buffer were prepared in a total volume of 5 ml. Samples of 100-μl supernatant were layered onto the top of the gradient and centrifugation was carried out at 40,000 rpm for 15 h at 4 °C in a Beckman L5-65 ultracentrifuge with an SW 50-1 rotor. After centrifugation, 175-μl fractions were collected and β-galactosidase assays with 1 mM 4-methylumbelliferyl-β-D-galactopyranoside as a substrate were performed on 10-μl samples.

## RESULTS

The intracellular β-galactosidase activity of the fibroblasts derived from all five patients with infantile $G_{M1}$-gangliosidosis was less than 1% of control values (normal range, 400–1400 nmol·h$^{-1}$·mg$^{-1}$ protein; mean activity, 760). The residual activity in the cells from the patient with Morquio syndrome and from three patients with the adult form of $G_{M1}$-gangliosidosis was 30–60 nmol·h$^{-1}$·mg$^{-1}$ protein (4–9% of controls). The $K_m$ measured with 4-methylumbelliferyl-β-D-galactopyranoside in $G_{M1}$-gangliosidosis fibroblasts was the same as in control fibroblasts, *i.e.* 0.33 mM. With the anti-β-galactosidase antiserum, 97% of the β-galactosidase enzyme activity was precipitated both in control fibroblasts and adult $G_{M1}$-gangliosidosis cells.

After $NH_4Cl$ stimulation, the activity of the secreted 88-kDa precursor β-galactosidase could be measured in the culture medium. The average activity above control fibroblasts was 376 pmol·h$^{-1}$/10 μl of medium and above cells from infantile or adult $G_{M1}$-gangliosidosis 11 to 13 pmol·h$^{-1}$/10 μl of medium. As a control, hexosaminidase activities were measured and these were the same in medium above control fibroblasts and the mutant cells tested (16,000–20,000 pmol·h$^{-1}$/10 μl medium). The $K_m$ value of the β-galactosidase in medium above $G_{M1}$-gangliosidosis fibroblasts could not be determined because of too low activities.

Control and mutant fibroblasts were labeled for 3 days with [$^3$H]leucine and the incorporation of radioactivity into β-galactosidase was studied by immunoprecipitation, followed by gel electrophoresis and fluorography (Fig. 1, *a–d*). Intracellularly, in control cells 85-kDa precursor β-galactosidase and the 64-kDa mature lysosomal β-galactosidase are seen; in addition, the 32-kDa "protective protein," a 20-kDa protein and their 54-kDa precursor react with the anti-β-galactosidase antiserum. In $NH_4Cl$-stimulated medium above control cells (Fig. 1*e*), only 88-kDa secreted precursor β-galactosidase and the 54-kDa secreted precursor of the protective protein are present.

In the infantile and adult $G_{M1}$-gangliosidosis cells (Fig. 1, *b* and *c*), no mature 64-kDa β-galactosidase can be detected. Scintillation counting of slices of gel cut from the 64-kDa position showed 4–12% radioactivity in both types of $G_{M1}$-gangliosidosis cells compared with controls.

Both in the infantile and adult $G_{M1}$-gangliosidosis cells, the 85-kDa precursor form of β-galactosidase is present (Fig. 1, *b* and *c*). After $NH_4Cl$ stimulation, equal amounts of the 88-kDa precursor are found in the culture medium above control fibroblasts (Fig. 1*e*) and the two types of $G_{M1}$-gangliosidosis cells (Fig. 1, *f* and *g*).

Intracellularly, equal amounts of 32-kDa protective protein

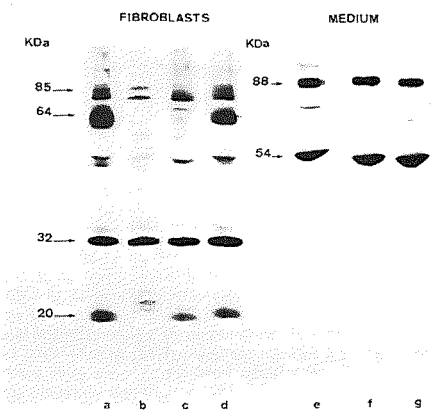



**FIG. 1. Immunoprecipitation studies of β-galactosidase after [$^3$H]leucine labeling of normal and mutant fibroblasts followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** *Lanes: a*, control fibroblasts; *b*, infantile $G_{M1}$-gangliosidosis fibroblasts; *c*, adult $G_{M1}$-gangliosidosis fibroblasts; *d*, Morquio B fibroblasts; *e*, medium above control fibroblasts after $NH_4Cl$ stimulation; *f*, medium above infantile $G_{M1}$-gangliosidosis fibroblasts; *g*, medium above adult $G_{M1}$-gangliosidosis fibroblasts.

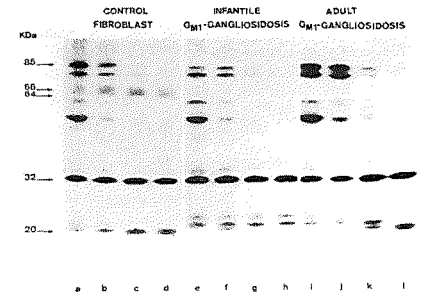



**FIG. 2. Pulse labeling of normal and mutant fibroblasts followed by different periods of chase, immunoprecipitation of β-galactosidase and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** *Lanes a–d*, control fibroblasts; *e–h*, infantile $G_{M1}$-gangliosidosis; *i–l*, adult $G_{M1}$-gangliosidosis. *a*, *e*, and *i*, 2-h labeling with [$^3$H]leucine; *b*, *f*, and *j*, 2-h labeling followed by 2-h chase; *c*, *g*, and *k*, 2-h labeling followed by 4-h chase; *d*, *h*, and *l*, 2-h labeling followed by 24-h chase.

are present in control and mutant fibroblasts; this is also true for its secreted 54-kDa precursor in the medium above both types of cells after $NH_4Cl$ treatment.

The immunoprecipitation pattern of cells from a Morquio B patient (Fig. 1*d*) is the same as in control cells, *i.e.* both 85-kDa precursor and 64-kDa mature β-galactosidase are present.

To study the dynamics of the processing of β-galactosidase in $G_{M1}$-gangliosidosis, pulse-chase experiments were carried out (Fig. 2). Control and mutant fibroblasts were labeled with [$^3$H]leucine for 2 h. The medium was then replaced by unlabeled medium and subsequently the cells were harvested after 0, 2, 4, and 24 h of chase, and immunoprecipitation studies were performed. In control fibroblasts, the 85-kDa precursor

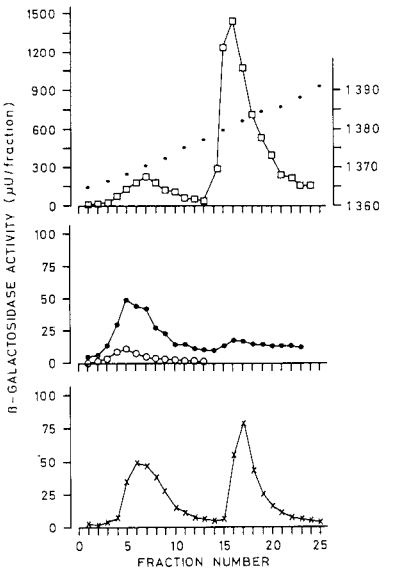



FIG. 3. **Sucrose density gradient centrifugation of lysosomal β-galactosidase in normal and mutant human fibroblasts.** *Top:* □, control fibroblasts; the *dotted line* indicates the refractive indices of the linear sucrose gradient from 20 to 40% in 20 mM Na phosphate, pH 6.0, containing 100 mM NaCl and 1% (w/v) zwitterionic detergent. *Middle:* ●, adult $G_{M1}$-gangliosidosis; ○, infantile $G_{M1}$-gangliosidosis. *Bottom:* ×, Morquio B syndrome.

β-galactosidase is observed at 0 and 2 h after labeling and then disappears because it is processed via intermediates into 64-kDa mature β-galactosidase within 24 h (Fig. 2, *a–d*). In the infantile and adult form of $G_{M1}$-gangliosidosis, the 85-kDa precursor is normally synthesized and disappears as in controls between 4 and 24 h. However, 64-kDa mature β-galactosidase does not appear in the $G_{M1}$-gangliosidosis cells (Fig. 2, *e–h* and Fig. 2, *i–l*).

To prevent intralysosomal proteolytic degradation, 0.02 mM leupeptin was added to the medium above $G_{M1}$-gangliosidosis cells. No effect on the immunoprecipitation pattern of β-galactosidase was observed nor did the intracellular β-galactosidase activity increase.

The aggregation pattern of the residual β-galactosidase activity in $G_{M1}$-gangliosidosis cells and Morquio B was studied by sucrose density gradient centrifugation (Fig. 3). In control fibroblasts at pH 6.0, about 85% of the β-galactosidase activity exists as a high molecular mass (600–700-kDa) multimer and 10–15% as a monomer of 64-kDa (Fig. 3, *top*). In infantile $G_{M1}$-gangliosidosis, only a minor quantity of monomeric β-galactosidase was detected. In adult $G_{M1}$-gangliosidosis, about 25% of the total activity was multimeric and the rest monomeric β-galactosidase (Fig. 3, *middle*). In Morquio B fibroblasts, the residual enzyme activity is equally divided over the monomeric and multimeric form (Fig. 3, *bottom*).

## DISCUSSION

The most remarkable finding in the immunoprecipitation studies reported here is the absence of 64-kDa mature β-galactosidase in $G_{M1}$-gangliosidosis fibroblasts. Previous investigations using immunotitration (2, 11) and immunodiffusion (12) showed the presence of normal quantities of cross-reactive material in fibroblasts and liver from patients with infantile and adult $G_{M1}$-gangliosidosis using anti-β-galactosidase antisera. In cells from patients with the juvenile form, some investigators also found normal amounts of cross-reactive material (2, 11) whereas others reported a reduction (18) or increase compared with controls. These discrepancies may be explained by different aggregation states of β-galactosidase, depending on the source of material and the way of its preparation (15). The precipitation of monomeric β-galactosidase might require relatively more antibody than that of enzyme present as a multimer. In addition, we now know that the antisera used not only react with mature β-galactosidase but also with its 85-kDa precursor and with the 32-kDa protective protein and its precursor (13), which were not known at the time of the immunological studies mentioned (11, 12, 18).

The absence of 64-kDa β-galactosidase in $G_{M1}$-gangliosidosis shown in the present study cannot be explained by an inability of the antiserum to react with mutated enzyme. Our antiserum does react with (mutated) 85-kDa precursor β-galactosidase in $G_{M1}$-gangliosidosis and also with mutated precursor and mature enzyme in Morquio B. Also, virtually all residual enzyme activity in adult $G_{M1}$-gangliosidosis can be precipitated with our antiserum, so we could not have missed a band at the 64-kDa position if there had been normal amounts of enzymatically deficient enzyme.

