LYSOSOMAL NEURAMINIDASE IN HUMAN GENETIC DISEASES

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Aan mijn ouders

CONTENTS

Publication I : Hoogeveen, A.T., Verheijen, F.W., d´Azzo, A. and Galjaard, H. (1980). Genetic heterogeneity in human neuraminidase deficiency. Nature 285, 500-502.

Publication II : 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.

Publication III: 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.

Publication IV : Verheijen, F.W., Palmeri, S., Hoogeveen, A.T. and Galjaard, H. (1985). Human placental neuraminidase. Activation, stabilization and association with ß-galactosidase and its ´protective´ protein. Eur. J. Biochem. 149, 315-321.

Publication V : Verheijen, F.W., Palmeri, S. and Galjaard, H. (1986) Purification and partial characterization of lysosomal neuraminidase from human placenta. Submitted for publication.

Publication VI : Verheijen, F.W., Janse, H.C., van Diggelen, O.P., Bakker, H.D., Loonen, M.C.B., Durand, P. and Galjaard, H. (1983). Two genetically different MU-NANA neuraminidases in human leucocytes. Biochem. Biophys. Res. Commun. 117, 470-478.

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CHAPTER I.
GENERAL INTRODUCTION

I.1. LYSOSOMES

Like many other important discoveries the lysosome was discovered by serendipity. Cell fractionation studies on rat liver by the group of de Duve led to the lysosomal concept as we know it nowadays. During the characterization of glucose 6-phosphatase, acid phosphatase activity was observed in the mitochondrial fraction of rat liver (Berthet and de Duve 1951), and this latter enzyme could be activated by a number of treatments such as hypotonic media, freezing, mechanical homogenization and detergents. This incidental finding is later commented by de Duve as "Our interest in this uninspiring enzyme was largely negative, extending only sofar as it provided a suitable contrast for glucose 6-phospatase" (de Duve 1969). Since latency of acid phosphatase was found to be associated with sedimentability, it was concluded that acid phosphatase was particle bound. Initially, it was thought to be related to mitochondria, but when better separation methods were developed, the association of acid phosphatase with a special class of cytoplasmic granules was established (Appelmans et al. 1955).
Simultaneously, similar observations were made by Walker (1952) for β-glucuronidase. De Duve and coworkers extended their observations to a total of five enzymes, all resembling acid phosphatase in distribution pattern and in their hydrolytic action on different substrates at low pH. Since all these enzymes were thought to play a role in intracellular digestion, the term "lysosome" (lytic particle) was introduced for the class of organelles containing these enzymes (de Duve et al. 1955; for reviews see de Duve 1969, 1983).

Lysosomes were visualized for the first time under the electronmicroscope as morphologically heterogeneous vesicles

surrounded by a membrane by Novikoff and coworkers (for review see Novikoff 1973). These data resulted in a better understanding of their role (for reviews see Holtzman 1976, Dingle et al. 1984, Barranger and Brady 1984). It is now well established that lysosomes are a constant feature of almost all eukaryotic cells and have a major function in intracellular digestion (chemically: hydrolysis) of substrates derived either from the extracellular environment by endocytosis, or from the cell's own constituents and organelles through cellular autophagy. By controlling these digestive processes lysosomes are important for metabolic regulation, nutrition, cell defence and developmental modulation (e.g. insect metamorphosis).

Lysosomal digestion is dependent upon substrates reaching the lysosomal compartment. For exogenous substrates this occurs via endocytosis (for review see Pastan and Willingham 1985). Three different types of endocytosis are distinguished:

1) Pinocytosis ("cell drinking"), the uptake of large bubbles of extracellulair medium;

2) Phagocytosis ("cell eating"), engulfment of large particles;

3) Receptor-mediated endocytosis, uptake of molecules via a specific recognition site.

The type of endocytosis is dependent on the cell type and extracellular environment. The complexity of the various pathways is illustrated in Fig. 1.

ad 1) Pinocytosis

(See left part of Fig. 1, arrows marked with 1).

Substrates taken up by pinocytosis are enclosed in pinosomes, which fuse with vesicles budding off from a specialized

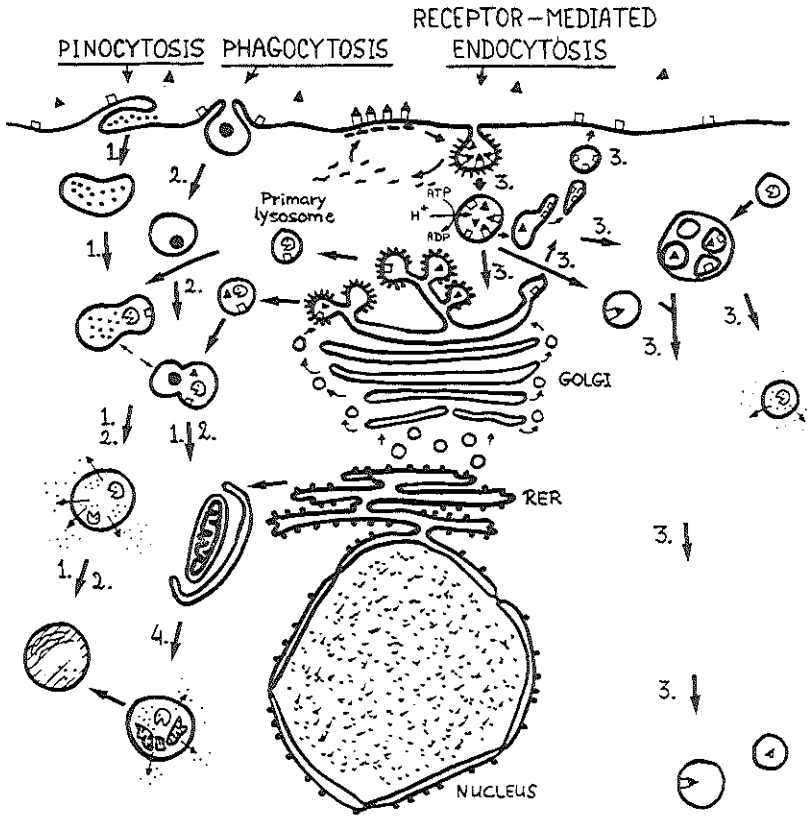

Fig. I.1. Schematic diagram of a human cell showing the three major pathways by which substrates enter the lysosomes. The figure also shows how the different cell organelles keep contact with each other. Numbered arrows refer to passages in the text. □ Receptors; ▲ Ligands; ◑ Lysosomal enzymes.

region of the golgi apparatus (TGR, Trans Golgi Reticulum). These vesicles contain hydrolytic enzymes and are called primary lysosomes. After fusion, which may involve many different vesicles, secondary lysosomes are formed and the breakdown of substrate can start. After hydrolysis, the resulting small molecules can leave the lysosome and may be reutilized in metabolic processes. The enzymatic process of hydrolysis will be discussed in more detail in the next chapter on lysosomal enzymes. Some undegradable substances accumulate in residual bodies.

ad 2) Phagocytosis

(See left part of Fig. 1, arrows marked with 2).

Substrates taken up by phagocytosis are enclosed in phagosomes. Phagocytosis is restricted to specialized cells such as macrophages. Larger particles (0.5-1µm) are internalized in this way. Phagosomes fuse with primary lysosomes and degradation can start.

ad 3) Receptor-mediated endocytosis

(See right part of Fig. 1, arrows marked with 3).

A wide variety of substrates has been shown to enter the cell via receptor-mediated endocytosis. Rapid developments have been made in this field (for reviews see Goldstein et al. 1979, Brown et al. 1983, Pastan and Willingham 1983, Wileman et al. 1985 and Stahl and Schwartz 1986). At least seven classes of ligand receptor systems have been recognized, functioning for e.g. nutrition, defense, transport and processing. Among the nutritional ligand-receptor systems the best known is the LDL system. The most important system for posttranslational modification of glycoproteins is the mannose 6-phosphate receptor system. This system which is essential for the processing of lysosomal enzymes, will be discussed in the next chapter.

Molecules (ligands) bind to specific cell surface receptors which cluster to a certain region of the plasma membrane. Cage-like structures are formed by specific protein complexes, the most important component of which is clathrin. Subsequently invaginations of the plasma membrane appear containing the clustered receptors:"coated pits". The clathrin coat is removed by an "uncoating" ATPase and endosomes (receptosomes) are formed. Acidification occurs by an endosomal proton pump and some receptors release their ligands. There are several different pathways depending on the function of the specific receptor-ligand system. Receptors may be recycled to the plasma membrane and the ligand is either targeted to the lysosome for breakdown, or to the opposite plasma membrane (transcytosis) in certain specialized cells (e.g. transport of nutrients by epithelial cells) (Mostov and Simister 1985). Some receptors even cotransfer together with their unreleased ligands. Other receptors are not recycled and are degraded in lysosomes after the release of their ligands. Morphologically this part of the route from "coated pit" via endosome to lysosome is still uncertain.

Several hypotheses all based on electronmicroscopical observations have been proposed. Endosomes are believed to be formed either from "coated pits" that pinch off and subsequently lose their "coat" within several minutes, or from invaginations from the "necks", that are narrow tubuli that still connect the "coated" vesicle with the plasma membrane. After endosome formation the "coated pit" is left on the plasma membrane. Endosomes may form extensions, in which recycling receptors are clustering and small vesicles pinch off and move back to the plasma membrane. Endosomes may also fuse with other endosomes to form big multivesicular endosomes. These may fuse with lysosomes to initiate the breakdown process. Another possibility is that endosomes fuse with the Trans Golgi Reticulum (TGR), to deliver ligand and/or receptor finally to primary lysosomes, which bud off probably by a mechanism similar to that for "coated pits" formed on the

plasma membrane. The intracellular release of ligand from their receptors is believed to occur in an acidifying compartment: the compartment for uncoupling of receptor and ligand (CURL) (Geuze et al. 1983). This CURL may be the same as the extension forming endosomes (Willingham and Pastan 1985), or may be even similar as the TGR. The morphological appearance seems to be dependent on the cell type studied. Details of the sorting mechanisms required for the recycling of membrane and receptors, are largely unknown necessitating future research in this area.

Autophagy is another way for substrates to reach lysosomes is (Fig.1, arrow marked with 4). In this process membranes, probably of the endoplasmic reticulum, form vacuoles (autophagosomes) by engulfing the cell constituents destined to be degraded. For this purpose autophagosomes fuse with lysosomes.

It is clear that lysosomes have a key function in cell processes requiring degradation. It is however also evident, that this function is performed by a highly sophisticated vesicle transport system, resembling the blood plasma as the extracellular transport system.

So far we have discussed by which routes substrates can enter the lysosome. In the next chapter we will focus on the special equipment of the lysosome, the lysosomal enzymes, and how they reach the lysosomal compartment after their synthesis.

I.2. LYSOSOMAL ENZYMES

Since digestion is the main function of lysosomes, it is not surprising that these organelles contain a large number of digestive enzymes: the lysosomal hydrolases. In fact, the presence of five of these enzymes led to the definition of the lysosome as discussed in chapter I.1. Digestion does not occur exclusively in the lysosomes, but in a few instances also outside the cell through lysosomal enzymes secreted by exocytosis (e.g. resorption of bone by osteoclasts) (Hers 1973, Vaes 1973, Lerner 1980). All lysosomal hydrolases have a low pH optimum and all have a specific function in the breakdown of complex biological substances into low molecular weight constituents which diffuse through the lysosomal membrane into the cytoplasm, where they may be further metabolized. For instance proteins are degraded into their aminoacids, carbohydrates into monosaccharides, phospholipids into fatty acids and glycerol or glycerophosphodiesters, sphingolipids into sphingosine, fatty acids, inorganic phosphate, free monosaccharides and choline, and nucleic acids into nucleosides and inorganic phosphate.

Lysosomal enzymes are not only involved in the breakdown of foreign substances taken up by endocytosis, but also in the degradation of the cell's own components by autophagy.

It is important point to consider that lysosomal enzymes themselves are (glyco) proteins. Immunocytochemical studies at the ultrastructural level have shown that in fibroblasts every lysosome contains all the different lysosomal hydrolases (van Dongen et al. 1984). A mechanism must therefore exist which protects the lysosomal enzymes against degradation by neighbouring hydrolases. The same applies to the lysosomal membrane, which like all biomembranes consists of proteins and phospolipids.

About seventy lysosomal hydrolases are known and they can be classified according to their digestive properties as fol-

lows: carboxyl/thiolester hydrolases, phosphatases, sulphuric esterases, glycosidases, peptidases and amidases. Of these groups the glycosidases represent the majority (Barrett and Heath 1977).

In general lysosomal hydrolases are specific for only one type of chemical linkage ("linkage specificity"), independent of the structure in which this linkage is present. Most are exoenzymes and different enzymes are therefore needed in a sequential action to degrade a certain substrate. However, a few lysosomal endoenzymes exist, such as cathepsins B and D. Some enzymes involved in the degradation of certain lipophilic substrates need so called "activator proteins" for a proper functioning. These activator proteins have a detergent-like function by serving the interaction between water-soluble hydrolases and their membrane-bound glycolipid substrates (for reviews see Sandhoff and Conzelmann 1979, Li and Li 1984). For the lysosomal enzymes β-galactosidase and arylsulfatase the existence of gene products (protective proteins), which directly or indirectly stabilize these enzymes has been demonstrated (D´Azzo et al. 1982, Waheed et al. 1982).

Lysosomal enzymes are glycoproteins that are synthesized like other proteins on ribosomes. Like secretory proteins, lysosomal enzymes are synthesized on ribosomes bound to the endoplasmic reticulum. For a long time it remained unexplained how the lysosomal enzymes find their way into the lysosome. In recent years the work of the groups of Neufeld, Blobel, Sly, Kornfeld, Von Figura and others has contributed significantly to the elucidation of this question, although some aspects of this mechanism are still unclear (for reviews see Neufeld 1981, Hasilik and Von Figura 1984, Goldberg et al. 1984, Creek and Sly 1984, Skudlarek et al. 1984, Erickson et al. 1984, Kornfeld 1986). During synthesis the precursor forms of lysosomal enzymes are translocated across the rough endoplasmic reticulum. Here, a proteolytic cleavage step is required (removing of "signal" sequence) together with glycosylation of asparagine residues (enbloc transfer of a large preformed

oligosaccharide, 3 glucose, 9 mannose, 2 N-acetylglucosamine residues, from a lipid-linked intermediate to the nascent polypeptide). The carbohydrate part is then trimmed by sequential removal of glucose and mannose residues, followed by formation of mannose 6-phosphate residues of a complex or hybrid structure during transport to and through the Golgi stacks. Then secretion or segregation from secretory products occurs by a specific receptor-ligand system as indicated in the first chapter. A crucial role is played by the mannose 6-phosphate residues present on the carbohydrate part of the lysosomal enzyme precursors, which function as ligand. These ligands are recognized by specific receptor proteins, mannose 6-phosphate receptors (MPR), that are present on the internal side of the Golgi stacks.

The receptor-bound enzymes are now clustered in vesicles ("coated pits"), that bud off from the Trans Golgi Reticulum (TGR). These vesicles ("pre-lysosomes") acidify by the working of a proton pump, resulting in release by MPR of their ligands (lysosomal enzyme precursors). The receptors then recycle back to the Golgi apparatus and may be reutilized. The released lysosomal enzyme precursors are now packed into primary lysosomes. At present it is unclear how these last steps are performed, but several mechanisms have been proposed (Geuze et al. 1985). The most likely hypothesis is that the TGR is the same as the so called CURL (Compartment for Uncoupling Receptor and Ligand), and primary lysosomes (containing lysosomal enzymes but no MPR !) are formed after acidification of coated vesicles. Within the lysosome the last posttranslational processing steps occur. These final proteolytic maturation steps may involve activation, as is the case for the lysosomal enzyme cathepsin D and more in general for several zymogens like trypsin and chymotrypsin (for review see Neurath and Walsh 1976). Another possibility is that the last proteolytic steps may involve changes which act as signals to allow conformational changes, which may be necessary for stabilization or protection against neighbouring hydrolases. The precise

mechanisms involved in proteolytic cleavage and the relationship between various lysosomal enzymes, other proteins and the lysosomal membrane are yet to be resolved.

Another possibility is that the last proteolytic processing step actually is the removal of a peptide which has been essential for lysosomal enzyme targeting independent of the MPR-ligand system. That such a MPR independent pathway exists is evident. For instance the membrane-bound lysosomal enzyme ß-glucocerebrosidase is transported into lysosomes by a mechanism independent of the MPR system, because no phosphorylation of oligosaccharide moieties occurs (for review see Tager 1985). If other membrane-bound lysosomal enzymes follow this MPR independent pathway, is unknown at present. Evidence for an alternative routing independent of MPR comes from studies of cultured cells from patients with the lysosomal storage disorder I-cell disease (for review see d´Azzo 1982). These cells are deficient in the Golgi associated enzyme N-acetylglucosaminylphosphotransferase, which normally transfers N-acetylglucosamine 1-phosphate to the 6-hydroxyl of mannose residues present in high mannose-type oligosaccharides of lysosomal enzymes (Hasilik et al. 1981, Reitman et al. 1981, Varki et al. 1982). Because of the lack of phosphomannosyl recognition markers on the enzyme protein, some cells (like fibroblasts) from I-cell disease patients, secrete most of their lysosomal enzymes extracellularly. Other cell types (e.g. hepatocytes and leucocytes), however, have a normal transport and are not affected, although the phosphotransferase activity is also deficient in these cells. Apparently the transport of lysosomal enzymes follows in these cells a mannose 6-phosphate independent mechanism.

Recently some cell types (endothelial cells and some murine tissue culture lines) were found to have no MPR protein, but despite this, these cells show normal levels of intralysosomal enzymes. However, these enzymes do not follow an MPR independent routing, but instead these cells contain a different MPR protein. This newly discovered MPR shares most

of its physiological properties, as ligand-binding and release, with the original described MPR; this new MPR can be discriminated by its cation-dependency (Hoflack and Kornfeld 1985a and 1985b).

Another interesting point is the fact that a small fraction (5-20%) of the precursors of soluble lysosomal enzymes synthesized in fibroblasts is secreted, instead of being transferred directly to the lysosomes (for review see Hasilik and Von Figura 1984). These secreted molecules may either be "lost" in the extracellular environment or they may be internalized again by receptor-mediated endocytosis, via MPR present on the plasma membrane. Such a routing may represent a route reminiscent of older evolutionary organisms, or it represents a route to deliver lysosomal enzymes to the extracellular environment, where they can exert an active function (e.g. exocytosis of lysosomal enzymes by osteoclasts during bone resorption), although most lysosomal enzyme precursor forms may not be very active in extralysosomal compartments.

For the unraveling of the complex process whereby newly synthesized lysosomal enzymes are segregated from secretory proteins and packaged into lysosomes, studies of patients with defects in this sorting pathway have been very important. Studies on cultured (mutant) cells derived from patients with lysosomal storage disorders have been crucial in the development of concepts in this area.

I.3. LYSOSOMAL STORAGE DISORDERS

Lysosomal storage disorders are relatively rare inborn (enzymatic) defects resulting from an inability to degrade or release certain metabolic products. The accumulation of non-degraded products into the lysosomes is thought to interfere with the cell´s functioning, and ultimately can lead to cell death. Accumulated material may originate from extracellular products internalized by endocytosis or from the cell´s own constituents by crinophagy or autophagy (see chapter I.1). Most of the lysosomal storage disorders show an autosomal recessive mode of inheritance, and a few are X- linked (Fabry´s disease and Hunter´s disease). Since lysosomes occur in nearly all cell types pathological and clinical features are quite generalized. There is however considerable heterogeneity, both among different lysosomal storage disorders and among different variants within one disease. This heterogeneity is related to different genetic and molecular aspects of the enzyme defects involved, and to the rate of storage under in vivo conditions (for reviews see Hers and van Hoof 1973, Neufeld et al. 1975, Galjaard 1980, Callahan and Lowden 1981, Stanbury et al. 1983, Conzelmann and Sandhoff 1983-1984, Galjaard and Reuser 1984).

The concept of lysosomal storage disorders was first defined by Hers (1965), in Glycogenosis type II (Pompe´s disease), due to a genetic deficiency of lysosomal acid α-glucosidase. During the next twenty years about 30 lysosomal storage disorders were recognized to be due to a certain lysosomal malfunctioning (for reviews see Galjaard 1980, Galjaard and Reuser 1984, in: Stanbury et al. 1983) (see Table I.1). The incidence of individual diseases varies from about 1:25000 (for all mucopolysaccharidoses together) to less than 1:100000 for others. For some lysosomal storage diseases a high incidence has been observed in certain ethnic groups. A

TABLE I.1

Lysosomal storage diseases and their molecular defects

Adapted from H. Galjaard, A.J.J. Reuser 1984

Disease	Defect
Glycogenosis II (Pompe disease)	acid α- glucosidase
Mucopolysaccharidosis	
IH (Hurler disease)	α-L-iduronidase
IS (Scheie syndrome)	α-L-iduronidase
II (Hunter disease)	sulfoiduronide sulfatase
III A (Sanfilippo disease)	heparan sulfate sulfamino hydrolase
III B (Sanfilippo disease)	α-N-acetyl-D-glucosaminidase
III C (Sanfilippo disease)	acetyl-CoA:α-glucosaminide-N-acetyltransferase
III D (Sanfilippo disease)	N-acetylglucosamine-6-sulfate sulfatase
IV A (Morquio A syndrome)	6-sulfo-N-acetylgalactosaminide sulfatase
IV B (Morquio B syndrome)	β-galactosidase
VI (Maroteaux-Lamy syndrome)	4-sulfo-N-acetylgalactosaminide sulfatase (arylsulfatase B)
VII (Sly disease)	β-glucuronidase
Sialidosis (previous mucolipidosis I)	α-sialidase (neuraminidase)
I-cell disease (mucolipidosis II)	UDP-N-acetylglucosamine-1-phosphotransferase
Mucolipidosis III	UDP-N-acetylglucosamine-1-phosphotransferase
Mucolipidosis IV	ganglioside neuraminidase
Galactosialidosis	β-galactosidase protective protein
Fucosidosis	α-fucosidase
Mannosidosis	acid α-mannosidase
Farber lipogranulomatosis	ceramidase
Sphingomyelin lipidosis (Niemann-Pick disease)	sphingomyelinase
Glucosylceramide lipidosis (Gaucher disease)	β-glucocerebrosidase
Galactosylceramide lipidosis (Krabbe disease)	β-galactocerebrosidase
Metachromatic leucodystrophy	arylsulfatase A
Mucosulfatidosis	multiple sulfatase deficiency
Ceramide trihexoside lipidosis (Fabry disease)	ceramide trihexoside α-galactosidase
GM1-gangliosidosis	GM1-ganglioside β-galactosidase
GM2-gangliosidosis type Tay-Sachs	β-N-acetyl hexosaminidase A
GM2-gangliosidosis type Sandhoff	β-N-acetyl hexosaminidase B
Wolman disease	acid lipase
Cystinosis	lysosomal membrane transport defect
Free N-acetylneuraminic acid storage disease	lysosomal membrane transport defect

* All diseases are inherited as autosomal-recessive traits except Fabry and Hunter disease, which are X-linked.

well known example is the high incidence of G_{M2}-gangliosidosis type Tay-Sachs among Ashkenazi jews, where 1 in 30 people is a carrier (Kaback 1977 and 1981, Galjaard and Reuser 1984).

A characteristic morphological feature in all these diseases is the abundancy of swollen lysosomes. In some instances electronmicroscopical studies reveal the presence of a quite characteristic morphology of the storage product, but in most instances one may only observe abnormal intralysosomal storage as an important diagnostic criterion. Because of the increasing vacuolization, ultimately cell function is thought to be disturbed, resulting in tissue- and organ failure. Depending on the type of lysosomal storage disease and on the clinical variant within a certain disorder the pathological and clinical features have a progressively fatal, or a later onset milder course (for reviews see Galjaard 1980, in: Stanbury et al. 1983).

Historically the discovery and classification of lysosomal storage diseases were based on a characteristic combination of clinical and pathological abnormalities and the presence of more affected relatives, sometimes in different generations in larger families. The earlier studies were focussed on the demonstration of a storage product and its chemical characterization. The accumulated material is often of a heterogeneous nature, and varies considerably in type and quantity depending on the disorder and the cell types investigated. In some instances, like in various types of mucopolysaccharidoses, the chemical analysis of storage products in the urine has contributed to the diagnosis and to the elucidation of the different genetic enzyme deficiencies involved (for review see McKusick and Neufeld 1983).

The expression of lysosomal enzyme activity and of the deficiency in patients in a variety of cell types including cultured fibroblasts has greatly facilitated the early diagnosis of patients and carriers. It also enabled biochemical and genetic research of normal and abnormal cell biology. The (enzyme) defects responsible for the lysosomal accumulation of

certain products in more than 30 genetic diseases could be elucidated (Table I.1).

The development of radioactive and fluorescent substrates and the simplification of analytical methods has offered the possibility of a rapid and early diagnosis using enzyme assays of total leucocytes, tissue biopsies or cultured skin fibroblasts (Galjaard 1980). Transport of tissues and cell material from patients and carriers all over the world allows biochemical diagnosis in specialized centres. The possibility of storing cultured fibroblasts in liquid nitrogen enables the use of these cells for diagnostic and research purposes also after death of the patient. This is especially important for genetic counseling and prenatal diagnosis in couples at increased genetic risk.

Various follow-up studies have shown that a high proportion of couples, who were timely informed about their high risk of affected offspring (25% in Mendelian disorders) will refrain from pregnancy. For those couples who still want children the option of prenatal diagnosis and termination at an early stage of pregnancy, in case the fetus is found to be affected, is an important alternative (for reviews see Milunsky 1979, Galjaard 1980).

During the last 18 years prenatal diagnoses of lysosomal storage disorders and other genetic metabolic diseases were based on biochemical (micro) analysis of cultured amniotic fluid cells and comparison of the analytical results with those from skin fibroblasts from an affected patient, the heterozygous parents and controls. The sampling of amniotic fluid by transabdominal amniocentesis is usually performed at 16 weeks gestation and followed by 2-4 weeks for cell- cultivation and biochemical analysis (for review see Galjaard 1980).

An important improvement is the recent introduction of chorionic villus sampling at 9-10 weeks of gestation via a thin polythene catheter introduced through the cervical canal under ultrasound control (for reviews see Brambati et al. 1985

and Fraccaro et al. 1985). In 10-20 mg. chorionic tissue, which is of fetal origin, direct biochemical demonstration of lysosomal enzyme deficiencies is possible (Kleijer 1986 and Galjaard 1986). Nearly all lysosomal storage disorders can now be diagnosed within 1-2 days after chorion sampling at 9-10 weeks. If the fetus is shown to be affected, the pregnancy can be terminated by vacuum aspiration, which is psychologically less stressful than an interruption at 18-20 weeks after amniotic fluid cell analysis.

Although the birth of subsequent affected children can be prevented to a certain extent by early diagnosis, carrier detection, genetic counseling and prenatal diagnosis, many patients will still be born. Unfortunately most attempts at treatment of lysosomal storage diseases by administration of purified normal enzyme (for review see Desnick 1980; Tager et al. 1980) or enzyme replacement using transplanted cells or organs (for review see Barranger 1984 and Adinolfi (personal communication, 1986)) have been unsuccessful. In part this is due to problems such as the blood brain barrier and immunological reactions, but also the exact molecular background of each of the (variants of) lysosomal storage diseases must be understood to prevent disappointing effects of administration of exogenous enzyme protein. The considerable clinical, pathological and biochemical heterogeneity among patients with a certain lysosomal enzyme deficiency may well be based on different mutations within the same gene resulting in quite different abnormalities at the molecular and cell biological level. These differences may be relevant for the success or failure of future attempts at treatment.

During the last few years much research has been focussed on the molecular background of the clinical and biochemical heterogeneity. Initially, complementation studies after somatic cell hybridization were used to differentiate between mutations in different genes, and allelic mutations in patients with different variants of "the same" disease (for review see Galjaard and Reuser 1984, Reuser 1984). Another

approach is the analysis of the biochemical and molecular properties of the normal lysosomal enzyme and of the enzyme protein in cells from different types of patients. In some instances certain variants do not show any residual enzyme activity, while cells from other variants have different levels of residual activity. The functional properties of the latter cell types can be studied and may be related to the different clinical phenotypes (Reuser 1984).