In $G_{M1}$-gangliosidosis, the mutation on chromosome 3 must affect the 85-kDa precursor β-galactosidase in such a way that the molecule is degraded at one of the early processing steps. Similar molecular defects have recently been reported in the late onset form of glycogenosis type II (19, 20), multiple sulfatase deficiency (21) and some variants of Tay-Sachs disease (22, 23). Pulse-chase studies in the different forms of $G_{M1}$-gangliosidosis indicate that the 85-kDa precursor β-galactosidase is normally synthesized but during its early processing the enzyme is degraded and hardly any 64-kDa mature enzyme is formed (see Fig. 2). A genetic deficiency of one of the enzymes involved in this processing is unlikely since there is no accumulation of the 85-kDa precursor and its rate of disappearance is similar as in controls.

Inhibition of proteolytic degradation by leupeptin does not result in the appearance of mature 64-kDa β-galactosidase or one of its precursors. Thiol cathepsins which are inhibited by leupeptin seem therefore not responsible for the degradation of the precursor enzyme. This is in contrast with the situation in galactosialidosis (24), where the β-galactosidase and sialidase deficiency was found to be due to the absence of a 32-kDa glycoprotein (13). This protein is normally required for the aggregation of monomeric β-galactosidase into high molecular weight aggregates which form a complex with the membrane-bound sialidase (25). The absence in galactosialidosis cells of the protective protein results in the rapid intralysosomal degradation of 66-kDa β-galactosidase, which degradation can be inhibited by leupeptin.

In $G_{M1}$-gangliosidosis and Morquio B, the 32-kDa protective protein and its 54-kDa precursor were found to be present and functional (26). This enables in principle the aggregation of mature monomeric β-galactosidase (Fig. 3). In Morquio B, the processing of β-galactosidase proceeds normally (27), mature 64-kDa enzyme is present in normal quantities (see Fig. 1*d*), and about half of these molecules aggregate into 60–70-kDa multimers, whereas in controls this is more than 85%. This difference may be due to the genetic alteration of the β-galactosidase molecule which may interfere with its aggrega-

tion despite the presence of a normal amount of protective protein. Previous work (27) has shown that the mutation in Morquio B mainly affects the hydrolysis of galactose from keratan sulfate.

Both in infantile and adult $G_{M1}$-gangliosidosis 4–12% radioactivity was found to be present in sodium dodecyl sulfate gels at the 64-kDa position. This might imply that not all mutated precursor β-galactosidase is degraded and that about 4–12% is processed into the mature form. In infantile $G_{M1}$-gangliosidosis, the mutation seems to affect the active site of the enzyme since there is less than 1% catalytic activity toward different natural and artificial substrates (2, 5). In adult $G_{M1}$-gangliosidosis, there is 5–10% residual β-galactosidase activity and the kinetic properties and the half-life of the enzyme (28) are normal. As is shown in Fig. 3, aggregation of β-galactosidase does occur in fibroblasts from adult $G_{M1}$-gangliosidosis; the large proportion of monomeric enzyme might be explained by a high amount of 85-kDa precursor enzyme relative to the 5–10% remaining 64-kDa mature enzyme.

It is possible that in the adult form the genetically altered part of the β-galactosidase molecule is removed during the last processing step resulting in normal 64-kDa mature β-galactosidase; the bulk of mutated precursor is degraded as in the infantile form. The ultimate experimental proof must await the identification of the exact nature of the molecular defect using sequencing of (mutant) β-galactosidase or of the gene on chromosome 3 coding for it.

REFERENCES

1. Okada, S., and O'Brien, J. S. (1968) *Science (Wash. D. C.)* **160,** 1002–1004
2. O'Brien, J. S. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Frederickson, D. S., Goldstein, J. L., and Brown, M. S., eds) 5th ed., pp. 945–972, McGraw-Hill, New York
3. O'Brien, J. S., Gugler, E., Giedion, A., Wiesmann, U., Hershkowitz, N., Meier, C., and Leroy, J. (1976) *Clin. Genet.* **9,** 495–504
4. Groebe, H., Krins, M., Schmidberger, H., von Figura, K., Harzer, K., Kresse, H., Paschke, E., Sewell, A., and Ullrich, K. (1980) *Am. J. Hum. Genet.* **32,** 258–272
5. Galjaard, H. (1980) *Genetic Metabolic Diseases: Early Diagnosis and Prenatal Analysis*, Elsevier/North-Holland, Amsterdam
6. Galjaard, H., Hoogeveen, A., de Wit-Verbeek, H. A., Reuser, A. J. J., Ho, M. W., and Robinson, D. (1975) *Nature (Lond.)* **257,** 60–62
7. Suzuki, Y., Furukawa, T., Hoogeveen, A., Verheijen, F., and Galjaard, H. (1979) *Brain Dev.* **1,** 83–86
8. Hoeksema, H. L., de Wit, J., and Westerveld, A. (1980) *Hum. Genet.* **53,** 241–247
9. Suzuki, Y., Nakamura, N., Fukuoka, K., Shimada, Y., and Uono, M. (1977) *Hum. Genet.* **36,** 219–229
10. Wenger, D. A., Sattler, M., Myers, G. G., Scheiman, R. S., and Nixon, G. W. (1980) *Clin. Genet.* **17,** 323–334
11. O'Brien, J. S. (1975) *Clin. Genet.* **8,** 303–313
12. Ben-Yoseph, Y., Burton, K. B., and Nadler, H. L. (1977) *Am. J. Hum. Genet.* **29,** 575–580
13. d'Azzo, A., Hoogeveen, A., Reuser, A. J. J., Robinson, D., and Galjaard, H. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 4535–4539
14. Skudlarek, M. D., and Swank, R. T. (1979) *J. Biol. Chem.* **254,** 9939–9942
15. Hoogeveen, A. T., Verheijen, F. W., and Galjaard, H. (1983) *J. Biol. Chem.* **258,** 12143–12146
16. Hasilik, A., and Neufeld, E. F. (1980) *J. Biol. Chem.* **255,** 4937–4945
17. Martin, R. G., and Ames, B. N. (1961) *J. Biol. Chem.* **236,** 1372–1379
18. Meisler, M., and Rattazzi, M. C. (1974) *Am. J. Hum. Genet.* **26,** 683–691
19. Steckel, F., Gieselmann, V., Waheed, A., Hasilik, A., von Figura, K., Oude Elferink, R., Kalsbeek, R., and Tager, J. M. (1982) *FEBS Lett.* **150,** 69–76
20. Reuser, A. J. J., and Kroos, M. (1982) *FEBS Lett.* **146,** 361–364
21. Waheed, A., Hasilik, A., and von Figura, K. (1982) *Eur. J. Biochem.* **123,** 317–321
22. Proia, R. L., and Neufeld, E. F. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 6360–6364
23. d'Azzo, A., Proia, R. L., Kolodny, E. H., Kaback, M. M., and Neufeld, E. F. (1983) *Am. J. Hum. Genet.* (abstr.), in press
24. Wenger, D. A., Tarby, T. J., and Wharton, C. (1978) *Biochem. Biophys. Res. Commun.* **82,** 589–595
25. Verheijen, F., Brossmer, R., and Galjaard, H. (1982) *Biochem. Biophys. Res. Commun.* **108,** 868–875
26. Hoogeveen, A., d'Azzo, A., Brossmer, R., and Galjaard, H. (1981) *Biochem. Biophys. Res. Commun.* **103,** 292–300
27. Paschke, E., and Kresse, H. (1982) *Biochem. Biophys. Res. Commun.* **109,** 568–575
28. Van Diggelen, O. P., Schram, A. W., Sinnott, M. L., Smith, P. J., Robinson, D., and Galjaard, H. (1981) *Biochem. J.* **200,** 143–151

# P A P E R VI

J. Biol. Chem.

(1986) 261, 5702-5704

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 261, No. 13, Issue of May 5, pp. 5702-5704, 1986


# $G_{M1}$-Gangliosidosis

## DEFECTIVE RECOGNITION SITE ON β-GALACTOSIDASE PRECURSOR*

(Received for publication, December 31, 1985)

**Andre T. Hoogeveen, Arnold J. J. Reuser, Marian Kroos, and Hans Galjaard‡**

*From the Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands*

**Cultured fibroblasts from different variants of $G_{M1}$-gangliosidosis synthesize normal amounts of 88-kDa β-galactosidase precursor. Yet the amount of the mature 64-kDa form is reduced to 5–15% of normal values. In this communication it is shown that the mutation in the infantile and adult form of $G_{M1}$-gangliosidosis interferes with the phosphorylation of precursor β-galactosidase. As a result the precursor is secreted instead of being compartmentalized into the lysosomes and further processed. The impaired phosphorylation might be due to conformational changes of the precursor molecule.**

Human lysosomal β-galactosidase (EC 3.2.1.23) is synthesized as a 88-kDa precursor form which is processed via intermediate forms into the 64-kDa mature enzyme (1). Within the lysosome the mature form aggregates into a high molecular weight complex of β-galactosidase, a 32-kDa "protective" protein and the lysosomal neuraminidase (2, 3). The β-galactosidase deficiency in a number of clinical variants of the inherited disease $G_{M1}$-gangliosidosis[1] (4) is due to allelic mutations of the gene on chromosome 3 coding for the 88-kDa polypeptide (5, 6). In recent biochemical studies on mutant cultured skin fibroblasts from different forms of $G_{M1}$-gangliosidosis 4–12% cross-reactive material of the mature β-galactosidase molecules was detected (7). Since the synthesis of the 88-kDa precursor was found to be normal it was hypothesized that most of the enzyme was degraded during one of the early steps of posttranslation modification.

The purpose of the present study was to elucidate the molecular nature of such defects in different β-galactosidase-deficient variants.

### EXPERIMENTAL PROCEDURES

*Materials*—Human skin fibroblasts from normal individuals and from patients with the infantile form of $G_{M1}$-gangliosidosis and I-Cell disease were obtained from the Rotterdam Cell Repository (Dr. M. F. Niermeijer); cells from patients with the adult form of $G_{M1}$-gangliosidosis were kindly provided by Dr. Y. Suzuki (Department of Pediatrics, University of Tokyo). The cells were cultured in Ham's F-10 medium (Flow Laboratories, Mclean, VA) supplemented with 10% fetal calf serum and antibiotics (Gibco). Early passages of the cell strains were grown in 25-$cm^2$ plastic culture flasks to a density of about 1 mg of protein/flask before analysis.

*Radioactive Labeling*—For labeling, Ham's F-10 medium was replaced by Dulbecco's modified Eagle's medium free of leucine, and 4 ml of this medium was supplemented with 2% fetal calf serum dialyzed against 0.9% NaCl and with 150 μl of 1 [4,5-$^3$H]leucine (150 μCi:135 Ci/mmol; Amersham Corp.) to study the biosynthesis of β-galactosidase. Secretion of precursor forms of lysosomal enzymes was induced by addition to the medium of $NH_4Cl$ in a final concentration of 10 mM during 2 days. Phosphorylation and glycosylation of the secreted precursor forms was studied by replacing Ham's F-10 medium by Dulbecco's modified Eagle's medium free of phosphate or glucose supplemented with dialyzed fetal calf serum. To a 25-$cm^2$ Falcon flask, 150 μCi of [2-$^3$H]mannose (10–20 Ci/mmol) or 200 μCi of carrier-free [$^{32}$P]phosphate was added.

*Immunoprecipitation*—After 48-h labeling, the medium was collected and mixed with an excess of polyclonal anti-β-galactosidase antibodies raised against human placental enzyme (1) and left for 15 h at 4 °C. The immunoprecipitates were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate on 10% slab gels and the radioactive bands were visualized by fluorography (8), with minor modifications (1).

*Uptake Studies*—Radioactively labeled β-galactosidase was prepared by the addition of 10 mM $NH_4Cl$ and [$^3$H]leucine to the medium above control and $G_{M1}$-gangliosidosis fibroblasts during 2 days. The radioactively labeled medium from a 25-$cm^2$ Falcon flask was concentrated and dialyzed against Ham's F-10 medium and added to control fibroblasts for 4 days. Subsequently, immunoprecipitation studies were carried out with anti-β-galactosidase antibodies to study the uptake and processing of labeled β-galactosidase after uptake in control fibroblasts.

### RESULTS

Secretion of precursor forms of β-galactosidase was studied in normal and mutant fibroblasts before and after treatment with $NH_4Cl$. The results of immunoprecipitation studies on culture media after [$^3$H]leucine labeling of the cells are shown in Fig. 1. After $NH_4Cl$ treatment normal fibroblasts are known to secrete the precursor forms of virtually all lysosomal glycoproteins and this is also the case for the 88-kDa precursor of β-galactosidase as well as for the 54-kDa precursor of the 32-kDa protective protein which also reacts with the conventional antiserum used (Fig. 1, *lane a*).

The enzymatic activity of β-galactosidase precursor secreted by control fibroblasts after $NH_4Cl$ treatment is 10–15 nmol of methylumbelliferon/h/ml of medium. In the medium above $G_{M1}$-gangliosidosis fibroblasts, no catalytic activity could be measured despite the presence of a considerable amount of β-galactosidase precursor molecules. Without $NH_4Cl$ treatment, control cells secrete only 10–20% of the β-galactosidase precursor as measured both by enzyme activity and scintillation counting of bands cut from the gels (Fig. 1, *lane b*).

$G_{M_1}$-gangliosidosis fibroblasts, however, secrete virtually all their 88-kDa β-galactosidase precursor even without $NH_4Cl$ treatment (Fig. 1, *lane c*), in a similar fashion as fibroblasts from patients with I-Cell disease (Fig. 1, *lane d*). The specificity of this effect is illustrated by the fact that the 54-kDa precursor of the protective protein is hardly secreted, this in contrast with $NH_4Cl$ treatment. No difference was found in the secretion pattern between cells from patients with the infantile and those with the adult form of $G_{M1}$-gangliosidosis (Fig. 2).

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Cell Biology and Genetics, Erasmus University, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands.

[1] The abbreviation used is: $G_{M1}$-gangliosidosis, *N*-acetylneuraminylgangliotetraglycosylceramide-gangliosidosis.

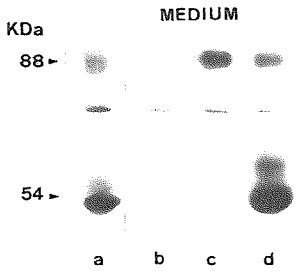



FIG. 1. **Immunoprecipitation studies of β-galactosidase after [$^3$H]leucine labeling of medium above normal and mutant fibroblasts followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** *Lane a*, medium above control cells after $NH_4Cl$ treatment; *lane b*, same without $NH_4Cl$ treatment; *lane c*, medium above $G_{M1}$-gangliosidosis cells; *lane d*, medium above I-Cells.

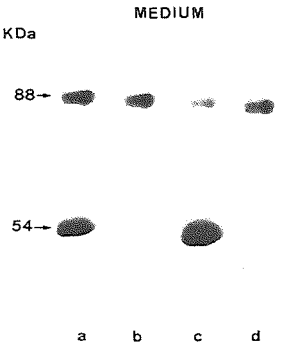



FIG. 2. **Immunoprecipitation studies of β-galactosidase after [$^3$H]leucine labeling of medium above $G_{M1}$-gangliosidosis fibroblasts, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** *Lane a*, medium above infantile $G_{M1}$-gangliosidosis cells after $NH_4Cl$ treatment; *lane b*, same without treatment; *lane c*, adult $G_{M1}$-gangliosidosis after $NH_4Cl$ treatment; *lane d*, same without treatment.

Uptake experiments were then performed to investigate the properties of the secreted precursor forms. After $NH_4Cl$ stimulation of [$^3$H]leucine-labeled cells, medium above normal and mutant fibroblasts was collected and used to incubate unlabeled fibroblasts for 4 days. The result in Fig. 3 shows that the 88-kDa β-galactosidase precursor secreted by normal fibroblasts is ingested and processed into the 64-kDa mature form (Fig. 3, *lane a*). The secreted 54-kDa precursor of the protective protein is also ingested and processed into a 32- and 20-kDa protein. The 88-kDa mutant β-galactosidase precursor secreted by $G_{M1}$-gangliosidosis fibroblasts, however, is not detected in the recipient cells (Fig. 3, *lane b*). This indicates a lack of uptake specific for β-galactosidase precursor since the 54-kDa precursor form of the protective protein secreted by the mutant cells is taken up and processed further.

To investigate whether the spontaneous secretion of the β-galactosidase precursor by $G_{M1}$-gangliosidosis fibroblasts and the impaired uptake of the precursor by normal fibroblasts was related to a defective binding to the mannose-6-phosphate receptor, phosphorylation studies were carried out. Normal

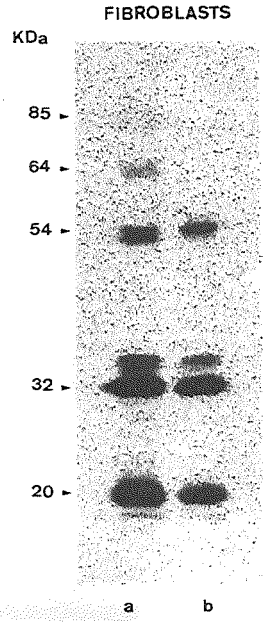



FIG. 3. **Immunoprecipitation of [$^3$H]leucine-labeled β-galactosidase in control fibroblasts followed by polyacrylamide gel electrophoresis.** *Lane a*, control fibroblasts after uptake of $^3$H-labeled proteins from medium above control fibroblasts; *lane b*, control fibroblasts after uptake of $^3$H-labeled proteins from medium above $G_{M1}$-gangliosidosis fibroblasts.

and mutant cells were labeled with carrier-free [$^{32}$P]phosphate and the secretion of the precursor protein was stimulated with $NH_4Cl$. Immunoprecipitation studies of β-galactosidase in the medium (Fig. 4) show that β-galactosidase precursor secreted by normal fibroblasts is phosphorylated (Fig. 4, *lanes a and b*), but no labeling is found for β-galactosidase precursor secreted by infantile or adult $G_{M1}$-gangliosidosis fibroblasts (Fig. 4, *lanes c and d*). The defective phosphorylation is restricted to β-galactosidase since the 54-kDa precursor of the protective protein is heavily phosphorylated both in normal and $G_{M1}$ cells.

The defective phosphorylation in the $G_{M1}$-gangliosidosis fibroblasts does not seem to be due to an altered glycosylation because immunoprecipitation studies after [$^3$H]mannose labeling gave a similar pattern in normal and mutant cells (Fig. 5).

## DISCUSSION

In the present study we provide an explanation for the marked reduction in the amount of 64-kDa mature β-galactosidase molecules in different variants of $G_{M1}$-gangliosidosis. The 88-kDa precursor, which is synthesized normally, is not phosphorylated probably as a result of a defective recognition of the mutant protein by *N*-acetylglucosaminylphosphotransferase. As a consequence there is no binding to the mannose-6-phosphate receptor, required for a correct lysoso-

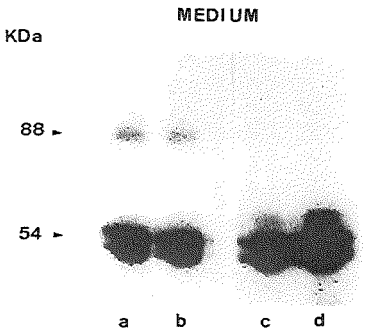



FIG. 4. **Immunoprecipitation studies of $^{32}$P-labeled β-galactosidase from medium above control and $G_{M1}$-gangliosidosis fibroblasts after $NH_4Cl$ treatment, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** *Lanes a* and *b*, medium above control fibroblasts; *lane c*, medium above infantile $G_{M1}$-gangliosidosis cells; *lane d*, medium above adult $G_{M1}$-gangliosidosis cells.

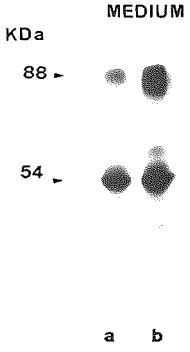



FIG. 5. **Immunoprecipitation studies of [$^3$H]mannose-labeled β-galactosidase from medium after $NH_4Cl$ stimulation, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** *Lane a*, medium above control fibroblasts; *lane b*, medium above $G_{M1}$-gangliosidosis fibroblasts.

mal compartmentalization (9, 10), instead the β-galactosidase precursor is secreted.

This defect is different from that in I-cell disease where the absence of the mannose-6-phosphate marker on a variety of lysosomal glycoproteins is caused by a deficiency of the enzyme *N*-acetylglucosaminylphosphotransferase (11–13).

In $G_{M1}$-gangliosidosis a mutation in the gene on chromosome 3 apparently alters the structure of the 88-kDa β-galactosidase precursor in such a way that it is not phosphorylated. The fact that this defect is found in all $G_{M1}$-gangliosidosis variants, may indicate that the various allelic mutations change the conformation of the β-galactosidase precursor in such a way that the recognition site for *N*-acetylglucosaminylphosphotransferase is lost.

Recent studies (14) indicate that the latter enzyme can only recognize lysosomal glycoproteins in their native conformation, suggesting the importance of protein conformation for the exposure of the recognition marker. The experimental data described in the present paper indicate that $G_{M1}$-gangliosidosis fibroblasts might be a useful model for further studies on this subject.