Recently methods were developed that enabled the study of the biosynthesis, posttranslational modification and intracellular fate of normal and mutant (lysosomal) proteins irrespective of their hydrolytic activity. The basis for this was immunoprecipitation after labeling of living cells (for review see Hasilik and Von Figura 1984). Normal human enzyme is purified from an easily available tissue, rich in the particular enzyme protein, usually human placenta. Subsequently antibodies are raised in animals, which are then used for immunoprecipitation studies of cultured fibroblasts that have previously been labeled in vivo with (^{3}H)-leucine, (^{32}P) or other labeled components. By compairing cells from normal individuals and patients with different variants of a lysosomal storage disease, after different time intervals of cultivation in non-labeled medium ("pulse chase" studies) immunoprecipitation allows detailed information about the biosynthesis of a specific lysosomal (enzyme) protein. SDS-polyacrylamide gel electrophoresis is used to identify the different forms of enzyme molecules produced during biosynthesis. Such studies have already provided important information about the molecular background in diseases like G_{M1}-gangliosidosis, G_{M2}-gangliosidosis, Glycogenosis type II, Gaucher´s disease, I-cell disease and Galactosialidosis (in: Barranger and Brady 1984, for review see Tager 1985).

The classification of lysosomal storage diseases based upon the nature of storage material and a specific enzyme (protein) deficiency (Table I.1), may now be extended with information on the molecular nature of the enzyme defect. Also

immunocytochemical studies at the electronmicroscope level now starts to provide information about the intracellular routing of normal lysosomal proteins and about the subcellular site where a mutant (enzyme) protein shows abnormal behaviour. Fig.I.2. shows at which level until now mutations have been demonstrated, during the different steps from transcription to the expression of mature active enzyme in the lysosome. The largest group of lysosomal disorders is caused by lysosomal enzyme dysfunctioning and a few diseases are caused by a defective transport of certain metabolites across the lysosomal membrane.

ad 1) Defective enzyme functioning.

These lysosomal enzyme deficiencies can be subdivided on the basis of the nature of the molecular defect as follows:

a) Disorders in which no precursor form of the defective enzyme is synthesized because of lack of the essential mRNA molecules (Fig I.2,1a). An example is classical Tay-Sachs' disease, associated with a ß-hexosaminidase A deficiency (Myerowitz and Proia 1984).

b) Disorders in which precursor enzyme is synthesized but the maturation process is impaired. Abnormal posttranslational modification may be expressed at different levels both biochemically and ultrastructurally (Fig I.2,1b):

For instance at the level of translation (Fig I.2,1b leftside), some variants of G_{M2}-gangliosidosis type Tay-Sachs can be distinguished where the precursor forms of the α-chain polypeptide of ß-hexosaminidase are insoluble, indicating that the mutation may prevent the transport of the precursor out of the endoplasmic reticulum (Proia and Neufeld 1982).

Examples at the posttranslational level (RER->GOLGI, Fig. I.2, 1b middle) are the mutations leading to defective modification of the carbohydrate chain, interfering with phosphorylation in some variants with Pompe's disease (Reuser et

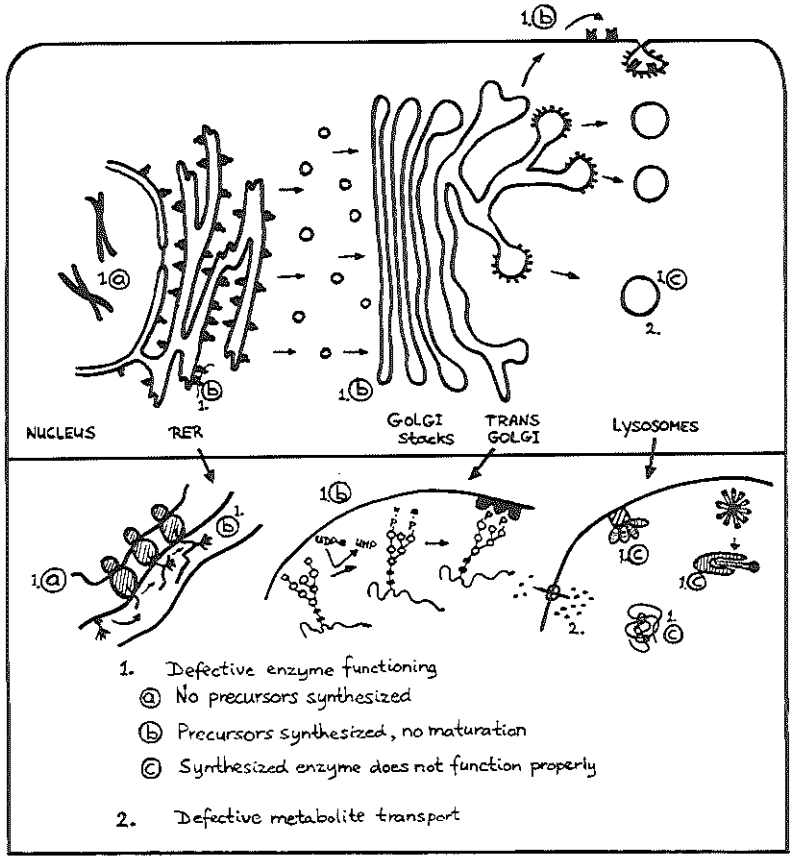

Fig. I.2. Two main classes of defects that lead to a lysosomal storage disorder can be distinguished. The first class can be further subdivided into three groups. The diagram shows schematically the level at which the different mutations interfere with the different steps by which a lysosomal enzyme is synthesized, processed in the Golgi-complex, and finally transported to lysosomes. The numbers refer to the examples mentioned in the text.

al. 1985) and in patients with G_{M1}-gangliosidosis (Hoogeveen et al. 1986). Other examples of defective posttranslational modification may be found at the GOLGI-Trans GOLGI level, where mutations may lead to rapid degradation of precursor molecules, e.g. in some variants with late onset forms of Pompe´s disease (Steckel et al. 1982). Especially interesting at this level are some non-classic forms of Tay-Sachs´ disease, showing a lack of α- and ß-chain association which is normally required for further proteolytic processing of the α-chain precursor (d´Azzo et al. 1984).

Normal precursor synthesis with defective maturation is the hall-mark of "I-cell" disease. (Fig. I.2,1b up). A defective phosphotransferase enzyme leads to non-phosphorylation and consequently secretion of many lysosomal enzymes, resulting in multiple intracellular lysosomal enzyme deficiencies (Hasilik and Neufeld 1980, for review see d´Azzo 1982).

c) Disorders with normal precursor synthesis and maturation but defective enzyme function or enhanced enzyme degradation. This may result from the lack of secondary essential proteins. This group of defects is expressed exclusively at the level of the lysosome (Fig. I.2,1c).

Examples are the deficiency of a protective protein for ß-galactosidase, which normally also serves as a subunit of neuraminidase, in galactosialidosis patients (d´Azzo et al. 1982, Hoogeveen et al. 1983, Verheijen et al. 1985) (Fig. I.2 lower part, 1c upper left), and the hexosaminidase activator protein deficiency in some variants with Sandhoff´s disease (Conzelmann and Sandhoff 1978) (Fig. I.2 lower part, 1c upper right).

Examples of another type of defect can be found in types of Morquio B disease and Gaucher´s disease where the mutation results in a change in secondary or tertiary structure which in turn interferes with full enzymatic activity or may lead to enhanced intralysosomal degradation. (Paschke and Kresse 1982, Ginns et al. 1982, van der Horst et al. 1983) (Fig. I.2 lower

part, lc middle).

ad 2) Defective metabolite transport.

A totally different group of lysosomal storage disorders are the disorders which are due to a defective transport of metabolite across the lysosomal membrane (Fig. I.2, 2).

Examples are cystinosis with a defective cystine transport mechanism (Gahl et al. 1982) and Salla disease and other free N-acetylneuraminic acid (NANA) storage disorders where the transport of NANA out of the lysosome is found to be impaired (Renlund et al. 1986, Mancini et al. 1986[b], Cantz 1986 personal communication).

As yet, the molecular defect has been elucidated of only a minority of the lysosomal enzyme deficiencies. The classification given above is therefore tentative but does provide an impression about the research that is currently in progress in the area of lysosomal storage diseases and other inborn errors of metabolism.

Another important technical approach is cloning of genes coding for the lysosomal (enzyme) proteins (Konings et al. 1984, Myerowitz and Proia 1984, Ginns et al. 1984, O´Brien et al. 1984, Fukushima et al. 1985). From the gene sequence the amino acid sequence can be derived and application of these methods to mutant cells will give insight in the exact nature of the different gene mutations (for review see Brown and Goldstein 1986). Knowledge of the amino acid sequence in normal and mutant cells may also provide information about the tertiary structure of the (mutant) protein. Such information is likely to contribute to a better understanding of the interaction between (enzyme) protein and its substrate(s).

Detailed knowledge on the exact nature of the molecular defect in the different variants of lysosomal storage diseases is also essential for the planning of future treatment. This is clearly demonstrated by model experiments with fibroblasts showing that the β-galactosidase deficiency can be corrected

in some types of mutant cells, whereas in others the exogenous enzyme is rapidly degraded after uptake by the deficient cells (Van Diggelen et al. 1982). Similar problems can be expected in attempts of gene replacement therapy (Williams and Orkin 1986).

The experimental work described in this thesis is focussed on the elucidation of the molecular nature of human lysosomal neuraminidase deficiencies, with special emphasis on Galactosialidosis, which is characterized by a combined deficiency of β-galactosidase and neuraminidase.

In the next chapter the biochemical aspects of neuraminidase will be discussed, which are important for the understanding of the involvement of lysosomal neuraminidase in human genetic disease.

CHAPTER II
BIOCHEMICAL ASPECTS OF NEURAMINIDASE

II.1. PHYSIOLOGICAL FUNCTION

Neuraminidases (EC 3.2.1.18 , sialidase, N-acetylneuraminosyl glycohydrolase) catalyze the hydrolysis of neuraminic acid residues (sialic acids) from a variety of neuraminic acid-containing compounds. They are widely distributed in nature and are found in microorganisms like viruses and bacteria, as well as in mammalian tissues. An exception in nature seems to be the plant kingdom in which no neuraminidases have been found (for reviews see Gottschalk and Bhargave 1971, Gottschalk and Drzeniek 1972, Rosenberg and Schengrund 1976, Corfield et al. 1981). Although different types of neuraminidases have been identified, only a few, mainly from microorganisms, have been studied in detail (for review see Drzeniek 1973). The mammalian neuraminidases have not yet been well characterized, not only because of the complex characteristics of mammalian tissues but also because most mammalian neuraminidases are membrane-bound and extremely labile.

Neuraminidases are likely to fulfil an important role in many different biological processes. They catalyze the hydrolysis of terminal neuraminic acid residues at the non-reducing end of carbohydrate chains from a large number of different substances like oligosaccharides, glycoproteins and glycolipids (gangliosides) in which these substances are involved. Neuraminidase was first discovered in a virus, and was initially described as RDE (receptor destroying enzyme). This RDE was recognized as a glycosidase (Gottschalk 1956), and later as O-glycosidase (Gottschalk 1957) and α-O-glycosidase (Kuhn and Brossmer 1958). As a product of interaction between mucoprotein and RDE, N-acetylneuraminic acid (NANA) could be isolated and the enzyme was termed neuraminidase. Its function was defined as cleaving the α-ketosidic linkage joining the keto groups of terminal NANA to adjacent sugar residues

(Gottschalk 1957).

Besides the receptor destroying activity of viral neuraminidase, neuraminidases may have many other different physiological functions. These functions will become clear after considering the influence of neuraminic acid residues present in different substances (for review see in Schauer 1982 en 1985).

Sialic acids contribute to the negative charge of a molecule, and are important in binding and transport processes with positively charged compounds, like aggregation and disaggregation of cells (for review see Reutter et al. 1982) and for instance synaptic transmission (Rahmann et al. 1976, Svennerholm 1980). They may also influence the conformation of glycoproteins and so fulfil a role in regulating the resistance to proteases. Another property is the formation of antigenic determinants (e.g. blood groups, differentiation antigens). Sialic acids are essential components of receptors and/or ligand molecules and may regulate receptor-mediated endocytosis (see chapter I.1.), as demonstrated by Ashwell and coworkers (Ashwell and Morell 1974, Harford et al. 1984), who found that sialylated serum glycoproteins are protected against breakdown by the presence of sialic acids on the proteins. These sialic acids mask the neighbouring carbohydrate galactose, which upon exposure binds to specific receptors present on the cell surface of rat hepatocytes. By this receptor-mediated endocytosis mechanism asialoglycoproteins enter the cell and may reach the lysosomes in which degradation occurs (see chapter I).

The "masking" of specific recognition sites on molecules and cells is probably the most important role of sialic acids. For instance tumor cells seem to be protected against the immune defense system by the presence of highly sialylated surfaces, and sialylated glycoproteins are protected from breakdown by these sialic acid masks. A similar role of sialic acids can be found in erythrocytes. Removal of sialic acids leads to a rapid disappearance of the red cells from the blood

stream.

Neuraminidases also have a key function in lysosomal degradation of sialic acid containing substances, since other lysosomal hydrolases can only perform their catalytic function after removal of the terminal NANA residues attached to the carbohydrate moieties of glycoproteins or gangliosides.

The great pathophysiological importance of neuraminidases is indicated by their wide distribution in nature (from microorganisms to humans), and the biological importance of their potential substrates, glycosidically-bound N-acetylneuraminic acids. This is also illustrated in man, by the existence of several severe genetic diseases associated with a neuraminidase deficiency (see chapter III).

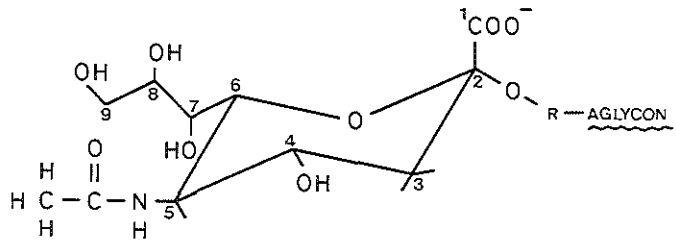

Fig. II.1. Structure of the N-acetylneuraminic acid containing substrates of neuraminidase. R-, represent other carbohydrate residues, which are bound to the AGLYCON part.

II.2. SUBSTRATE SPECIFICITY

The substrate specificity of neuraminidases is determined by several features of the substrate (for review see Corfield et al. 1981) (Fig. II.1.)

- -The structure of the neuraminic acid residue itself (N-acetyl, N-glycolyl, N-acyl, O-acetyl derivatives).
- -The nature of neighbouring sugars (usually D-galactose, but also N-acetyl-D-galactosamine, N-acetyl-D-glucosamine or other neuraminic acid residues).
- -The type of linkage by which NANA is linked to neighbouring sugars (2-3, 2-4, 2-6 or 2-8).
- -The size of the carbohydrate portion (Keilich et al. 1979).
- -The structure of the aglycon part to which the neuraminic acid residues containing carbohydrate chains are linked.