REFERENCES

1. d'Azzo, A., Hoogeveen, A. T., Reuser, A. J. J., Robinson, D., and Galjaard, H. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 4535–4539
2. Hoogeveen, A. T., Verheijen, F. W., and Galjaard, H. (1983) *J. Biol. Chem.* **258,** 12143–12146
3. Verheijen, F. W., Palmeri, S., Hoogeveen, A. T., and Galjaard, H. (1985) *Eur. J. Biochem.* **149,** 315–321
4. O'Brien (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., and Brown, M. S., eds) 5th Ed., pp. 945–972, McGraw-Hill, New York
5. Shows, T. B., Scrafford-Wolf, Brown, J. A., and Meisler, M. (1978) *Cytogenet. Cell Genet.* **22,** 219–222
6. Sips, H. J., de Wit-Verbeek, H. A., de Wit, J., Westerveld, A., and Galjaard, H. (1985) *Hum. Genet.* **69,** 340–344
7. Hoogeveen, A. T., Graham-Kawashima, H., d'Azzo, A., and Galjaard, H. (1984) *J. Biol. Chem.* **259,** 1974–1977
8. Hasilik, A., and Neufeld, E. F. (1980) *J. Biol. Chem.* **255,** 4937–4945
9. Neufeld, E. F., and Ashwell, G. (1980) *The Biochemistry of Glycoproteins and Proteoglycans*, pp. 252–257, Plenum Press, New York
10. Creek, K. E., and Sly, W. S. (1984) in *Lysosomes in Pathology and Biology* (Dingle, J. T., Dean, R. T., and Sly, W., eds) pp. 63–82, Elsevier/North-Holland, New York
11. Hickman, S., and Neufeld, E. F. (1972) *Biochem. Biophys. Res. Commun.* **49,** 992–999
12. Reitman, M. L., and Kornfeld, S. (1981) *J. Biol. Chem.* **256,** 4275–4281
13. Hasilik, A., Waheed, A., and von Figura, K. (1981) *Biochem. Biophys. Res. Commun.* **98,** 761–767
14. Lang, L., Reitman, M., Tang, J., Roberts, R. M., and Kornfeld, S. (1984) *J. Biol. Chem.* **259,** 14663–14671

P A P E R  VII

Eur. J. Cell Biol.

(1986) 40, 9-15

European Journal of Cell Biology 40, 9-15 (1986) 

# Immunoelectron microscopical localization of lysosomal β-galactosidase and its precursor forms in normal and mutant human fibroblasts

Rob Willemsen[1], André T. Hoogeveen, Herman J. Sips, Johan M. van Dongen, Hans Galjaard

Department of Cell Biology and Genetics, Erasmus University, Rotterdam/The Netherlands

Received October 10, 1985
Accepted December 12, 1985

*Immunocytochemistry — fibroblasts — β-galactosidase — $G_{MI}$-gangliosidosis — galactosialidosis*

Immunoelectron microscopy was performed to study the biosynthesis of lysosomal β-galactosidase (β-gal) in normal and mutant human fibroblasts. Using polyclonal and monoclonal antibodies we show in normal cells precursor forms of β-gal in the rough endoplasmic reticulum (RER) and in the Golgi apparatus throughout the stack of cisternae. In the lysosomes virtually all β-gal exists as a high molecular weight multimer of mature enzyme. In the autosomal recessive disease $G_{MI}$-gangliosidosis caused by a β-gal deficiency and in galactosialidosis, associated with a combined deficiency of lysosomal neuraminidase and β-gal, precursor forms of the latter enzyme are found in RER, Golgi and some labeling is present at the cell surface. The lysosomes remain unlabeled, indicative for the absence of enzyme molecules in this organelle. In galactosialidosis fibroblasts also no mature β-gal is found in the lysosomes but in these cells the presence of the monomeric form can be increased by leupeptin (inhibition of proteolysis) whereas addition of a partly purified 32 kDa "protective protein" results in the restoration of high molecular weight β-gal multimers in the lysosomes.

## Introduction

About 30 autosomal recessive disorders in man are based on a lysosomal enzyme deficiency [5]. During the last few years it has become clear that a number of these deficiencies are due to abnormal posttranslational processing of the precursor forms of the enzymes. Such processing defects can be related to a structural abnormality of the polypeptide itself or they can be caused by a genetic deficiency of other proteins involved in the modification and compartmentalization of the enzyme(s) (for reviews see [1, 4]).

The β-galactosidase deficiency in $G_{MI}$-gangliosidosis [17] appears to be an example of the former. The 85 kDa precursor of β-galactosidase is synthesized normally but no mature 64 kDa enzyme is detected in the lysosome [13]; recently we have found that the structural mutation interferes with the phosphorylation of the precursor form which is then secreted into the medium (to be published) as is the case in some variants of α-glucosidase deficiency in glycogenosis II [20].

Galactosialidosis, an inherited disease associated with deficiencies of lysosomal β-galactosidase and neuraminidase [26], was found to be due to a deficiency of a 32 kDa "protective protein" [2] which normally is required for the aggregation of monomeric β-galactosidase into high molecular weight aggregates [12] and which forms a complex with neuraminidase [24].

These studies thus far are all based on biochemical analyses mostly on homogenates from normal and mutant fibroblasts. The availability of polyclonal and monoclonal antibodies against β-galactosidase in its different aggregation forms [21] and the possibility of immunoelectron microscopy on frozen sections [7, 23] enabled us to relate the different steps of posttranslational processing of β-galactosidase to the various subcellular compartments in normal and mutant human fibroblasts.

## Materials and methods

Human skin fibroblasts from normal individuals and from patients with the adult type $G_{MI}$-gangliosidosis [22] and the early infantile variant of galactosialidosis [14] were obtained from the Rotterdam Cell Repository (Prof. Dr. M. F. Niermeijer). The cells were cultivated in Ham's F10 medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum and antibiotics (Gibco). Early passages of the cell strains were grown in 75 $cm^2$ Falcon flasks to a density of 1 to 2 mg of protein per flask. After trypsinization cells were harvested, rinsed twice with fresh medium and kept in suspension in a rotating tube for 2 h at 37°C to recover from possible damage by trypsin.

### *Antibodies*

For immunocytochemistry rabbit polyclonal antibodies against human placental β-galactosidase described by d'Azzo et al. [2] were used; these react with monomeric and multimeric forms of mature enzyme, its 85 kDa precursor and with the 32 kDa protective protein required for the aggregation of monomeric 64 kDa β-galactosidase and its 54 kDa precursor.

Mouse monoclonal antibodies (3C7/B4) react with the 64 kDa monomeric β-galactosidase and its 85 kDa precursor only, as reported by Sips et al. [21]. Additional data concerning the isolation

[1] Rob Willemsen, Department of Cell Biology and Genetics, Erasmus University, P. O. Box 1738, 3000 DR Rotterdam/The Netherlands.

and characterization of monoclonal antibodies against β-galactosidase will be published separately. In applying the monoclonal antibodies for immunocytochemistry a rabbit anti-(mouse IgG) was used as an intermediate step.

*Immunocytochemistry*

Cells were collected by centrifugation and taken up in 0.1 M phosphate buffer (PB), *p*H 7.3, containing 1% acrolein and 0.4% glutaraldehyde. After fixation for 60 min at 4°C, the cells were rinsed twice in 0.1 M PB and resuspended in 10% (w/v) gelatin in 0.1 M PB at 37°C. After centrifugation the pellet was solidified on ice, and postfixation to cross-link the gelatin was performed in the same fixative as used before. The gelatin-embedded cells were stored in 1% paraformaldehyde in 0.1 M PB containing 1 M sucrose, at 4°C [6]. Ultracryotomy was carried out as described by Tokuyasu [23] using an LKB Nova ultramicrotome equipped with the Cryo Nova.

The methods used for immunoelectron microscopy were those described by Geuze et al. [7] with the following minor modifications. Prior to incubation with the first antibody, the sections were treated for 10 min with sodium borohydride (2 mg/ml PB) to reduce residual aldehyde groups of the fixative [3, 25]. Antigen-antibody complexes were visualized by using goat anti-(rabbit IgG) coupled to 10 nm colloidal gold (GAR 10, Janssen Pharmaceutica, Beerse/Belgium). Sections were stained with uranyl acetate and embedded in 1.5% methylcellulose [9]. Control sections were treated with rabbit anti-(human albumin) or rabbit anti-(mouse IgG) and GAR 10 only. Background labeling appeared to be negligible.

*Correction experiments*

Correction of the combined β-galactosidase and neuraminidase deficiency in galactosialidosis cells was established in two ways. Firstly, the intralysosomal proteolytic degradation of the lysosomal enzymes was inhibited by the addition of leupeptin (Sigma, final concentration 0.02 mM) to culture medium above the mutant fibroblasts for 4 days.

Secondly, a concentrate of the 54 kDa precursor of the 32 kDa protective protein [12] was prepared by addition of 10 mM $NH_4Cl$ to the medium above $G_{M1}$-gangliosidosis fibroblasts during 2 days followed by concentration on an Amicon $PM_{10}$ filter to a final volume of 1 ml as described [11]. An aliquot of 40 µl of this concentrate was added per ml medium of galactosialidosis cells during 4 days.

## Results

*Processing of β-galactosidase in normal fibroblasts*

The polyclonal antibodies against β-galactosidase are known to react with the 85 kDa precursor, the 64 kDa mature monomeric enzyme, the 600 to 700 kDa multimeric aggregate and with the 32 kDa protective protein and its 54 kDa precursor. Using this antibody, immunogold labeling of ultrathin frozen sections of normal fibroblasts show a random distribution of gold particles within the lysosomes (Fig. 1). The rough endoplasmic reticulum (RER) (Fig. 1) and the Golgi-complex (Fig. 2) are labeled too. The Golgi complex was labeled from cis to trans throughout the stack of cisternae. Also a weak labeling at the plasma membrane and occasionally of a small vesicle just below was observed (not shown).

With the monoclonal antibody, known to react with the 85 kDa β-galactosidase precursor and the monomeric mature enzyme only, gold labeling was found in the RER (Fig. 3), the Golgi complex (not shown), at the plasma membrane and in small vesicles just below the plasma membrane (Fig. 4). No gold particles were detected in the lysosomes (Fig. 3).

*Processing of β-galactosidase in mutant fibroblasts*

In fibroblasts from a patient with $G_{M1}$-gangliosidosis no difference was found between the labeling pattern using the polyclonal or the monoclonal antibodies. Most lysosomes remained unlabeled although occasionally a single gold particle was observed (Figs. 5, 6). The labeling of the RER and the Golgi complex is similar to that shown for normal cells.

Cells from a patient with galactosialidosis had a similar labeling pattern as described above for $G_{M1}$-gangliosidosis: again no labeling of virtually all lysosomes (Fig. 7) and weak labeling of the RER, Golgi complex and plasma membrane.

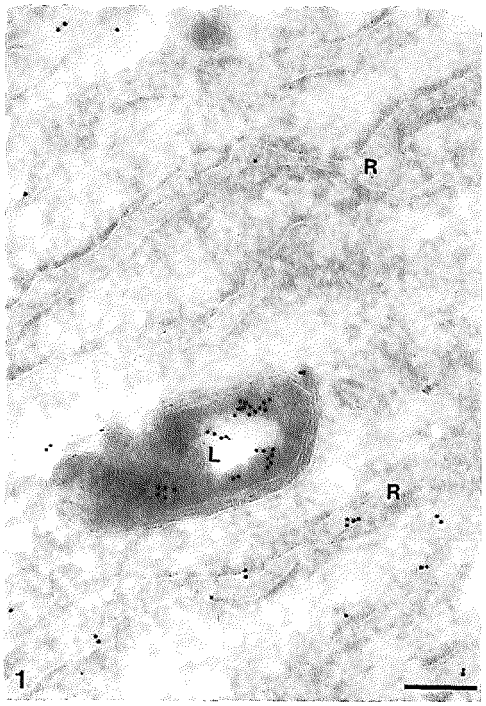
*Correction of the defect*

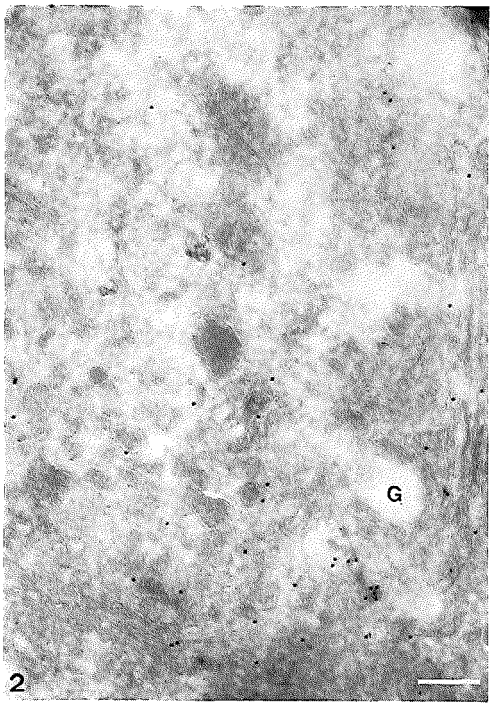
Addition of leupeptin which inhibits thiolcathepsins, blocks the enhanced degradation of monomeric β-galactosidase molecules. The latter is responsible for the β-galactosidase deficiency in galactosialidosis [2]. Addition of leupeptin to galactosialidosis fibroblasts causes a marked increase of gold labeling in the lysosomes using monoclonal antibodies (compare Figs. 7 and 8); similar results were obtained with polyclonal antibodies, as expected.

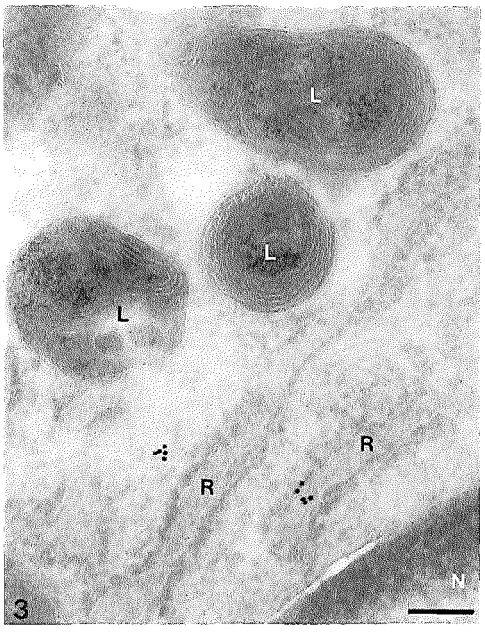
Addition of the precursor form of the 32 kDa protective protein to galactosialidosis cells is known to aggregate monomeric β-galactosidase into high molecular weight aggregates and to activate neuraminidase, thereby correcting the combined enzyme deficiency [12]. At the subcellular level ingestion of exogenous 54 kDa precursor of the protective protein results in a marked increase of gold labeling using polyclonal anti-β-galactosidase antibodies. However, when monoclonal antibodies were used no labeling was observed (Figs. 9, 10).

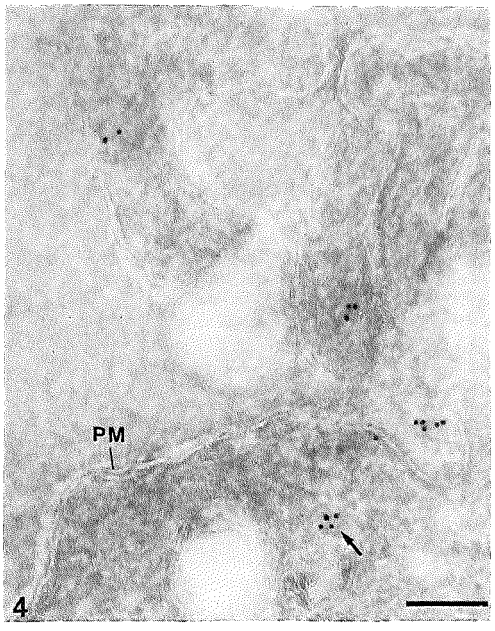
**Figs. 1 to 10.** Ultrathin cryosections of normal and mutant human skin fibroblasts. The sections were indirectly labeled with goat anti-(rabbit IgG) conjugated with 10 nm colloidal gold for the demonstration of β-galactosidase. — Bars 0.2 µm.

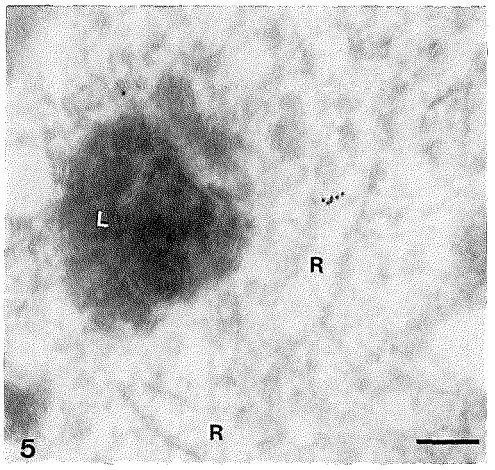
**Figs. 1 to 4.** Subcellular localization of β-galactosidase in normal fibroblasts. — **Fig. 1.** Polyclonal anti β-galactosidase. Labeling is observed in the RER (R) and in lysosomes (L). — **Fig. 2.** Polyclonal anti β-galactosidase. Labeling can be seen throughout the stack of Golgi cisternae (G). — **Fig. 3.** Monoclonal anti β-galactosidase. Gold particles are present only in the RER (R), and the lysosomes (L) remain unlabeled. — N Nucleus. — **Fig. 4.** Monoclonal anti β-galactosidase. Labeling is observed at the plasma membrane (PM) and occasionally in a small vesicle just below (*arrow*).

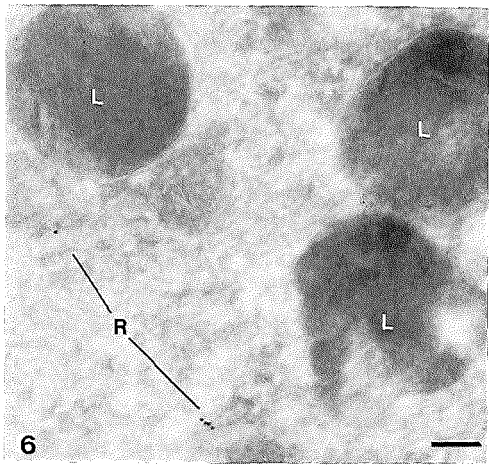

R
L
R
1


G
2


L
L
L
R
R
N
3


PM
4




**Figs. 5, 6.** Subcellular localization of β-galactosidase in fibroblasts from the adult form of $G_{M1}$-gangliosidosis. — **Fig. 5.** Polyclonal anti β-galactosidase. — **Fig. 6.** Monoclonal anti β-galactosidase. In both instances the RER is labeled whereas the lysosomes remain unlabeled although occasionally a single gold particle is seen in a lysosome (Fig. 5).

## Discussion

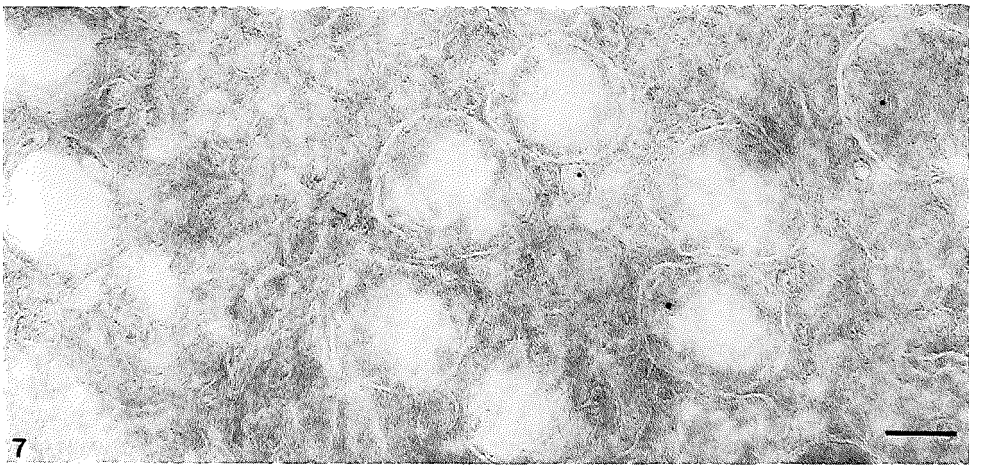
The present study is the first to follow the intracellular processing of β-galactosidase in normal and mutant human fibroblasts. The labeling of the RER and Golgi complex most likely represents the 85 kDa precursor form of β-galactosidase, since an identical labeling is observed using either monoclonal or polyclonal antibodies against β-galactosidase. The fact that labeling is observed from cis to trans throughout the stack of cisternae is in agreement with the biochemical data about the glycosylation, phosphorylation and the uncovering of the mannose-6-P-recognition site of lysosomal (enzyme) proteins [8, 19]. On the basis of the combined data with polyclonal and monoclonal antibodies, the labeling of lysosomes in normal fibroblasts using polyclonal antibodies is indeed the result of the presence of high molecular weight aggregates of the 64 kDa mature β-galactosidase. The immunocytochemical findings are thus in accordance with the biochemical evidence for such aggregates in the lysosomes, as previously described [12]. Furthermore, no lysosomal labeling is observed with the monoclonal antibodies that react with monomeric forms of β-galactosidase only.

The weak labeling at the plasma membrane and very small vesicles below might indicate a minor second pathway of precursor molecules leaving the cell via the plasma membrane. Such a pathway has been suggested previously on the basis of biochemical studies [10].