Knowledge about the substrate specificity may be important for the understanding of (patho) physiological functions (chapter II.1.). Experiments with different natural or synthetic neuraminic acid residues have provided information on the parts of the neuraminic acid residue that are important for enzyme-substrate recognition, binding and eventually hydrolysis (for review see Drzeniek 1973, Corfield et al. 1981, Schauer 1982). Only α-glycosides of neuraminic acid are cleaved. The only existing β-glycoside of neuraminic acid exists in cytidine-monophosphate (CMP)-neuraminic acid, which is not a substrate for neuraminidase, independent of the source of the neuraminidase. Substrate specificity of neuraminidases was identical in different species independent of the neuraminic acid residue itself. The same holds for the α-linkage specificity which is a general characteristic for all neuraminidases. After hydrolysis the α-anomer mutarotates in water to the β-anomer (Friebolin et al. 1980).

The naturally occurring neuraminic acids bear on C-atom 5 an acetyl (N-acetylneuraminic acid, NANA) or glycolyl (N-glycolylneuraminic acid) residue; NANA is hydrolysed prefer-

entially (Corfield et al. 1980). Synthetic modification of N-substitution with e.g. N-formyl or N-propionyl derivatives results in reduced cleavage rates (Brossmer and Nebelin 1969).

Further important structural features for the hydrolysis of bound neuraminic acid residues by neuraminidases are the presence of a negative charge on C-atom 1 (Brossmer and Holmquist 1971), and a free OH-group on C-atom 4, with exceptions for some viral neuraminidases (Schauer 1982). For maximum activity also the side chain (C7-C9) is important. Shortening leads to progressive decrease in the rate of hydrolysis (Veh et al. 1977). When neuraminic acid residues are present at a terminal position of a carbohydrate side chain cleavage may not occur directly because of steric hindrance (for instance in G_{M1} ganglioside) (Drzeniek 1973, Svennerholm 1980), but only after removal of the other carbohydrate chain.

Neuraminidases are heterogeneous in their specificity towards the aglycon part. Viral and bacterial neuraminidases appear to be rather unselective, in contrast to the mammalian neuraminidases which at this point are very selective. By using immobilized N-(4-nitrophenyl)-oxamic recognition of substrates by neuraminidases was found to be influenced by a hydrophobic centre in the enzyme, distinct from the catalytic site (Brossmer et al. 1977, Keilich et al. 1979).

The next section of this chapter will mainly discuss mammalian neuraminidases. Substrate specificity studies of these enzymes have been hampered by the impurity of the neuraminidase preparations and accordingly reliable information is only scarcely available. The data obtained sofar will be discussed in the next section describing the evidence for the existence of different neuraminidases.

II.3. DIFFERENT NEURAMINIDASES

In contrast with neuraminidases from microorganisms mammalian neuraminidases form a heterogeneous group. This is shown by the many forms of neuraminidase that have been found, with rather different substrate specificities concerning the aglycon part of the substrates, depending on species, tissue, or even subcellular localization. Whether all these neuraminidases are genetically related or present different gene products remains still an open question. (for reviews see Corfield et al. 1981, Veh and Sander 1981).

Mammalian neuraminidases have been studied in various species (rat, pig and cow) and tissues such as heart muscle (Tallman and Brady 1973), brain (Schengrund and Rosenberg 1970, Carubelli and Tulsiani 1971, Venerando et al. 1975, 1985; Cruz and Gurd 1981), liver (Aldaheff and Wolfe 1981, Michalski et al. 1982) spleen (Schengrund et al. 1979), kidney (Cohen-Forterre et al. 1984), thyroid gland (Van Dessel et al. 1984), placenta (McNamara et al. 1982), leucocytes (Nguyen Hong et al. 1980, Schauer et al. 1984), and cultured fibroblasts (Caimi et al. 1979, Zeigler and Bach 1981).

However, it has been hardly possible to obtain sufficiently pure preparations allowing reliable studies on substrate specificity and genetic and molecular characterization.

The neuraminidases are very heterogeneous in respect to their subcellular localization. Soluble and particulate forms of the enzyme have been found, but it cannot always be excluded that one form is derived from the other as a result of the experimental manipulation. Mammalian neuraminidases have been localized in the plasma membrane, microsomes, Golgi apparatus, lysosomes and the cytosol (Tulsiani and Carubelli 1972, Visser and Emmelot 1973, Kishore et al. 1975, Venerando et al. 1975). Different biochemical properties such as the K_m value and sensitivity to certain treatments have been reported. These different neuraminidase activities may vary in relative

amounts among different species and tissues.

Concerning the substrate specificity two groups of neuraminidases can be distinguished:

-neuraminidases with specificity for sialoglycolipids (gangliosides).

-neuraminidases with specificity for sialyl linkages in oligosaccharides and glycoproteins.

Evidence has accumulated that these two groups of neuraminidases are derived from different gene products by the discovery of two different genetic diseases in man: Mucolipidosis IV, a ganglioside specific neuraminidase deficiency, sialidosis, due to lysosomal oligosaccharide/glycoprotein specific neuraminidase deficiency (see chapter III)

Neuraminidases of the first group seem to be primarily located in the plasma membrane of various cells of which brain tissue has been studied best. The highest specific activity has been found in the synaptosomal membrane (Ohman et al. 1970, Schengrund and Rosenberg 1970, Tettamanti et al. 1972, Yohe and Rosenberg 1977). However this ganglioside specific neuraminidase activity is also found in the cytosolic fractions of brain tissue and liver (Tulsiani and Carubelli 1970 and 1972, Venerando et al. 1975 and 1978).

Neuraminidases of the second group, acting on sialyl linkages in oligosaccharides and glycoproteins, seem to be primarily located in lysosomes (Mahadevan et al. 1967, Horvat and Touster 1968, Tulsiani and Carubelli 1970) Some activity has also been attributed to the plasma membrane (Visser and Emmelot 1973) , Golgi (Kishore et al. 1975), and cytosol (Tulsiani and Carubelli 1970). It is not yet clear whether this is of physiological importance, or is due to experimental manipulation or contamination of the subcellular fractions.

It is evident that reliable data about biochemical, molecular, physiological (substrate specificity), and genetic characteristics of neuraminidases can only be obtained once sufficiently purified preparations are available.

A main problem in the research of the past has been the

lack of a reliable and sensitive assay for neuraminidase. The most frequently used chromogenic assay is not only laborious, but also prone to misinterpretations, especially when used for crude preparations, with interfering substances. Recent methods enabled a more sensitive and reliable assay. The artificial fluorogenic substrates have been of great value in the (prenatal) diagnosis of genetic disease involving neuraminidase deficiency (Kleijer et al. 1979). In basic research these substrates have also been useful during purification procedures (Ngyuen Hong et al. 1980, McNamara et al. 1982, Spaltro and Aldaheff 1984, Van Dessel et al. 1984). Radiolabeled natural substrates or the use of adapted chromogenic procedures after removal of interfering substances have greatly improved studies on substrate specificity (Cohen-Forterre et al. 1984).

Recently, a cytosolic neuraminidase from rat liver has been purified to homogeneity (Miyagi and Tsuiki, 1985). This enzyme (molecular mass 43 kDa) appeared to be active towards sialyloligosaccharides, sialoglycoproteins as well as sialoglycolipids (gangliosides), and shows a pH optimum around pH 6.5, suggesting a physiological function in desialylation in extralysosomal compartments. This demonstrates that a classification in oligosaccharide/glycoprotein specific neuraminidases and ganglioside specific neuraminidases is not satisfactory. Some neuraminidases apparently have activity towards both types of substrates whereas others have a higher specificity.

In cultured human fibroblasts at least two different neuraminidases can be distinguished on the basis of their subcellular localization and defined substrate specificity (Cantz and Messer 1979, Zeigler and Bach 1981, Mendla and Cantz 1984). First of all there is a lysosomal neuraminidase with an acid pH optimum around pH 4.0, probably membrane-bound and extremely labile and its activity primarily towards oligosaccharides and glycoproteins, although some investigators also reported some activity towards gangliosides (Chigorno et

al. 1986, Mancini et al. 1986[a]). Secondly there is a neuraminidase probably located in the plasma membrane that acts exclusively on gangliosides (Cantz and Messer 1979, Zeigler and Bach 1981). The linkage specificity (α2-3 or α2-6) suggested earlier (Strecker and Michalski 1978) seems not to exist (Mendla and Cantz 1984).

The interest in different human neuraminidases has been stimulated by the discovery of several inherited human diseases associated with a neuraminidase deficiency. (see chapter III). At least two neuraminidases are involved in these diseases: one acting on oligosaccharides and glycoproteins and one acting on gangliosides.

An exact characterization of mammalian (preferably human) neuraminidases is important both for a reliable prenatal diagnosis of the different diseases, and for a better understanding of the pathophysiological features in the different clinical variants of each of these diseases. On the other hand the study of human cell material with a neuraminidase deficiency may be of great help in defining and characterizing the normal neuraminidases and for attributing specific physiological functions to them.

CHAPTER III
NEURAMINIDASE INVOLVEMENT IN HUMAN GENETIC DISEASE

III.1. CLINICAL AND GENETIC ASPECTS

Research on neuraminidases has been stimulated by the discovery of different genetic disorders associated with a deficiency of neuraminidase activity. A neuraminidase deficiency was first demonstrated in patients with an autosomal recessive disease originally described as Mucolipidosis I (Spranger and Wiedemann 1968) by Cantz et al. (1977), Spranger et al. (1977) and Kelly and Graetz (1977).

Biochemical investigations showed an accumulation of sialic acid containing compounds in cultured fibroblasts and leucocytes from these patients, excessive urinary excretion of sialyl oligosaccharides, and a profound deficiency of an acid neuraminidase which normally cleaves sialic acid residues from oligosaccharides (sialyllactose) and glycoproteins (fetuin). Clinically these patients exhibited features of a lysosomal storage disorder resembling Hurler disease, with an early onset (signs of illness developed in the first year of life).

In the same period some patients were described with similar biochemical features, but with a milder clinical phenotype: absence of mental retardation and longer survival. These patients were described as new types of Mucolipidosis I or as cherry-red spot-myoclonus syndrome (Strecker et al. 1977, Durand et al. 1977, O'Brien 1977, Thomas et al. 1978, Rapin et al. 1978).

Several patients originally classified as atypical forms of G_{M1}-gangliosidosis variants (Pinsky et al. 1974, Loonen et al. 1974, Galjaard et al. 1975, Suzuki et al. 1977, Andria et al. 1978) were later found to have a combined deficiency of lysosomal β-galactosidase and neuraminidase (Wenger et al. 1978, Hoogeveen et al. 1980). Within each of these syndromes

the clinical heterogeneity is considerable.

Since clinical manifestations have been used for classification without considering the genetic and molecular background, a lot of confusion about the nomenclature of disorders associated with neuraminidase deficiency was introduced (Lowden and O'Brien 1979).

At this moment the term Sialidosis (Durand et al. 1977) has been accepted for the category of patients with an isolated neuraminidase deficiency (the former Mucolipidosis I and cherry-red spot-myoclonus syndrome), and patients with a combined β-galactosidase/neuraminidase deficiency are now classified as Galactosialidosis (Andria et al. 1980). Somatic cell hybridization studies demonstrated that these two syndromes are caused by mutations in different genes (see also chapter IV) (for reviews see Lowden and O'Brien 1979, Wiesmann and Herschkowitz 1980, Cantz 1980, in: Tettamanti et al. (eds) 1981, O'Brien 1982, Galjaard et al. 1984a and 1984b, Cantz et al. 1984).

Patients with Mucolipidosis II (I-cell disease) (Leroy and Spranger 1970), although presenting a severe neuraminidase deficiency, should not be classified among the Sialidoses, since the neuraminidase defect is only of one many lysosomal enzyme defects, all due to the same defective posttranslational modification (Reitman et al. 1981, Hasilik et al. 1981).

Two other types of neuraminidase deficiency associated disorders have been recognized, which also do not fit into the Sialidosis group, and which cannot be properly classified until the primary defect has been elucidated: Mucolipidosis IV with a supposed ganglioside-specific neuraminidase deficiency (Bach et al. 1979, Caimi et al. 1982, Ben-Yoseph et al. 1982) and some variants of Morquio disease type A with a partial oligosaccharide neuraminidase deficiency, which is probably a secondary effect of the primary defect N-acetylgalactosamine-6-sulphate sulphatase (Glössl et al. 1984).

III.2. SIALIDOSIS

The Sialidoses can be divided into two main groups with distinct clinical phenotypes:

1. A mild, normomorphic form.
2. A severe, dysmorphic form.

ad 1) The mild normomorphic form is characterized by the absence or a minimal mental retardation, cherry red spots, myoclonus and progressive blindness, and no dysmorphic features or bony involvement.

In the past these patients have been classified as adult variants of Mucolipidosis I or cherry-red spot-myoclonus syndrome. A high proportion of these patients is of Italian ethnic origin. (for review see Lowden and O´Brien 1979).

ad 2) The severe dysmorphic form is characterized by moderate to severe mental retardation, Hurler like facial features, kidney involvement, bony involvement, cherry-red spots, myoclonus and a rapidly progressive clinical course.

Previously these patients were described as Mucolipidosis I or the Goldberg syndrome. (for review see Spranger and Wiedeman 1970, Spranger 1981).

Biochemically all patients are characterized by elevated levels of bound N-acetylneuraminic acid in urine and cultured fibroblasts. The responsible protein defect was elucidated as an oligosaccharide/ glycoprotein specific lysosomal neuraminidase deficiency (Cantz et al. 1977, O´Brien, 1977). The normal enzyme cleaves α2-3 as well as α2-6 linkages but the mutant enzyme retains higher residual activity towards the former, resulting in preferential storage and urinary excretion of α2-6 linked oligosaccharides (Cantz et al. 1977, O´Brien 1982, Cantz et al. 1984). Further support for neuraminidase deficiency as the primary gene defect was the observation of half-normal enzyme activities in the parents of patients (gene-

dosage effect) (for review see Lowden and O´Brien 1979). The neuraminidase acting on gangliosides is not affected (Cantz and Messer 1979, Cantz et al. 1984).

Considerable clinical heterogeneity is found, not only between the two groups, but also within each of them (infantile juvenile and adult forms) (Lowden and O´Brien 1979, Spranger 1981, Beck et al. 1984). This heterogeneity is probably due to different allelic mutations affecting the neuraminidase gene(s), which may lead to different residual activities in vivo.

Recent gene localization studies (Mueller et al. 1986) suggest the presence of two genes essential for neuraminidase activity. This is in agreement with our studies presented in chapter IV, which provide evidence for the existence of two different neuraminidase subunits essential for its catalytic activity.