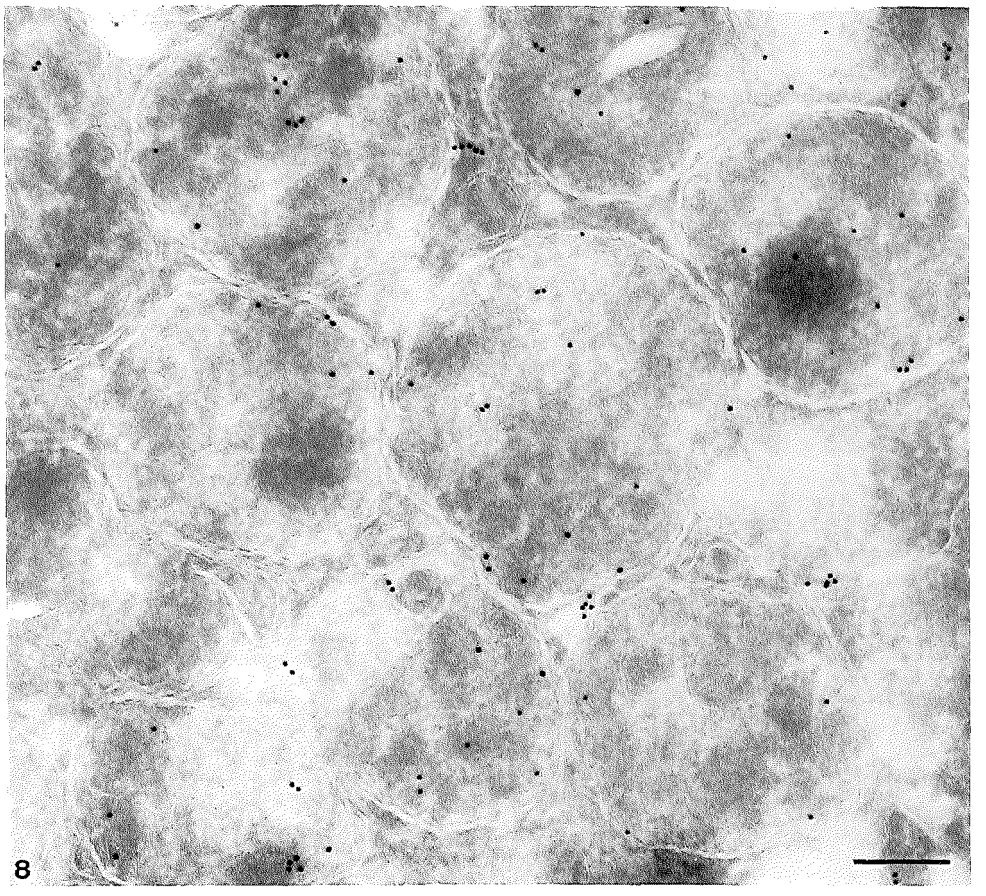
In $G_{M1}$-gangliosidosis fibroblasts a normal synthesis of 85 kDa precursor β-galactosidase and absence of the 64 kDa mature form has been described [13]. Our immunocytochemical studies support this observation: no cross-reactive material is seen in the lysosomes. It has been demonstrated that $G_{M1}$-gangliosidosis fibroblasts also contain a normal amount of functional 32 kDa protective protein [11]; apparently the 32 kDa protective protein is not or hardly immunoreactive under the conditions appropriate for immunoelectron microscopy. Recently, evidence has been obtained that the structural mutation of the β-galactosidase precursor molecule interferes with its phosphorylation and results in secretion of the precursor by $G_{M1}$-gangliosidosis fibroblasts (to be published). In previous studies, however, the presence of cross-reactive material was reported in different variants of $G_{M1}$-gangliosidosis [15, 16] but in these studies human liver was used. It is known that the intracellular routing is different in various cell types which will of course determine the ultimate effect of a genetic processing defect [18].

Our results on galactosialidosis are especially interesting since they show the absence of β-galactosidase molecules in the lysosomes, an increase of monomeric form after addition of leupeptin and a correction of the metabolic defect after administration of the 32 kDa protective protein precursor i. e. aggregation into high molecular weight complex of β-galactosidase molecules. The latter finding indicates that the aggregation of 64 kDa monomeric β-galactosidase takes place in the lysosome. Since it is known that

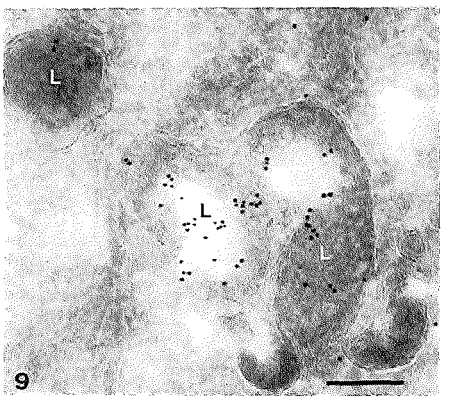
**Figs. 7, 8.** Subcellular localization of β-galactosidase in galactosialidosis fibroblasts. — **Fig. 7.** Monoclonal anti β-galactosidase. Most lysosomes show no labeling, occasionally a single gold particle is detected in a lysosome. — **Fig. 8.** Monoclonal anti β-galactosidase. After addition of leupeptin a marked increase of the number of gold particles is observed in the lysosomes.

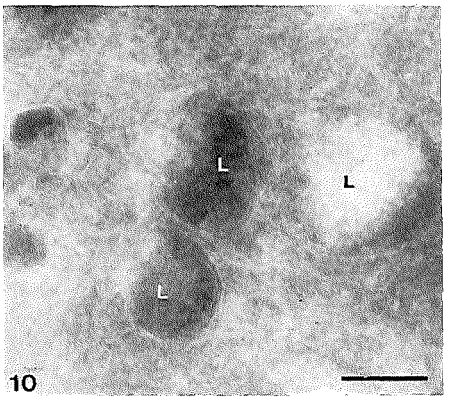


7



8




**Figs. 9, 10.** Subcellular localization of β-galactosidase in galactosialidosis fibroblasts corrected by addition of the precursor of the 32 kDa protective protein. — **Fig. 9.** Polyclonal anti β-galactosidase. In the lysosomes (L) the number of gold particles is normalized. — **Fig. 10.** Monoclonal anti β-galactosidase. The lysosomes (L) remain unlabeled.

the 32 kDa protective protein is essential for this aggregation our data on correction also suggest that the processing of 54 kDa precursor to 32 kDa protective protein also occurs within the lysososome. Studies to corroborate this with an affinity-purified monospecific antibody preparation against the 32 kDa protective protein are in progress.

*Acknowledgements.* We acknowledge the help in photography of T. van Os and the secretarial assistance of Mrs. P. C. van Sluijsdam. We thank Dr. A. J. J. Reuser for stimulating discussions.

## References

[1] Barranger, J. A., R. O. Brady (eds.): Molecular basis of lysosomal storage disorders. Academic Press, Inc. Orlando 1984.
[2] D'Azzo, A., A. T. Hoogeveen, A. J. J. Reuser, D. Robinson, H. Galjaard: Molecular defect in combined β-galactosidase and neuraminidase deficiency in man. Proc. Natl. Acad. Sci. USA **79**, 4535-4539 (1982).
[3] De Vries, J. W. A., R. Willemsen, H. J. Geuze: Immunocytochemical localization of acrosin and hyaluronidase in epididymal and ejaculated porcine spermatozoa. Eur. J. Cell Biol. **37**, 81-88 (1985).
[4] Dingle, J. T., R. T. Dean, W. Sly (eds.): Lysosomes in biology and pathology. Elsevier Science Publishers. Amsterdam 1984.
[5] Galjaard, H., A. J. J. Reuser: Genetic aspects of lysosomal storage diseases. In: J. T. Dingle, R. T. Dean, W. Sly (eds.): Lysosomes in biology and pathology, pp. 315-345. Elsevier Science Publishers. Amsterdam 1984.
[6] Geuze, H. J., J. W. Slot: Disproportional immunostaining patterns of two secretory proteins in guinea pig and rat exocrine pancreatic cells. An immunoferritin and fluorescence study. Eur. J. Cell Biol. **21**, 93-100 (1980).
[7] Geuze, H. J., J. W. Slot, P. A. van der Ley, R. C. T. Scheffer, J. M. Griffith: Use of colloidal gold particles in double labeling immunoelectron microscopy of ultrathin frozen tissue sections. J. Cell Biol. **89**, 653-665 (1981).
[8] Goldberg, D. E., S. Kornfeld: Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. J. Biol. Chem. **258**, 3159-3165 (1983).
[9] Griffiths, G., R. Brands, B. Burke, D. Louvard, G. Warren: Viral membrane proteins acquire galactose in trans Golgi cisternae during intracellular transport. J. Cell Biol. **95**, 781-792 (1982).
[10] Hasilik, A., E. F. Neufeld: Biosynthesis of lysosomal enzymes in fibroblasts. Synthesis as precursor of higher molecular weight. J. Biol. Chem. **255**, 4937-4945 (1980).
[11] Hoogeveen, A. T., A. d'Azzo, R. Brossmer, H. Galjaard: Correction of combined β-galactosidase/neuraminidase deficiency in human fibroblasts. Biochem. Biophys. Res. Commun. **103**, 292-300 (1981).
[12] Hoogeveen, A. T., F. W. Verheijen, H. Galjaard: The relation between human lysosomal β-galactosidase and its protective protein. J. Biol. Chem. **258**, 12143-12146 (1983).
[13] Hoogeveen, A. T., H. Graham-Kawashima, A. d'Azzo, H. Galjaard: Processing of human β-galactosidase in $G_{M1}$-gangliosidosis and Morquio B syndrome. J. Biol. Chem. **259**, 1974-1977 (1984).
[14] Kleijer, W. J., A. T. Hoogeveen, F. W. Verheijen, M. F. Niermeijer, H. Galjaard, J. S. O'Brien, T. G. Wanner: Prenatal diagnosis of sialidosis with combined neuraminidase and β-galactosidase deficiency. Clinical Genetics **16**, 60-61 (1979).
[15] Meisler, M., M. C. Rattazzi: Immunological studies of β-galactosidase in normal human liver and $G_{M1}$-gangliosidosis. Am. J. Hum. Genet. **26**, 683-691 (1974).
[16] O'Brien, J. S.: Molecular genetics of $G_{M1}$-β-galactosidase Clin. Genet. **8**, 303-313 (1975).
[17] Okada, S., J. S. O'Brien: Generalized gangliosidosis: β-galactosidase deficiency. Science **160**, 1002-1004 (1968).
[18] Owada, M., E. F. Neufeld: Is there a mechanism for introducing acid hydrolases into liver lysosomes that is independent of mannose-6-phosphate recognition? Evidence from I-cell disease. Biochem. Biophys. Res. Commun. **105**, 814-820 (1982).
[19] Pohlmann, R., A. Waheed, A. Hasilik, K. von Figura: Synthesis of phosphorylated recognition marker in lysosomal enzymes is located in the cis part of Golgi apparatus. J. Biol. Chem. **257**, 5323-5325 (1982).

[20] Reuser, A. J. J., M. Kroos, R. P. J. Oude Elferink, J. M. Tager: Defects in synthesis, phosphorylation and maturation of acid α-glucosidase in glycogenosis type II. J. Biol. Chem. **260**, 8336–8342 (1985).

[21] Sips, H. J., H. A. de Wit-Verbeek, J. de Wit, A. Westerveld, H. Galjaard: The chromosomal localization of human β-galactosidase revisited: a locus for β-galactosidase on human chromosome 3 and for its protective protein on human chromosome 22. Hum. Genet. **69**, 340–344 (1985).

[22] Suzuki, Y., T. Furukawa, A. Hoogeveen, F. Verheijen, H. Galjaard: Adult type $G_{M1}$-gangliosidosis: A complementation study on somatic cell hybrids. Brain Dev. **1**, 83–86 (1979).

[23] Tokuyasu, K. T.: A technique for ultracryotomy of cell suspensions and tissues. J. Cell Biol. **57**, 551–565 (1973).

[24] Verheijen, F., R. Brossmer, H. Galjaard: Purification of acid β-galactosidase and acid neuraminidase from bovine testis: Evidence for an enzyme complex. Biochem. Biophys. Res. Commun. **108**, 868–875 (1982).

[25] Weber, K., P. C. Rathke, M. Osborn: Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electron microscopy and by immunofluorescence microscopy. Proc. Natl. Acad. Sci. USA **75**, 1820–1824 (1978).

[26] Wenger, D. A., T. J. Tarby, C. Wharton: Macular cherry-red spots and myoclonus with dementia: coexistent neuraminidase and β-galactosidase deficiencies. Biochem. Biophys. Res. Commun. **82**, 589–595 (1978).

# P A P E R  VIII

Hum. Genet.

(1986) 73, 35-38

Hum Genet (1986) 73:35–38

Human Genetics



# Ganglioside $G_{M1}$ metabolism in living human fibroblasts with β-galactosidase deficiency

G.M.S. Mancini[1,*], A.T. Hoogeveen[1], H. Galjaard[1], J.E. Mansson[2], and L. Svennerholm[2]

[1] Department of Cell Biolgy and Genetics, Erasmus University Rotterdam, Postbus 1738, NL-3000 DR Rotterdam, The Netherlands
[2] Department of Neurochemistry, Psychiatric Research Centre, University of Göteburg, St. Jörgen Hospital, S-422 03 Hisings Backa, Sweden

**Summary.** The uptake and catabolism of [$^3$H-ceramide]-$G_{M1}$ was followed in living fibroblasts from patient with different forms of β-galactosidase deficiency. Gangliosides are identified according to the nomenclature of Svennerholm (1963). A total inability to metabolize the ingested substrate was found in infantile $G_{M1}$-gangliosidosis whereas cells from an adult $G_{M1}$-gangliosidosis variant showed a slower rate of degradation, compared with controls. Morquio B fibroblasts had a comparable catabolism of $G_{M1}$ as controls. Fibroblasts from different types of galactosialidosis, a recessive disease associated with a coexistent β-galactosidase/neuraminidase deficiency all showed degradation of ingested $G_{M1}$. In view of the molecular defect in this disease, this catabolism must be due to the 10–20% of monomeric β-galactosidase molecules present in the lysosomes. Unexpectedly, in these cells an impaired metabolism of $G_{M3}$ was found. The same finding was observed when cells with an isolated neuraminidase deficiency (mucolipidosis I) were loaded with $G_{M1}$. A hypothesis is presented to explain these results.

## Introduction

Several human genetic diseases are associated with a deficiency of lysosomal β-galactosidase (EC.3.2.1.23) alone like $G_{M1}$-gangliosidosis (Okada and O'Brien 1968) and Morquio B syndrome (O'Brien et al. 1976; Groebe et al. 1980) or in combination with a deficiency of neuraminidase (EC.3.2.1.18) as in galactosialidosis (Wenger et al. 1978; Hoogeveen et al. 1980). During the last few years progress has been made to elucidate the exact nature of the molecular defect in these lysosomal storage diseases. In $G_{M1}$-gangliosidosis the structural mutation in chromosome 3 affects the 85 kilodalton precursor β-galactosidase so that it is secreted instead of being properly processed and compartmentalized into the lysosome (Hoogeveen et al. 1986). In Morquio B syndrome the mutation affects the affinity and catalytic properties towards glycosaminoglycans and ganglioside $G_{M1}$ (Paschke and Kresse 1982; Van der Horst et al. 1983).

The combined β-galactosidase and neuraminidase deficiency in patients with galactosialidosis appears to be due to a deficiency of a 32 kilodalton "protective protein" (d'Azzo et al. 1982) which normally is required for the aggregation of β-galactosidase monomers into a high molecular weight complex with neuraminidase (Verheijen et al. 1982; Hoogeveen et al. 1983). Deficiency of this protein results in enhanced proteolytic degradation of β-galactosidase monomers whereas neuraminidase remains inactive because the 32 kilodalton protein is an essential subunit for this enzyme (Verheijen et al. 1985).

For each of the genetic diseases mentioned above different clinical variants, concerning age of onset, involvement of different organs, and mental retardation, have been reported (Andria et al. 1981; O'Brien 1983; Van Gemund et al. 1983; Galjaard et al. 1984). These are probably based on different rates of ganglioside $G_{M1}$ catabolism due to different molecular types of the deficient enzyme (Conzelmann and Sandhoff 1983/1984). The synthesis of [$^3$H-ceramide] $G_{M1}$-ganglioside enabled us to investigate under in vivo conditions the metabolism of ganglioside $G_{M1}$ in normal fibroblasts and in the various types of mutant cells mentioned above.

## Materials and methods

Fibroblasts from patients with the infantile or adult form of $G_{M1}$-gangliosidosis, with the infantile or adult form of galactosialidosis, with mucolipidosis I, and with Morquio B syndrome were obtained from the Rotterdam Cell Repository (Professor Dr. M.F. Niermeijer). Early passages of the cell strains were cultured in Ham's F10 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. The activities of β-galactosidase and neuraminidase were measured with the appropriate 4-methylumbelliferyl substrates as described by Galjaard (1980).

### *$G_{M1}$-β-galactosidase activity in cell homogenates*

The preparation of [$^3$H-$G_{M1}$]-ganglioside labeled in the galactose moiety and the determination of $G_{M1}$-β-galactosidase activity was performed as previously described (Svennerholm et al. 1979).

### *Metabolism in living fibroblasts of [$^3$H-ceramide] ganglioside-$G_{M1}$*

Ganglioside $G_{M1}$ was labeled in the ceramide portion with tritium (specific activity: 57 mCi/mmol) (Schwarzmann 1978). Labeled $G_{M1}$ ganglioside was dissolved in Ham's F10 medium

* On leave from Department of Pediatrics, University of Siena, Italy
*Offprint requests to:* H. Galjaard, Department of Cell Biology and Genetics, Erasmus University Rotterdam, Postbus 1738, NL-3000 DR Rotterdam, The Netherlands

at a concentration of 5 μmol/l and added to 25 $cm^2$ Falcon flasks with fibroblasts grown to confluency. After 16 h of incubation in labeled medium and 8–48 h of "chase", the cells were harvested using 0.25% trypsin, washed extensively with phosphate buffered saline (PBS) to remove unspecifically bound ganglioside and, after centrifugation, the pellet was disrupted by suspending in 500 μl of water. A small sample of the pellet (25 μl) was dissolved in Soluene 350 (Packard) and counted in Dimilume 30 (Packard) to assess the total ganglioside uptake. The lipids were extracted from 400 μl of cell suspension by adding methanol and chloroform to a final ratio of C-M-W 4:8:3 (v/v/v). The insoluble material was removed by centrifugation. The clear supernatant was evaporated to dryness under nitrogen, dissolved in C-M-W 60:30:4.5 (v/v/v), and desalted through a small Sephadex G25 column. From different samples equal amounts of counts were applied to a HP-TLC plate (Merck) and run in C-M-0.25 KCL (50:40:10:v/v/v). The radiolabeled compounds were visualized by fluorography.

*Correction studies*

Correction of the defect in galactosialidosis cells was established in two ways. First, the intralysosomal proteolytic degradation of β-galactosidase monomers was inhibited by addition to the medium of leupeptin (Sigma), a thiol protease inhibitor, at a final concentration of 0.02 m*M* for 5 days, as described previously (Hoogeveen et al. 1983), prior to labeling with [$^3$H]-$G_{M1}$. Second, both the aggregation of galactosidase and activation of neuraminidase was established by adding the precursor form of the 32 kilodalton protective protein. This was prepared by addition of 10 m*M* $NH_4Cl$ to the medium above $G_{M1}$-gangliosidosis fibroblasts for 2 days, followed by concentration on an Amicon PM10 filter to a final volume of 1 ml, as previously described (Hoogeveen et al. 1981). A sample of 40 μl of the concentrate was added per ml of medium above galactosialidosis fibroblasts. After 5 days the cells were labeled with [$^3$H]-$G_{M1}$.

## Results and discussion

The residual activities in the different types of mutant fibroblasts used are summarized in Table 1. In the infantile form of $G_{M1}$-gangliosidosis the residual activity with both natural and artificial substrate is less than 1%, in Morquio B 3–4%, and in adult $G_{M1}$-gangliosidosis the activity is of the order of 10%, as it is in both galactosialidosis variants. The apparent $K_m$ towards [$^3$H]-galactose $G_{M1}$ was found to be 1.2 m*M* in control fibroblasts which is in agreement with previous findings (Svennerholm et al. 1979). The same values were observed in adult $G_{M1}$-gangliosidosis and in both galactosialidosis variants. We could not obtain reliable data on the $K_m$ for the other mutant cell strains with a very low residual enzyme activity. Various investigators have, however, questioned the reliability of $K_m$ studies on gangliosides in aqueous solutions (Scheel et al. 1982; Venerando et al. 1985).

To study the metabolism of gangliosides under in vivo conditions, $G_{M1}$ $^3$H-labeled in its ceramide portion was administered to the various mutant fibroblasts and after uptake the ganglioside pattern was analyzed by thin layer chromatography. After a period of 16 h 5–7% (0.9 nmol) of the total amount of ganglioside $G_{M1}$ in the medium was ingested and part of it ought to be localized in the lysosomes since the degradation into $G_{M2}$ and $G_{M3}$ is known to be catalyzed by the lysosomal enzymes β-galactosidase and β-N-acetylglucosaminidase (E.C.3.2.1.52) respectively (Sonderfeld et al. 1985). As is shown in Fig. 1 after 48 h incubation in non-labeled medium ("chase") much of $G_{M1}$ is metabolized and in all subsequent analyses of mutant fibroblasts a period of 16 h labeling followed by 48 h chase was used.

The results in Fig. 2 indicate that cells from the patient with infantile $G_{M1}$-gangliosidosis do not metabolize any of the ingested ganglioside $G_{M1}$; the same was true after longer periods of labeling (data not shown). Adult $G_{M1}$-gangliosidosis fibroblasts, however, do show some metabolism of $G_{M1}$ into $G_{M2}$ and $G_{M3}$ (compare Fig. 2a, c). It is known that in adult $G_{M1}$-gangliosidosis 5–10% of the normal amount of β-galactosidase molecules is present in the lysosomes and that these molecules

**Table 1.** Enzyme activities in fibroblast homogenates

| | β-Galactosidase[a] | | Neuraminidase[a] |
|---|---|---|---|
| | 4-MU-β-GAL | [$^3$H]-$G_{M1}$ | 4-MU-NANA |
| Controls Range: | 450–1250 | 60–120 | 40–150 |
| | ($N = 20$) | ($N = 3$) | ($N = 20$) |
| Mean: | 850 | 90 | 95 |
| Infantile $G_{M1}$-gangliosidosis | 2.0 | 0.02 | |
| Adult $G_{M1}$-gangliosidosis | 30.0 | 10.0 | |
| Morquio disease Type B | 35.0 | 2.5 | |
| Adult galactosialidosis | 79.0 | 9.1 | |
| Infantile galactosialidosis | 70.0 | 17.8 | 0.5 |
| Infantile galactosialidosis + leupeptin | 270 | | 0.7 |
| + protective protein precursor | 300 | | 20 |

[a] Specific activities expressed in nmol/h/mg protein

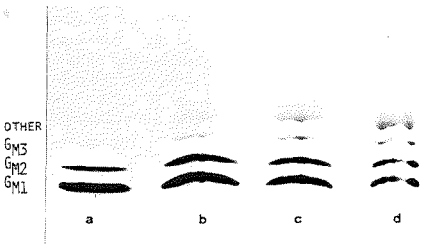



**Fig. 1.** Loading of living fibroblasts with [$^3$H-ceramide] $G_{M1}$. Control fibroblasts: 16 h labeling (*a*) followed by 8 h (*b*), 24 h (*c*), and 48 h (*d*) of "chase". For experimental conditions see text