Molecular characterization of the defects and their allelic variants will only be possible when purification and characterization of the neuraminidase involved has been performed, and when antibodies become available (see chapter IV).

III.3. GALACTOSIALIDOSIS

Several patients originally described as variant forms of G_{M1}-gangliosidosis, were later found to have a combined deficiency of an oligosaccharide neuraminidase and β-galactosidase. These patients had earlier been classified as Sialidosis type 2, because of a supposed primary neuraminidase deficiency. Somatic cell hybridization studies (see chapter IV) demonstrated, however, that the combined β-galactosidase-neuraminidase deficiency was genetically unrelated to the isolated neuraminidase deficiency in Sialidosis. (for reviews see Andria et al. 1981, Galjaard et al. 1984a, b).

As in most other lysosomal storage diseases, different clinical phenotypes of Galactosialidosis have been recognized. Patients with the early-infantile form, show severe edema, ascites, skeletal dysplasia and ocular abnormalities, and die shortly after birth (Kleijer et al. 1979, Gravel et al. 1979). Patients with the late-infantile form present after 6-12 months with dysmorphism, dysostosis and skeletal dysplasia, visceromegaly, macular cherry-red spot and mild mental retardation (Pinsky et al. 1974, Andria et al. 1981, Strisciuglio et al. 1984). The largest number of patients, mainly of Japanese origin are those with the juvenile/adult form where symptoms appear in late childhood or early adulthood. The major features are skeletal dysplasia, dysmorphism, corneal clouding, macular cherry-red spot, angiokeratomata, neurological manifestations and mental retardation (Loonen et al. 1974, 1984; Suzuki et al. 1983, 1984).

The responsible molecular defect in Galactosialidosis appeared to be a deficiency of a 32 KDa glycoprotein (d'Azzo 1982, d'Azzo et al. 1982), subsequently shown to be required for the aggregation of β-galactosidase monomers (see chapter IV). The high molecular weight aggregate was found to be essential to protect the β-galactosidase against rapid intralysosomal proteolytic degradation. The explanation for the neuraminidase deficiency is discussed in chapter IV.

Gene localization experiments have assigned the structural gene for this 32 kDa protein on chromosome 22 (Sips et al. 1985).

Recently molecular characterization of the different variants of Galactosialidosis was performed by immunoprecipitation studies of the 32 kDa protective protein and its 54 kDa precursor form (Palmeri et al. 1986, see also chapter IV).

III.4. MUCOLIPIDOSIS IV

Mucolipidosis type IV is a lysosomal storage disorder, first recognized in 1974 (Berman et al. 1974), which clinically is characterized by congenital cloudy cornea and slow progressive, severe mental and motor deterioration, without visceral involvement.

Patients reported until now are mainly of Jewish Ashkenazi origin and range in age from 6 months to the mid-twenties.

Lysosomal storage of gangliosides, phospholipids and acid mucopolysaccharides has been found (for review see Bach and Zeigler 1981).

The neuraminidase active towards gangliosides has been reported to be partially deficient in cultured amniotic fluid cells and fibroblasts (Bach et al. 1979, Caimi et al. 1982). Other investigators, however, could not reproduce this finding (Sandhoff, personal communication). Ganglioside neuraminidases have been localized primarily in plasma membranes but a lysosomal localization cannot be ruled out completely (Zeigler and Bach 1985, Chigorno et al. 1986).

Recently evidence has been presented that subcellular distribution of the ganglioside neuraminidase is abnormal in fibroblasts from patients with Mucolipidosis IV (Zeigler and Bach 1985).

Accurate subcellular fractionation studies and a sensitive and reliable assay for ganglioside neuraminidase are required before definite conclusions will be possible. Purification of ganglioside neuraminidase(s) will be the first essential step, followed by a molecular characterization in normal and mutant fibroblasts.

CHAPTER IV
THE EXPERIMENTAL WORK

IV.1. AIM OF THE WORK PRESENTED IN THIS THESIS

Several autosomal recessive inheritable lysosomal storage disorders are known which are associated with a deficiency of lysosomal β-galactosidase (G_{M1}-gangliosidosis), a lysosomal neuraminidase (Sialidosis), or of both β-galactosidase and neuraminidase (Galactosialidosis) (see chapter III). Within each syndrome there are progressive infantile, somewhat later-onset juvenile, and still milder adult forms.

The aim of the work presented in this thesis was the elucidation of the genetic and biochemical background of the three syndromes mentioned above and their variant forms.

The emphasis of the experimental work is on the molecular characterization of neuraminidase and on the clarification of its deficiency in combined β-galactosidase/neuraminidase deficiency (Galactosialidosis).

IV.2. DISCUSSION

a. Genetic background

Somatic cell hybridization studies on fibroblasts from classical infantile forms of G_{M1}-gangliosidosis and cells from atypical patients at that time also classified as variant forms of G_{M1}-gangliosidosis, showed that two different genes were involved in these two categories of diseases (Galjaard et al., 1975). Later, Wenger et al. (1978) found in another atypical patient with β-galactosidase deficiency a coexistent neuraminidase deficiency.

Subsequently we have tested fibroblasts from different atypical patients (Pinsky et al. 1974, Loonen et al. 1974, Andria et al. 1978, Kleijer et al. 1978) all of which were found to have a combined β-galactosidase and neuraminidase deficiency.

Subsequently, somatic cell hybridization studies have been performed to elucidate the genetic background of this combined deficiency, the isolated neuraminidase deficiency in Sialidosis and the multiple lysosomal enzyme deficiency in "I-cell disease".

The results of these studies are reported in publication I. Restoration of neuraminidase activity was found in heterokaryons after fusion of cells from patients with different clinical variants of Mucolipidosis I (Sialidosis) and cells from patients with different clinical variants of combined β-galactosidase/neuraminidase deficiency. It was therefore concluded that the single neuraminidase deficiency in Sialidosis is caused by a mutation in a different gene than that involved in β-galactosidase/neuraminidase deficiency (Galactosialidosis). The results of these complementation experiments have subsequently been confirmed using natural glycoprotein substrates (Swallow et al. 1981).

Together with the earlier observations on β-galactosidase complementation (Galjaard et al. 1975) it could be concluded

that the three disorders associated with β-galactosidase and/or neuraminidase deficiency are caused by mutations in three different genes.

Recent gene-localization studies (Sips et al. 1985) have confirmed and extended this observation: the structural gene coding for β-galactosidase is on chromosome 3 and the gene coding for the 32 kDa protein, which is responsible for the combined β-galactosidase and neuraminidase deficiency is on chromosome 22. Genes coding for neuraminidase have been assigned to chromosome 10 and 20 (Mueller et al. 1986).

No complementation was observed after somatic cell hybridization of different variants within one disease, suggesting the involvement of different mutations within the same gene (allelic mutations).

Another important finding in publication I is the increase of neuraminidase deficiency during cocultivation with neuraminidase deficient sialidosis cells. This was the first indication for the existence of a corrective factor.
Finally publication I demonstrates that I-cell disease is genetically different from the other neuraminidase deficiencies. Subsequent biochemical analysis showed that I-cell disease is due to a defect in a posttranslational modification step (phosphorylation), which is essential for a proper lysosomal compartmentalization of lysosomal proteins (see chapter I.3).

b. Biochemical background Galactosialidosis (β-gal$^-$/neur$^-$) and Sialidosis (neur$^-$).

d'Azzo et al. (1982) demonstrated that in normal human fibroblasts β-galactosidase is synthesized as a 85 kDa precursor form which is "processed" by a number of steps to a mature 64 kDa protein. β-Gal$^-$/neur$^-$fibroblasts were found to synthesize this 85 kDa precursor form normally, but mature 64 kDa β-galactosidase is not or barely detectable.

Turn-over studies of human β-galactosidase and uptake-

experiments using bovine testis β-galactosidase suggested rapid proteolytic degradation of β-galactosidase in Galactosialidosis cells (van Diggelen et al. 1981, 1982). Studies by d'Azzo et al. (1982) provided evidence that this rapid degradation is correlated with the deficiency of an until then unknown 32 kDa protein ("protective protein"). The reason for the neuraminidase deficiency in Galactosialidosis could not be clarified at that stage. A relation between β-galactosidase, the protective protein and neuraminidase was, however, evident.

1. Relation β-galactosidase-protective protein.

The experimental work described in publication II clarifies the relation between the protective protein and β-galactosidase. Sucrose-density gradient studies revealed that in normal fibroblasts 80-90% of the β-galactosidase exists as high molecular weight multimer. The protective protein was found to play an essential role in the aggregation of monomeric β-galactosidase (64 kDa) into multimeric β-galactosidase (600-700 kDa). Because of the absence of protective protein molecules in Galactosialidosis, no multimers can be formed and apparently monomeric β-galactosidase is rapidly degraded by lysosomal cathepsins. This explains the observed β-galactosidase deficiency.

With ammonium chloride, secretion of the 54 kDa precursor of the protective protein can be stimulated (Hoogeveen et al. 1981), enabling its partial purification. When this 54 kDa precursor is added to the medium above $\beta\text{-gal}^-/\text{neur}^-$ cells a correction of β-galactosidase activity is found. This correction is accompanied by the formation of multimeric forms of β-galactosidase. Subsequent immuno-electron-microscopical studies indicated that this restoration of multimers also occurs in vivo (Willemsen et al. 1986).

2. Relation β-galactosidase-protective protein-neuraminidase.

The relation between the 32 kDa protective protein and neuraminidase was still unclear. Addition of a concentrate of medium above human fibroblasts, to Galactosialidosis cells resulted in a restoration of both β-galactosidase and neuraminidase activity (Hoogeveen et al. 1981). The mechanism, however, remained unexplained, especially since ingested neuraminidase from bovine testis was not rapidly degraded by Galactosialidosis cells (Verheijen, unpublished data). In experiments described in publication III it was demonstrated that in bovine testis lysosomal neuraminidase exists in a complex together with β-galactosidase and the 32 kDa protein. Later, it could be demonstrated that this β-galactosidase-protective protein-neuraminidase complex is also present in other cell types like human fibroblasts (unpublished data), human leucocytes (see publication VI), and human placenta (see publication IV).

3. Purification of human lysosomal neuraminidase.

In publication IV a start has been made with the purification of the labile lysosomal human neuraminidase. This became possible because part of the lysosomal neuraminidase activity in human placenta, present in a soluble inactive form, could be activated and stabilized by excessive concentration followed by preincubation at 37°C. Now, partial purification of human lysosomal neuraminidase analogous to the purification described for bovine testis neuraminidase (publication III) could be performed, as well as further studies on the relation between β-galactosidase and neuraminidase.

Polyclonal antibodies raised against β-galactosidase and protective protein coprecipitate the neuraminidase activity. Sucrose density gradient centrifugation demonstrated that neuraminidase activity is exclusively present in high-density fractions associated with β-galactosidase.

Monospecific antibodies against 64 kDa β-galactosidase

precipitate ß-galactosidase activity both in the monomeric fractions as in the multimeric fractions. However, purified monospecific antibodies against the 32 kDa protective protein precipitate only ß-galactosidase present in multimeric forms. In addition they precipitate the neuraminidase activity associated with the high-density multimeric form of ß-galactosidase. Since neuraminidase activity can be restored upon formation of high-density multimeric forms, evidence is obtained for a direct relation between neuraminidase activity and the presence of the 32 kDa protective protein.

The direct relation between neuraminidase activity and 32 kDa protective protein clarifies the deficiency of neuraminidase activity in Galactosialidosis, where the 32 kDa protective protein is absent (d´Azzo et al. 1982).

Additional evidence for a direct role of the protective protein in neuraminidase activity was obtained by Cantz´ group (Mendla 1983), who demonstrated the presence of a macromolecular factor essential for the oligosaccharide/glycoprotein specific neuraminidase activity from pig kidney. This factor could bind reversibly to the enzyme and showed characteristics of a protein. It is likely that this activating factor in pig kidney is identical with the protective protein in human tissues.

The data in publication IV further suggest that association of different subunits is essential for neuraminidase activity. One of these is the 32 kDa protective protein, but the others remained to be identified.

In publication V the purification of these different neuraminidase subunits is described using a procedure based on the findings in publication IV. Activated and stabilized human placental neuraminidase is copurified with ß-galactosidase using ß-galactosidase specific affinity chromatography. Subsequently neuraminidase is dissociated from the ß-galactosidase-protective protein-neuraminidase complex by the chaotropic agent KSCN. The ß-galactosidase specific affinity chromatography step is repeated to remove the ß-galactosidase-pro-

tective protein complex. The resulting inactive neuraminidase preparation was used to raise antibodies. Finally the 32 kDa protective protein was dissociated from the complex with β-galactosidase and purified to homogeneity.

This experimental work shows that besides the 32 kDa subunit another neuraminidase subunit of 76 kDa is essential for the activity of lysosomal neuraminidase. This 76 kDa subunit is probably the clue to the elucidation of the biochemical background of Sialidosis variants in which an isolated neuraminidase deficiency has been demonstrated. The antibodies against this subunit may provide a valuable tool to probe the nature of the molecular defect in Sialidosis.

c. Biochemical diagnosis of neuraminidase related disorders

When (Galacto)sialidosis is assumed on the basis of clinical symptoms usually enzyme assays on total leucocytes or cultured skin fibroblasts are performed for a definite diagnosis.

In publication VI it is, however, shown that a total leucocyte preparation contains two different types of lysosomal neuraminidases both acting on the artificial substrate 4-methylumbelliferyl-N-acetylneuraminic acid (4 MU-NANA). Only one of which is genetically deficient in (Galacto)sialidoses. This makes a total leucocyte preparation an inadequate source of diagnostic material, because these cells from patients with (Galacto)sialidoses may show neuraminidase activities as high as 40% of normal values. In isolated lymphocytes, however, the lysosomal neuraminidase involved in genetic disease, comprises more than 85% of the total neuraminidase activity and these cells are therefore a reliable tool for diagnosis.

Many investigators have tried to relate residual enzyme activity to the severity of the clinical features. In Sialidoses and Galactosialidoses the heterogeneity can be summarized as follows:

Sialidosis:

- infantile, progressive forms : neur. activity $< 1\%$
- milder forms without severe mental retardation : $1\% <$ neur. act. $< 10\%$

Galactosialidosis:

- early infantile forms	β-gal.act. 5-15%	neur. act. 5%
- late infantile forms	β-gal.act. 5-15%	neur. act. 1%
- juvenile/adult forms	β-gal.act. 5-15%	neur. act. 1%

An attempt to relate clinical symptoms and residual (enzyme) protein activity is likely to be a simplification of a much more complex situation. First of all most biochemical assays are performed in vitro on homogenates of a selected cell type with an artificial substrate. Furthermore the clinical symptoms reflect a complex disturbance in the pathophysiology of different organs and cell types. A genetic (enzyme) protein deficiency may have very different consequences in different cell types and organs within one organism. In addition there may be considerable clinical and pathological heterogeneity among different patients with the same genetic metabolic disease.