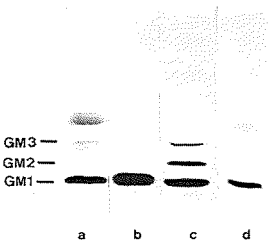



**Fig. 2.** Loading of living fibroblasts with [$^3$H-ceramide] $G_{M1}$. *Lane a:* control fibroblasts; *lane b:* infantile $G_{M1}$-gangliosidosis; *lane c:* adult $G_{M1}$-gangliosidosis; *lane d:* Morquio disease type B

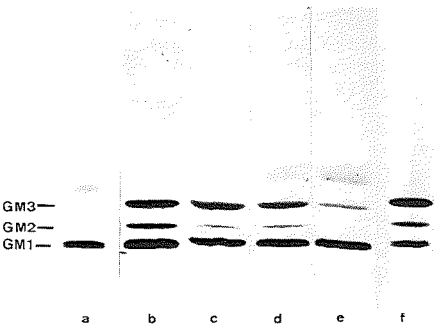



**Fig. 3.** Loading of living fibroblasts with [$^3$H-ceramide] $G_{M1}$. *Lane a:* control fibroblasts; *lane b:* galactosialidosis, adult form; *lanes c–e:* galactosialidosis, infantile form; *c:* without treatment; *d:* after addition of leupeptin; *e:* after correction of the 32 kilodalton protective protein deficiency; *lane f:* mucolipidosis I

have normal properties (van Diggelen et al. 1981; Hoogeveen et al. 1986). The early onset of neurologic symptoms in infantile $G_{M1}$-gangliosidosis and the late onset in adult $G_{M1}$-gangliosidosis seem to be related to the difference in the number of β-galactosidase molecules available in the lysosome.

Morquio B fibroblasts exhibit a similar catabolism of $G_{M1}$ as control cells (Fig. 2d). Here, the lysosomes are known to contain β-galactosidase molecules, which have a decreased affinity for their substrates. In test tube assays using ganglioside $G_{M1}$ as a substrate a residual activity of 20–50% is found when physiologic amounts of activator protein are present (Paschke and Kresse 1982).

In both variants of galactosialidosis (Fig. 3), ingested $G_{M1}$ is degraded to $G_{M2}$ and $G_{M3}$, but unexpectedly, this latter ganglioside is not further metabolized. The catabolism of $G_{M1}$ to $G_{M3}$ must be due to the 10–20% of monomeric β-galactosidase known to be left in the lysosomes (Hoogeveen et al. 1983). Administration of leupeptin, which inhibits intralysosomal proteolysis results in an increase of monomeric β-galactosidase and this accounts for the higher enzyme activity in cell homogenate (Table 1). Under in vivo conditions, however, leupeptin does not seem to affect the catabolism of ingested $G_{M1}$ (Fig. 3d). The high molecular weight aggregates, predominantly present in control cells, seem therefore to be not essential for the hydrolysis of $G_{M1}$. This implies that the 10–20% monomeric β-galactosidase is sufficient for the $G_{M1}$ catabolism under the experimental conditions. In normal cells, however, β-galactosidase is predominantly present as a high molecular weight aggregate which normally must be responsible for the catabolism of $G_{M1}$ (Fig. 1).

When the basic genetic defect in galactosialidosis is corrected by administrating a preparation containing the 54 kilodalton precursor of the 32 kilodalton protective protein, the in vivo metabolism of $G_{M1}$ almost normalizes (Fig. 3e). This might be related to the restoration of high molecular weight β-galactosidase complexes (Hoogeveen et al. 1983) which in turn are essential for the expression of lysosomal neuraminidase activity (Table 1) (Verheyen et al. 1985). The latter is probably responsible for the normalization of $G_{M3}$ catabolism. Further support for a role of this neuraminidase in lysosomal ganglioside degradation was obtained by the observation that mucolipidosis I fibroblasts, which have an isolated lysosomal neuraminidase deficiency, also show a clear degradation of ingested $G_{M1}$ to $G_{M2}$ and $G_{M3}$ and an accumulation of the latter (Fig. 3f).

This finding is surprising because previous studies on cell homogenates has shown that sialic acid is released from different types of gangliosides, including $G_{M3}$, by both mucolipidosis I cells (Cantz and Messer 1979) and galactosialidosis fibroblasts (Wenger et al. 1978; Miyatake et al. 1979). The latter report mentions, however, a marked accumulation of gangliosides in sympathetic ganglia of the patient. This might either be compatible with the hypothesis of an in vivo inhibition of neuraminidase by storage products (protein-bound sialic acid) or our experiments indicate that lysosomal neuraminidase plays a role in ganglioside degradation in living fibroblasts.

## References

Andria G, Strisciuglio P, Pontarelli G, Sly WS, Dodson WE (1981) Infantile neuraminidase and β-galactosidase deficiencies (galactosialidosis) with mild clinical courses. In: Durand P, et al. (eds) Perspectives in inherited metabolic diseases: Sialidases and sialidoses, vol 4. Edi Ermes srl, Milano, pp 379–395

Cantz M, Messer H (1979) Oligosaccharide and ganglioside neuraminidase activities of mucolipidosis I (sialidosis) and mucolipidosis II (I-cell disease) fibroblasts. Eur J Biochem 97:113–118

Conzelmann E, Sandhoff K (1983/84) Partial enzyme deficiencies: residual activities and the development of neurological disorders. Dev Neurosci 6:58–71

D'Azzo A, Hoogeveen AT, Reuser AJJ, Robinson D, Galjaard H (1982) Molecular defect in combined β-galactosidase and neuraminidase deficiency in man. Proc Natl Acad Sci USA 79:4535–4539

Galjaard H (1980) Genetic metabolic diseases: early diagnosis and prenatal analysis. Elsevier/North-Holland, Amsterdam, pp 817–825

Galjaard H, d'Azzo A, Hoogeveen AT, Verheijen FW (1984) Combined β-galactosidase-sialidase deficiency in man: genetic defect of a "protective protein". In: Barranger JA, Brady RO (eds) Molecular basis of lysosomal storage disorders. Academic Press, New York London, pp 113:131

Groebe H, Krins M, Schmidberger H, von Figura K, Harzer K, Kresse H, Paschke E, Sewell A, Ullrich K (1980) Morquio syndrome (mucopolysaccharidosis (IV-B) associated with β-galacto-

sidase deficiency. Report of two cases. Am J Hum Genet 32:258–272

Hoogeveen AT, Verheijen FW, d'Azzo A, Galjaard H (1980) Genetic heterogeneity in human neuraminidase deficiency. Nature 285: 500–502

Hoogeveen A, d'Azzo A, Brossmer R, Galjaard H (1981) Correction of combined β-galactosidase/neuraminidase deficiency in human fibroblasts. Biochem Biophys Res Commun 103:292–300

Hoogeveen AT, Verheijen FW, Galjaard H (1983) The relation between human lysosomal β-galactosidase and its protective protein. J Biol Chem 258:12143–12146

Hoogeveen AT, Reuser AJJ, Kroos M, Galjaard H (1986) $G_{M1}$-Gangliosidosis: defective recognition site on beta-galactosidase precursor. J Biol Chem (in press)

Miyatake T, Yamada T, Suzuki M, Pallman B, Sandhoff K, Ariga T, Atsumi T (1979) Sialidase deficiency in adult-type neuronal storage disease. FEBS Lett 97:257–259

O'Brien JS (1983) The gangliosidoses. In: Stanbury JB, Wijngaarden JB, Frederickson DS, Goldstein JL, Brown MS (eds) The metabolic basis of inherited disease, 5th edn. McGraw-Hill, New York pp 945–972

O'Brien JS, Gugler E, Giedion A, Wiesmann U, Hershkowitz N, Meier C, Leroy J (1976) Spondyloepiphyseal dysplasia, corneal clouding normal intelligence and acid-β-galactosidase deficiency. Clin Genet 9:495–504

Okada S, O'Brien JS (1968) Generalized gangliosidosis β-galactosidase deficiency. Science 160:1002–1004

Paschke E, Kresse H (1982) Morquio disease, type B: activation of $G_{M1}$-β-galactosidase by $G_{m1}$-activator protein. Biochem Biophys Res Commun 109:568–575

Scheel G, Acevedo E, Conzelmann E, Nehrkorn H, Sandhoff K (1982) Model for the interaction of membrane-bound substrate and enzymes, hydrolysis of ganglioside $G_{M1}$ by sialidase of neuronal membranes isolated from calf brain. Eur J Biochem 127: 245–253

Schwarzmann G (1978) A simple and novel method for tritium labeling of gangliosides and other sphingolipids. Biochim Biophys Acta 529:106–114

Sonderfeld S, Conzelmann E, Swarzmann G, Burg J, Hinrichs U, Sandhoff K (1985) Incorporation and metabolism of ganglioside $G_{M2}$ in skin fibroblasts from normal and $G_{M2}$ gangliosidosis subjects. Eur J Biochem 149:247–255

Svennerholm L (1963) Chromatographic separation of human brain gangliosides. J Neurochem 10:613–623

Svennerholm L, Hakansson G, Månsson JE, Vanier MT (1979) The assay of sphingolipid hydrolases in white blood cells with labeled natural substrates. Clin Chim Acta 92:53–64

Van der Horst GTJ, Kleijer WJ, Hoogeveen AT, Huijsmans JGM, Blom W, Van Diggelen OP (1983) Morquio B syndrome: a primary defect in β-galactosidase. Am J Med Genet 16:261–275

Van Diggelen OP, Schram AW, Sinnott ML, Smith PJ, Robinson D, Galjaard H (1981) Turnover of β-galactosidase in fibroblasts from patients with genetically different types of β-galactosidase deficiency. Biochem J 200:143–151

Van Gemund JJ, Giesberts MAH, Eerdmans RF, Blom W, Kleijer WJ (1983) Morquio B disease, spondyloepiphyseal dysplasia associated with acid β-galactosidase deficiency. Report of three cases in one family. Hum Genet 64:50–54

Venerando B, Fiorilli A, Masserini M, Giuliani A, Tettamanti G (1985) Interactions of pig brain cytosolic sialidase with gangliosides. Formation of catalytically inactive enzyme-ganglioside complexes. Biochim Biophys Acta 833:82–92

Verheijen FW, Brossmer R, Galjaard H (1982) Purification of acid β-galactosidase and acid neuraminidase from bovine testis: evidence for an enzyme complex. Biochem Biophys Res Commun 108: 868–875

Verheijen FW, Palmeri S, Hoogeveen AT, Galjaard H (1985) Human placental neuraminidase. Activation, stabilization and association with β-galactosidase and its "protective" protein. Eur J Biochem 149:315–321

Wenger DA, Tarby TJ, Wharton C (1978) Macular cherry-red spots and myoclonus with dementia: coexistent neuraminidase and β-galactosidase deficiencies. Biochem Biophys Res Commun 82: 589–696

Received December 19, 1985 / Revised February 12, 1986