Publication VII reports on the molecular background of such clinical heterogeneity in patients with Galactosialidosis.

In all three clinical phenotypes (see table above) the β-galactosidase and neuraminidase deficiencies are secondary phenomena to the lack of the 32 kDa protective protein (see previous section b). We have therefore studied the biosynthesis of this 32 kDa protein in the different clinical variants. In the early infantile form no 32 kDa protein can be detected and only very little of its 52 kDa precursor.

Cells from patients with the late infantile form, however, do show an excessive amount of 52 kDa precursor and a small amount of 32 kDa protein. Different experiments demon-

strated that in this clinical variant the mutation interferes with the posttranslational modification of the 52 kDa precursor. The small residual amount of 32 kDa protein is apparently sufficient to yield a 1-5% neuraminidase activity, which could be stimulated by leupeptin treatment. This latter substance inhibits proteolytic degradation which explains the increase of the amount of 32 kDa protein. The experimental work described in publications IV and V demonstrated that this 32 kDa protein is an essential part of neuraminidase.

In the juvenile/adult form no 32 kDa protein can be detected, but the amount of 52 kDa precursor seems to be somewhat higher than in the early infantile form. Despite the further molecular characterization of the different clinical phenotypes we cannot yet provide an explanation for the mild clinical course of the juvenile/adult form.

Further studies on the exact primary, secundary and tertiary structure of the neuraminidase subunits and on the stoichiometry of the components of the lysosomal β-galactosidase-protective protein-neuraminidase complex are now required. The results of such studies will contribute to the understanding of the mechanisms involved in the protection of lysosomal proteins against the hydrolytic activity of neighbouring enzymes and they may provide a better insight in the pathogenesis of the clinical heterogeneity among the neuraminidase related disorders.

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SUMMARY

Since the discovery of the lysosome as a distinct subcellular compartment important for intracellular digestion, by the group of De Duve in 1955, more than 70 lysosomal hydrolases have been described. A genetically determined deficiency of one of these enzymes may result in the intralysosomal accumulation of cellular constituents or extracellular products. Depending on the function of the enzyme, the rate of accumulation and the interference with the cellular metabolism, a variety of clinical and pathological manifestations will occur. Up to now more than 30 lysosomal storage disorders are known, nearly all of which are of autosomal recessive inheritance.

This thesis deals with the genetic and molecular characterization of genetic diseases associated with a deficiency of lysosomal neuraminidase.

Neuraminidases (EC 3.2.1.18, sialidase, N-acetyl-neuraminosyl glycohydrolase) catalyze the hydrolysis of neuraminic acid residues (sialic acids) from a variety of neuraminic acid - containing compounds. These enzymes are widely distributed in nature and in mammalian cells they form a heterogeneous group as far as their subcellular localization and substrate specificity are concerned. Our experimental work has focussed on the lysosomal neuraminidases which catalyze the cleavage of N-acetylneuraminic acid residues from glycoproteins, oligosaccharides and glycopeptides. The availability of an artificial fluorogenic substrate (4-methylumbelliferyl-N-acetyl-neuraminic acid) permitting a sensitive and reliable enzyme assay, has greatly facilitated both the diagnostic work and the research described in this thesis.

Patients with the lysosomal storage disease Sialidosis have a generalized deficiency of neuraminidase which can be reliably demonstrated in cultured skin fibroblasts or in isolated blood lymphocytes. Patients excrete increased amounts of oligosaccharide-bound N-acetylneuraminic acid in urine which

compound is also elevated in (cultured) fibroblasts and other cell types. Clinically, two forms are being distinguished:

- a severe, dysmorphic form characterized by severe mental retardation, skeletal deformities, Hurler-like facial features, macular cherry-red spot, myoclonus, kidney involvement and a rapidly progressive clinical course.
- a milder, normomorphic form with a later onset, mild mental retardation or normal mental development, macular cherry-red spot, progressive visual impairment, and myoclonus.

Patients with Galactosialidosis are characterized by a combined deficiency of lysosomal neuraminidase and β-galactosidase and urinary excretion of sialyloligosaccharides. Clinically three different forms have been reported:

- an early infantile form with severe edema, ascites, skeletal dysplasia and ocular abnormalities where patients die shortly after birth.
- a late infantile form where symptoms occur after 6-12 months such as dysmorphic facial features, skeletal dysplasia, visceromegaly, macular cherry-red spot and mild mental retardation.
- a juvenile/adult form where clinical features occur during late childhood or early adulthood; the major symptoms are skeletal dysplasia, dysmorphism, corneal clouding, macular cherry-red spot, angiokeratomata, neurological manifestations and mental retardation.

In addition there are different types of patients with G_{M1}-gangliosidosis who are characterized by an isolated deficiency of β-galactosidase resulting in intralysosomal accumulation of G_{M1}-ganglioside and a variety of physical abnormalities and mental retardation.

One of the aims of the experimental work in our group is a better understanding of the molecular basis of clinical heterogeneity within certain lysosomal storage disorders. In

this context it is important to realize that gene mutations may interfere with a variety of molecular processes involved in the biosynthesis, modification and functioning of a lysosomal (enzyme) protein. Mutations may interfere with the synthesis of the precursor (enzyme) protein, they may block one of the many steps involved in the translocation, the phosphorylation and the correct compartmentalization into the lysosome. Furthermore, mutations may interfere with the correct aggregation of subunits or with other proteins, within the lysosome and also the molecular structure of the mature enzyme may be altered in such a way that the catalytic site does not function properly. Finally, lysosomal disorders are known where the defect concerns the interaction between enzyme and substrate (defective activator protein) or impairment of the lysosomal release of metabolites (such as N-acetylneuraminic acid storage disorders).

One approach towards the elucidation of the genetic background of different disorders or of different clinical variants of the same disorder is complementation analysis after hybridization of two mutant fibroblast strains. Such experiments are described in publication I for different neuraminidase related diseases. It was shown that the neuraminidase deficiencies in Sialidosis, Galactosialidosis and "I-cell" disease are based on mutations in different genes. The clinical variants within these diseases seem to be caused by different mutations within the same gene (allelic mutations).

Previous work by our group has revealed that the combined deficiencies of β-galactosidase and neuraminidase in Galactosialidosis are caused by the lack of a 32 kDa glycoprotein which normally seems to be required for the protection of β-galactosidase against rapid intralysosomal proteolytic degradation. In publication II we show the molecular basis for this protection. In normal fibroblasts β-galactosidase exists mainly as a high density multimer (600-700 kDa) of β-galactosidase monomers (64 kDa). The 32 kDa "protective protein" was found to be essential for the aggregation of β-galactosidase mono-

mers. The absence of the 32 kDa protein in Galactosialidosis results in rapid proteolytic degradation of normally synthesized 64 kDa β-galactosidase monomers.

The neuraminidase deficiency in Galactosialidosis remained, however, unexplained and this was elucidated by the experimental work in publications III, IV and V. First (publication III) it was found for bovine testis that lysosomal neuraminidase exists in a complex together with β-galactosidase and the 32 kDa protective protein. A similar complex was later found in human placenta, cultured fibroblasts and leucocytes.

The purification and characterization of human neuraminidases had so far been hampered by the extreme lability of this membrane-bound enzyme. In publication IV a method is described which enabled us to purify neuraminidase. Inactive neuraminidase present in the soluble fraction of homogenized human placenta could be activated and stabilized by excessive concentration and 90 min. incubation at 37°C. Monospecific antibodies against the 32 kDa protein were prepared and their use together with sucrose-density gradient centrifugation showed that this active neuraminidase and the 32 kDa protein are present only in a fraction containing high density β-galactosidase multimer. These results suggested that the 32 kDa protective protein also is essential for the catalytic activity of lysosomal neuraminidase.

In publication V we report the purification and partial characterization of human lysosomal neuraminidase. The results indicate that in addition to the 32 kDa protein another subunit (76 kDa) is associated with neuraminidase activity. Antibodies reacting with this subunit have been prepared.

These results not only explain the neuraminidase deficiency in Galactosialidosis but also form the basis for future studies aimed at the elucidation of the exact molecular defect in different variants of Sialidosis.

The importance of selecting the correct cell material for biochemical diagnosis of neuraminidase related disorders is

illustrated by observations described in publication VI. A total leucocyte preparation (granulocytes and lymphocytes) was found to contain two types of lysosomal neuraminidase, only one of which is genetically deficient. As a consequence such cell material is not an adequate source for diagnostic purposes in contrast with fibroblasts and isolated blood lymphocytes, which mainly contain the neuraminidase defective in (Galacto)sialidosis.

Several investigators have attempted to relate the severity of clinical features and the residual enzyme activity in selected cell types from patients with different variants of the same genetic disease. Such attempts are complicated by the fact that clinical features are the result of complex interactions of pathophysiological processes in a variety of organ systems and cells. Also, most enzyme assays are carried out on a homogenate of one cell type using artificial substrate(s). The difficulty in explaining the clinical heterogeneity is illustrated by the work on different variants of Galactosialidosis, described in publication VII.

Immunoprecipitation studies on fibroblasts from patients with the early infantile form and the juvenile/adult form show an absence of the 32 kDa protective protein and a small amount of its 52 kDa precursor. The milder clinical course in the juvenile/adult form cannot yet be explained on the basis of these molecular studies. In cells from patients with the late infantile form, however, an excessive amount of this 52 kDa precursor was found which appeared to be due to a defective processing into the 32 kDa protein. In the latter patients the small residual amount of 32 kDa protein is apparently sufficient to yield 1-5% neuraminidase.

The experimental work reported in this thesis has led to the explanation of the neuraminidase deficiency in the lysosomal storage disorder Galactosialidosis. It has also contributed to our understanding of the relationships in normal and mutant cells of the β-galactosidase, neuraminidase and the 32

kDa protective protein complex. Further studies on the primary, secundary and tertiary structure of these components, as well as their stoichiometry, will hopefully lead to a better understanding of the normal protective mechanism of (enzyme) protein in the lysosome and of the exact molecular defects in the different variants of Sialidoses.

SAMENVATTING

Sinds de Duve's groep in 1955 het voor intracellulaire afbraakprocessen belangrijke celorganel, het lysosoom, ontdekte, zijn meer dan 70 lysosomale hydrolases beschreven. Een erfelijk bepaalde deficiëntie van één van deze enzymen kan tot gevolg hebben dat allerlei intra- of extracellulaire componenten in de lysosomen ophopen. Afhankelijk van de funktie van het enzym, mate van ophoping en de invloed op het cellulaire metabolisme, zullen een groot aantal verschillende klinische en pathologische symptomen optreden. Tot nu toe zijn meer dan 30 lysosomale stapelingsziekten bekend, die bijna allemaal autosomaal recessief overerven.

Dit proefschrift beschrijft de genetische en moleculaire karakterisering van erfelijk bepaalde ziekten met een deficiëntie van lysosomaal neuraminidase.

Neuraminidases (EC 3.2.1.18, sialidase, N-acetylneuraminosyl-glycohydrolase) katalyseren de hydrolyse van neuraminezuur (siaalzuur) residuen van een groot aantal neuraminezuur-bevattende stoffen. Deze enzymen zijn wijd verbreid in de natuur en vormen in zoogdiercellen wat betreft hun subcellulaire localisatie en substraat specificiteit een heterogene groep. Ons experimentele werk heeft zich toegespitst op de lysosomale neuraminidases die de splitsing van N-acetylneuraminezuur residuen van glycoproteïnen, oligosacchariden en glycopeptiden katalyseren. Het beschikbaar komen van een kunstmatig fluorogeen substraat (4-methylumbelliferyl-N-acetylneuraminezuur) heeft een gevoelige en betrouwbare enzym activiteits bepalingsmethode mogelijk gemaakt en heeft diagnostisch werk en het onderzoek beschreven in dit proefschrift vergemakkelijkt.

Patiënten met de lysosomale stapelingsziekte Sialidose hebben een gegeneraliseerde deficiëntie van het enzym neuraminidase, die bepaald kan worden in gekweekte huidfibroblasten of in uit het bloed geïsoleerde lymfocyten. De patiënten scheiden grote hoeveelheden gebonden N-acetylneuraminezuur in

de urine uit en vertonen ook een verhoging van deze stof in (gekweekte) huidfibroblasten en andere celtypen. Gebaseerd op klinische verschijnselen kunnen twee vormen van de ziekte worden onderscheiden:

- een ernstige, dysmorfe vorm, gekarakteriseerd door ernstige mentale retardatie, skeletafwijkingen, gelaatstrekken zoals bij het syndroom van Hurler, "Cherry-red spots" op de macula, myoclonieën, nierafwijkingen en een snel progressief verloop.
- Een mildere, normomorfe vorm, gekenmerkt door later optredende symptomen, een normale mentale ontwikkeling of een milde retardatie, "cherry-red spots" op de macula, progressief visuele afwijkingen en myoclonieën.

Patiënten met Galactosialidose zijn biochemisch gekenmerkt door een samengestelde deficiëntie van de lysosomale enzymen neuraminidase en β-galactosidase en door de uitscheiding in de urine van sialyloligosacchariden. Gebaseerd op klinische verschijnselen kunnen drie verschillende vormen worden onderscheiden:

- Een vroeg-infantile vorm, met ernstig oedeem, ascites en oogafwijkingen. De patiënten overlijden kort na de geboorte.
- Een laat infantiele vorm waarin de verschijnselen optreden tussen 6 en 12 maanden na de geboorte; zoals vervormde gelaatstrekken, skeletafwijkingen, visceromegalie, "cherry-red spots" op de macula en milde mentale retardatie.
- Een juveniele/adulte vorm waarin de klinische verschijnselen optreden in de adolescentie periode; de belangrijkste symptomen zijn skeletafwijkingen, dysmorfieën, vertroebeling van de cornea, "cherry-red spots" op de macula, angiokeratomata, neurologische verschijnselen vaak gepaard gaande met mentale retardatie.

Een andere categorie patiënten bestaat, met de ziekte $GM1$-gangliosidose, gekenmerkt door een enkelvoudige deficiëntie van β-galactosidase, die resulteert in lysosomale stape-

ling van G_{M1}-ganglioside en een aantal verschillende fysieke en mentale afwijkingen.

Een van de doelstellingen van het experimentele werk binnen onze groep is het verkrijgen van een breder inzicht in de moleculaire achtergrond van de klinische heterogeniteit binnen bepaalde lysosomale stapelingsziekten. In dit verband is het belangrijk om zich te realiseren dat genmutaties invloed kunnen hebben op een groot aantal verschillende moleculaire processen die betrokken zijn bij de biosynthese, modificatie en werking van een lysosomaal enzym (eiwit). Mutaties kunnen invloed hebben op de synthese van het precursor (enzym) eiwit, of kunnen een van de vele stappen blokkeren die tijdens de translocatie, glycosylering en fosforylering, en uiteindelijke lysosomale bestemming van een nieuw gesynthetiseerd (enzym) eiwit plaatsvinden. Mutaties kunnen bovendien in het lysosoom zelf nog invloed hebben op de correcte associatie van subeenheden, of andere eiwitten, en mutaties kunnen een zodanige verandering van de moleculaire structuur teweegbrengen, dat de plaats op het enzym betrokken bij de katalyse ("catalytic site") niet goed meer functioneert. Andere lysosomale stapelingsziekten zijn bekend waarin de interactie tussen enzym en substraat niet goed plaatsvindt (activator deficiënties), of waarin de enzymatische afbraakprocessen wel normaal verlopen, maar de ontstane metabolieten het lysosoom niet kunnen verlaten (zoals in vrij N-acetyl-neuraminezuur stapelingsziekten).

Een benaderingswijze voor de opheldering van de genetische achtergrond van verschillende ziekten of verschillende klinische varianten van één ziekte is de complementatie analyse. Zulke experimenten voor de verschillende met neuraminidase geassocieerde ziektebeelden zijn beschreven in publikatie I. Hierin wordt aangetoond dat de neuraminidase deficiëntie in Sialidose, Galactosialidose en "I-cell disease" wordt veroorzaakt door mutaties in verschillende genen. De klinische varianten binnen één ziekte daarentegen worden waarschijnlijk veroorzaakt door verschillende mutaties binnen hetzelfde gen

(allele mutaties).

Uit eerder werk van onze groep is gebleken, dat de gecombineerde β-galactosidase en neuraminidase deficiëntie in galactosialidose wordt veroorzaakt door het ontbreken van een 32 kDa glycoproteïne dat essentieel is voor de bescherming van β-galactosidase tegen snelle intralysosomale proteolytische afbraak. In publikatie II wordt getoond hoe deze bescherming op moleculair niveau plaatsvindt. In normale fibroblasten bestaat β-galactosidase hoofdzakelijk als een multimeer van β-galactosidase (64 kDa) monomeren met een hoge dichtheid (600-700 kDa). Het 32 kDa "beschermend eiwit" bleek essentieël voor aggregatie van β-galactosidase monomeren. De afwezigheid van het 32 kDa eiwit leidt tot versnelde proteolytische afbraak van de normaal gesynthetiseerde 64 kDa β-galactosidase monomeren.

De neuraminidase deficiëntie in Galactosialidosis bleef echter onbegrepen tot het experimentele werk in publikatie III, IV enV. Ten eerste (publikatie III) werd gevonden dat lysosomaal neuraminidase uit rundertestis zich in een complex bevindt samen met β-galactosidase en het 32 kDa beschermend eiwit. Later kon zo'n zelfde complex worden aangetoond in menselijke leucocyten, fibroblasten en placenta.

Zuivering en karakterisering van menselijke neuraminidases was tot nu to niet mogelijk door de extreme labiliteit van dit membraan-gebonden enzym. In publicatie IV beschrijven we een methode die ons in staat stelde om met de zuivering van neuraminidase te beginnen. Een inactieve vorm van neuraminidase, die aanwezig is in de oplosbare fractie van gehomogeniseerde menselijke placenta's, kon worden omgezet in een actieve en stabiele vorm d.m.v. sterke concentrering gevolgd door een incubatie bij 37°C. Monospecifieke antilichamen tegen het 32 kDa eiwit konden worden gemaakt en gebruikt in combinatie met sucrose-gradient experimenten, om aan te tonen dat actief neuraminidase en het 32 kDa eiwit alleen aanwezig zijn in een fractie van β-galactosidase multimeren met een hoge dichtheid. Deze resultaten suggereerden dat het 32 kDa be-

schermend eiwit ook nodig is voor de katalytische activiteit van lysosomaal neuraminidase.

In publikatie V wordt gerapporteerd over de zuivering en karakterisering van lysosomaal neuraminidase van de mens. De resultaten laten zien dat naast het 32 kDa eiwit nog een andere subeenheid (76 kDa) is betrokken bij het tot stand komen van neuraminidase activiteit. Vervolgens konden antilichamen tegen deze subeenheid worden gemaakt.

Deze resultaten verklaren niet alleen de neuraminidase deficiëntie in Galactosialidose, maar vormen ook een beginpunt voor verdere studies naar de preciese moleculaire achtergrond in de verschillende varianten van Sialidose.

Alvorens een biochemische diagnose wordt uitgevoerd, is een goede celmateriaal keuze van belang, hetgeen geïllustreerd wordt door waarnemingen in publikatie VI. Leukocyten (een mengsel van granulocyten en lymfocyten) bevatten twee verschillende neuraminidases, waarvan er slechts één deficiënt is in sialidose. Dit celmateriaal is dan ook geen geschikte bron voor diagnostische doeleinden. Dit in tegenstelling met fibroblasten en uit leukocyten geïsoleerde lymfocyten die hoofdzakelijk die neuraminidase bevatten, die deficiënt is in (Galacto)sialidose.

Verscheidene onderzoekers hebben geprobeerd om relaties te leggen tussen de ernst van de klinische verschijnselen en de residuele enzymactiviteit, gevonden in bepaalde cellen van patiënten met verschillende variante vormen van dezelfde genetische ziekte. Zulke pogingen worden gecompliceerd door het feit dat de klinische symptomen een gevolg zijn van complexe interacties van pathofysiologische processen in verscheidene organen en cellen. Bovendien worden de meeste enzymbepalingen uitgevoerd met artificiële substraten op homogenaten gemaakt van slechts één celtype. De moeilijkheid in het verklaren van de genetische heterogeniteit is geïllustreerd door het werk met Galactosialidose varianten beschreven in publikatie VII.

Immunoprecipitatie studies met fibroblasten van patiënten met de vroege-infantiele vorm en de juveniele/adulte vorm

toonden aan dat het 32 kDa beschermend eiwit afwezig is en dat slechts een zeer geringe hoeveelheid van de 52 kDa "precursor" aanwezig is. Het mildere klinisch verloop in de juveniele/adulte vorm kan op basis van deze moleculaire studies nog niet worden verklaard. In cellen van patiënten met de laat-infantiele vorm werd een verhoogde hoeveelheid 52 kDa "precursor" gevonden, welke veroorzaakt lijkt te worden door een defect in de "processing" naar de 32 kDa vorm. Blijkbaar is de kleine hoeveelheid 32 kDa eiwit genoeg om te zorgen voor geringe neuraminidase activiteit (1-5%).

Het in dit proefschrift beschreven experimentele werk heeft geleid tot een verklaring van de neuraminidase deficiëntie in de lysosomale stapelingsziekte Galactosialidose. Bovendien heeft dit werk bijgedragen tot een beter begrip van het ß-galactosidase, neuraminidase, 32 kDa beschermend eiwit complex in normale- en mutante cellen. Toekomstige studies aan de primaire, secundaire en tertiaire structuur van deze componenten en aan de stoichiometrie van het complex zullen hopelijk kunnen leiden tot een beter begrip van het normale beschermingsmechanisme van lysosomale (enzym) eiwitten en van de preciese moleculaire defecten in de verschillende Sialidose varianten.

NAWOORD

Ik wil graag iedereen bedanken die op hun eigen wijze hebben meegewerkt aan het tot stand komen van dit proefschrift. Ik realiseer mij nu dat het hierbij om vele mensen gaat, die meer of minder direct bij het werk betrokken zijn geweest.

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Lieve Grazia, jouw wil ik als laatste, bedanken voor jouw geduld, steun en vertrouwen.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 13 februari 1956 te Rotterdam geboren. In 1974 werd het diploma Atheneum B behaald aan het Franciscuscollege te Rotterdam. In datzelfde jaar werd, na uitloting voor de studie diergeneeskunde, begonnen met de studie biologie aan de Gemeente Universiteit van Amsterdam. Na het Kandidaatsexamen medische biologie (B_1') in september 1977 werd op 18 juni 1980 "Cum laude" het doctoraalexamen afgelegd, met als hoofdvak celbiologie (Prof. Dr. H. Galjaard, Rotterdam) en als bijvakken biochemie (Prof. Dr. J.M. Tager, Amsterdam) en immunologie (Prof. Dr. K.W. Pondman, Amsterdam).

Van 1 juli 1980 tot 31 december 1985 is hij in dienst geweest als wetenschappelijk assistent op de afdeling Celbiologie en Genetica (Prof. Dr. H. Galjaard) van de Erasmus Universiteit te Rotterdam. In deze periode werd het in dit proefschrift beschreven onderzoek verricht en werd practicum onderwijs in de celbiologie en histologie gegeven aan 1e jaars studenten geneeskunde.

Op 1 januari 1986 volgde een aanstelling als wetenschappelijk onderzoeker bij bovengenoemde afdeling.

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Genetic heterogeneity in human neuraminidase deficiency

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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 (×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	350–1,050	43–129	—
	(*n* = 17)	(*n* = 36)	(*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, *p*H 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, *p*H 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 2×10^6 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.

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

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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

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[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

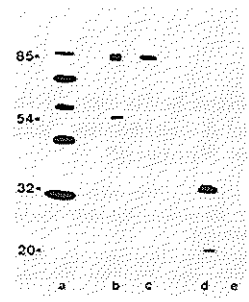

FIG. 1. **Polyacrylamide gel electrophoresis of immunoprecipitated β-galactosidase.** *a*, Standards. *b*, NH_4Cl-stimulated medium above control fibroblasts. *c*, NH_4Cl-stimulated medium above galactosialidosis (β-gal$^-$/neur$^-$) cells. *d*, Galactosialidosis fibroblasts after addition of medium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

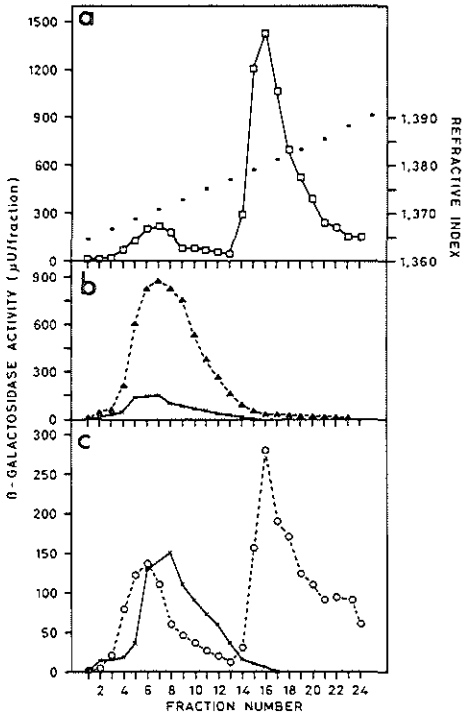

FIG. 2. **Sucrose density gradient centrifugation of lysosomal β-galactosidase in normal and mutant human fibroblasts.** *a*, Density pattern of β-galactosidase in normal fibroblasts (□——□); and ··· indicate the respective indices of the linear sucrose gradient from 20 to 40% in 20 mM Na phosphate, pH 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 (○- - -○).

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

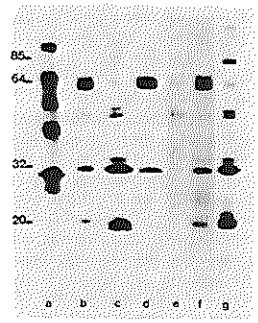

FIG. 3. **Polyacrylamide gel electrophoresis of immunoprecipitated β-galactosidase in pooled fractions after sucrose density gradient centrifugation.** *a*, Standards. *b*, Immunoprecipitate of the total lysate of control fibroblasts. *c*, Cell lysate after sucrose density gradient centrifugation; pooled 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.

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.

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PURIFICATION OF ACID β-GALACTOSIDASE AND ACID NEURAMINIDASE FROM BOVINE TESTIS: EVIDENCE FOR AN ENZYME COMPLEX

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The isolation of an acid neuraminidase from bovine testis is described. Under all experimental conditions this neuraminidase copurifies with acid β-galactosidase, but not with other lysosomal hydrolases. Immunotitration with an antiserum raised against purified human placental β-galactosidase results in the coprecipitation of both enzyme activities. Our data indicate that acid neuraminidase and β-galactosidase are present as an enzyme complex. The possible physiological relevance is discussed.

INTRODUCTION

Neuraminidases catalyze the hydrolysis of sialic acid residues α-ketosidically bound to oligosaccharides, glycoproteins, glycopeptides and glycolipids. They are widely distributed in eukaryotic and prokaryotic cells (1).

The neuraminidases of prokaryotic cells are very well studied (1,2), but relatively little information is available on eukaryotic neuraminidases (3-6).

In the last few years a number of different genetic defects of lysosomal acid neuraminidase have been found in man (for reviews see 7,8). Especially interesting are patients who have a combined deficiency of two lysosomal enzymes, neuraminidase and β-galactosidase (9,10). To understand the possible relationship between neuraminidase and β-galactosidase, it is necessary to have more information about the structure and properties of the different molecular forms of mammalian neuraminidases. Such information will also help to elucidate the molecular nature of genetic diseases associated with a neuraminidase deficiency.

Despite the recent availability of sensitive assay procedures with methylumbelliferyl substrate, the lability and strongly membrane bound character of the enzyme hampered studies on mammalian neuraminidases (3,4) (for review, see 6).

In the present report we describe the copurification of a bovine testis acid neuraminidase with lysosomal β-galactosidase throughout the isolation procedure developed for the latter enzyme. Evidence is presented that β-galactosidase and neuraminidase molecules exist as an enzyme complex.

MATERIALS AND METHODS

Purification procedure

All purification steps were carried out at 4°C, unless otherwise noted. The procedure used is a modification of the isolation method developed for bovine testis β-galactosidase as described earlier (11).

Bovine testes were obtained at the local slaughterhouse and kept frozen at -70°C until use. Testes devoid of epididymes (0.5 kg) were homogenized in 2 vol. distilled water in a Waring blendor for 3 min. The homogenate was brought to pH 4.0 by dropwise addition of 2N HCl. After 30 min stirring the acid homogenate was centrifuged at 25000 x g for 30 min. The clear supernatant was adjusted to pH 6.2 with 2N NaOH. This supernatant was applied to a Concanavalin A-Sepharose column (60 ml bed volume) (Pharmacia) that was previously equilibrated with 50 mM sodium phosphate buffer pH 6.2. After loading, the column was washed overnight with equilibration buffer containing 100 mM sodium chloride. The column was brought to room temperature and washed with equilibration buffer containing 1M sodium chloride and eluted with the same buffer with 1M α-methylglucoside added to it. The eluate was concentrated on an Amicon Hollow Fiber Concentrator and dialysed overnight against 10 vol. of 20 mM sodium acetate buffer pH 5.2 containing 100 mM sodium chloride. This preparation was applied to a 2 ml p-aminophenyl-thiogalactoside-CH-Sepharose column (prepared as described earlier (12)), equilibrated with the same buffer as used for dialysis. The column was then washed extensively with 10 times the bed volume of equilibration buffer and subsequently with the same volume of equilibration buffer containing 1M sodium chloride. β-Galactosidase and neuraminidase were eluted by adding 100 mM γ-galactonolacton to the last buffer. The eluate was concentrated and dialysed in an Amicon Ultrafiltration cell with a PM-10 filter. Dialysis was against 25 mM sodium acetate buffer containing 100 mM sodium chloride. The final preparation was frozen and stored at -70°C.

Assay of protein and enzyme activity

Protein was measured by the method of Lowry (13).

The activity of neuraminidase and the other enzymes was measured with 4-methylumbelliferyl substrates, according to procedures described earlier (8,10). One unit of activity is defined as the amount of enzyme forming 1 μmol of 4-methylumbelliferone per minute at 37°C.

SDS-Polyacrylamide gel electrophoresis

Electrophoresis was carried out on a 12% polyacrylamide slab gel as described by Laemmli (14), with a modified acrylbisacryl ratio of 30 : 0.315.

Gel filtration

A 1 x 95 cm column of Sephadex G-200 (Pharmacia) was equilibrated at 4°C with 25 mM sodium acetate buffer pH 5.2 containing 100 mM sodium chloride. 2 mg of the purified enzyme preparation was mixed with glycerol (final conc. 10%) and applied to the column. The column was run overnight and fractions of 1 ml were collected. All fractions were assayed for both neuraminidase and β-galactosidase.

Immunotitration

10 µl Concanavalin A-Sepharose eluate containing 6.03 mU β-galactosidase and 0.066 mU neuraminidase was added to 10 µl 1M sodium phosphate buffer pH 6.0 containing 1M sodium chloride and 5% bovine serum albumin. An increasing amount of an IgG preparation (5-100 µl) (prepared from an antiserum raised against purified human placental β-galactosidase (15) using Protein A-Sepharose) was added to this mixture. 20 mM sodium phosphate buffer pH 6.0 containing 100 mM sodium chloride was added to a final volume of 120 µl. After 3 hours incubation at 4°C 20 µl of a 1 : 1 protein A-Sepharose suspension in 20 mM sodium phosphate buffer pH 6.0 containing 100 mM sodium chloride was added. After 2 hours all tubes were centrifuged at 10,000 x g for 1 minute. Supernatants were assayed for both neuraminidase and β-galactosidase.

RESULTS AND DISCUSSION

In our initial attempts to purify active acid neuraminidase from a mammalian source, we observed that bovine testis contains large amounts of acid neuraminidase. We also found a considerable neuraminidase activity in the 25000 x g supernatant after acid precipitation, which enabled us to purify an active mammalian neuraminidase. This neuraminidase has a pH optimum around 4.5 and a K_m value of 3 mM using the 4-methylumbelliferyl-N-acetylneuraminic acid substrate.

In the course of studies on the molecular properties of β-galactosidase we found that neuraminidase copurified during purification of bovine testicular β-galactosidase, after two subsequent affinity chromatography steps. The purification and recovery of neuraminidase and β-galactosidase at different stages of a representative purification is summarized in table 1.

Chromatography on Concanavalin A-Sepharose yields a preparation in which neuraminidase and β-galactosidase are purified 25- and 30-fold respectively.

After p-aminophenylthiogalactoside-CH-Sepharose affinity chromatography, which seemed to be specific for β-galactosidase purification, not only a 512-fold purification of β-galactosidase was achieved, but also a 316-fold purification of neuraminidase. Neuraminidase had a somewhat lower overall recovery (17%) than β-galactosidase (28%). However, no neuraminidase activity was present in the unretained material and no residual neuraminidase could be detected on the column after elution. The lower recovery of neuraminidase is therefore probably due to a more pronounced denaturation during the last affinity chromatography step. The activity of neuraminidase is measured with a substrate concentration below the K_m, since higher substrate concentrations result in high blancs, which disturb accurate measurements. The specific

Table 1

Copurification of Neuraminidase (Neur) and β-Galactosidase (Gal).
Results are for 500 g wet weight testes.

Step	Volume	Protein	Activity		Specific activity		Activity	Purification		Yield	
			Neur.	Gal.	Neur.	Gal.	Neur/Gal	Neur.	Gal.	Neur.	Gal.
	ml	mg	mU[a]		mU/mg		$x10^{-2}$	-fold		%	
25000 xg Supernat. pH4	730	5542	2008	99604	0.36	18.0	2.0	1	1	100	100
Con A Sepharose	5.0	87.5	796	47033	9.10	537.5	1.7	25	30	40	47
PAPS-gal-Sepharose[b]	2.5	3.05	347	28083	113.8	9208	1.2	316	512	17	28

[a] nmol 4-methylumbelliferone released/min.

[b] PAPS-gal-Sepharose: p-aminophenylthiogalactoside-CH-Sepharose.

Table 2

Lysosomal hydrolase activities in the purified bovine testicular neuraminidase preparation

	25000 xg Supernatant mU/mg	Purified mU/mg	Purification factor
Neuraminidase	0.36	113.8	x 316
β-Galactosidase	18.0	9208	x 512
β-Glucuronidase	5.7	13.8	x 2
α-Galactosidase	2.4	17.4	x 7
β-Glucosidase	2.6	13.8	x 5
β-Hexosaminidase	204	562	x 3
α-Glucosidase	1.6	1.1	x 1
α-Mannosidase	1.2	1.5	x 1

activities of neuraminidase are therefore an underestimation of the real values.

Table 2 demonstrates that only β-galactosidase and neuraminidase are copurifying during our procedure: none of the other hydrolases tested were substantially purified. The overall yield of these other acid hydrolases varied from 0.02% to 1%. This indicates that the copurification is specific for neuraminidase and β-galactosidase.

Both neuraminidase and β-galactosidase elute in the void volume of a Sephadex G-200 column (Fig. 1), suggesting that both enzymes are present as a high molecular weight aggregate ($>$ 300 kd).

This observation prompted us to try and separate the two enzymes via a variety of separation techniques, e.g. sucrose gradients, aceton fractionation, cellogel electrophoresis and hydrophobic chromatography. However, under all conditions tested, β-galactosidase and neuraminidase could not be separated, which fits the assumption, that both enzymes are present as a complex.

Additional evidence for the existence of an enzyme complex was derived from immunotitration experiments (Fig. 2). In the presence of antibodies raised against purified human placental β-galactosidase, both neuraminidase and β-galactosidase activities were precipitated. The precipitation curves for both enzyme activities are identical, which indicates the simultaneous removal of

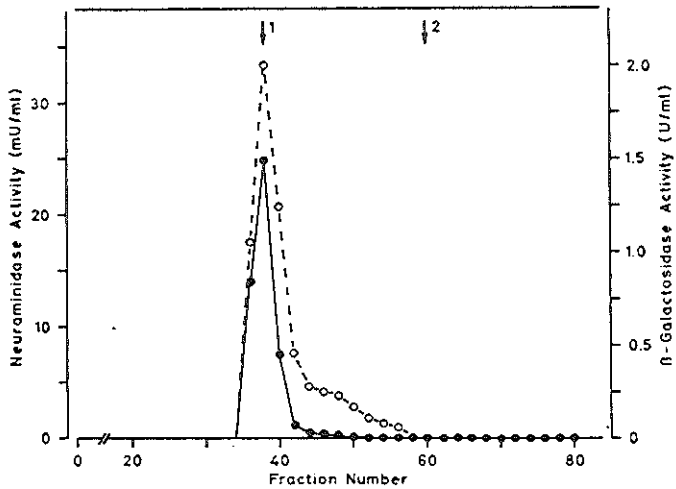

Fig. 1 Elution profile obtained after Sephadex G-200 column chromatography of the purified enzyme preparation.
● Neuraminidase activity
○ β-galactosidase activity
Arrow 1 denotes position of the void volume
Arrow 2 denotes position of bovine serum albumin

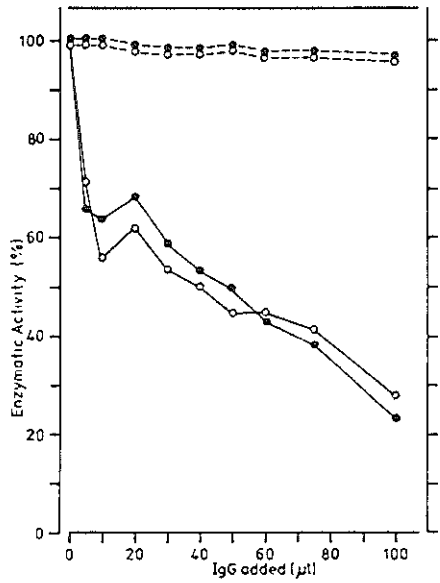

Fig. 2 Immunotitration of bovine testicular neuraminidase and β-galactosidase

●——●	Neuraminidase	Titrated with IgG raised against purified human placental β-galactosidase
○——○	β-galactosidase	
●– –●	Neuraminidase	Titrated with IgG derived from pre-immune serum
○– –○	β-galactosidase	

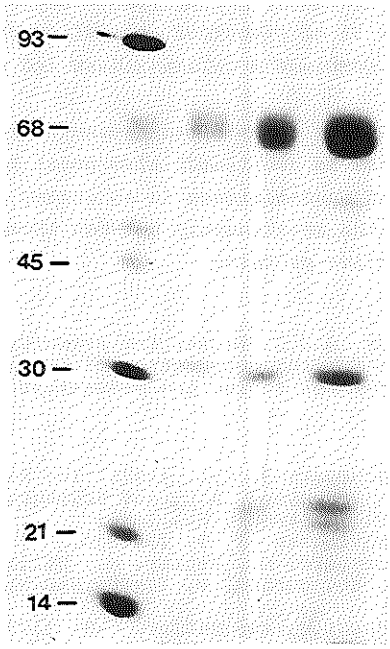

Fig. 3 Polyacrylamide gel electrophoresis of the purified enzyme preparation in the presence of SDS.
From left to right: marker proteins, 10, 20 and 40 µg of enzyme preparation, respectively.

both enzymes and that β-galactosidase and neuraminidase react with the same antibody population.

Polyacrylamide gel electrophoresis of the purified preparation in the presence of SDS shows three major protein bands with molecular weights of 64 kd, 32 kd and 22 kd (Fig. 3). Since bovine testis β-galactosidase monomer has a molecular weight of 64 kd, it is likely that the neuraminidase activity in our purified preparation is associated with the 32 kd and/or 22 kd protein band(s). Distler et al. (16) also reported a 64 kd band for bovine testis β-galactosidase, but did not mention any other components. In human liver, Norden et al. (17), and human fibroblasts, Hoeksema et al. (18) β-galactosidase monomer (64 kd) and high molecular weight aggregates (600-700 kd) were found. Recently our group (d'Azzo et al., 15) using immunoprecipitation, discovered that mature β-galactosidase (64 kd) is formed from a precursor (85 kd) and that in addition a protective glycoprotein (32 kd) is required

to prevent excessive intralysosomal proteolytic degradation. This protective protein and its 54 kd precursor are deficient in fibroblasts from patients with combined β-galactosidase and neuraminidase deficiency.

Preliminary experiments on solubilized neuraminidase from normal human fibroblasts (to be published) also point to the presence of β-galactosidase and neuraminidase in an enzyme complex. We are currently studying the relationship between these two enzymes and the 32 kd protective protein.

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Human placental neuraminidase

Activation, stabilization and association with β-galactosidase and its 'protective' protein

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Supernatant of homogenized human placenta hardly contains lysosomal neuraminidase activity. It is, however, possible to generate remarkably high activity by concentration of a partially purified glycoprotein fraction. This activity is labile to dilution, but can be stabilized by incubation at 37 °C and acid pH.

Using β-galactosidase specific affinity chromatography and immunotitration, we show that the activated and stabilized human lysosomal neuraminidase exists in a complex with β-galactosidase. Sucrose density gradient centrifugation experiments demonstrate that the neuraminidase activity is exclusively present in a high density multimeric form of β-galactosidase.

The formation of multimeric forms of β-galactosidase is known to require a 32000-M_r 'protective' protein. Monospecific antibodies against this 'protective' protein were purified from a conventional antiserum containing a mixture of antibodies against the 64000-M_r β-galactosidase protein and against the 32000-M_r 'protective' protein, using a nitrocellulose blot immunoaffinity purification procedure. Immunotitration experiments with these antibodies show that the 32000-M_r 'protective' protein is present both in association with the β-galactosidase multimer and with the high-density multimeric form together with neuraminidase.

Our data further suggest that association of the 32000-M_r 'protective' protein and another yet unidentified subunit is essential for the catalytic activity of lysosomal neuraminidase. These results explain the absence of neuraminidase activity in the autosomal recessive human lysosomal storage disorder galactosialidosis, where the 32000-M_r 'protective' protein is known to be absent.

A lysosomal glycoprotein and oligosaccharide substrate specific neuraminidase is known to be deficient in the human inherited lysosomal storage disorders, sialidosis and galactosialidosis. In sialidosis it is the only deficient enzyme, whereas in galactosialidosis there is a combined deficiency of lysosomal neuraminidase and β-galactosidase [1–4]. The latter disease was found to be due to a deficiency of a protein with a relative molecular mass of 32000, which function is to protect β-galactosidase from rapid proteolytic degradation [5]. Recently it was shown that this is realized by aggregation of monomeric β-galactosidase into high molecular mass complexes, which apparently are less sensitive to intralysosomal proteases [6].

Very little is, however, known about the molecular background of the neuraminidase deficiency in galactosialidosis. One of the major reasons is that the lability and membrane-bound character of human lysosomal neuraminidase have complicated its purification and characterization [7–9].

Our earlier observation that a lysosomal neuraminidase from bovine testis copurifies with lysosomal β-galactosidase [10], pointed to a complex of these two enzymes. On the basis of this observation and of studies on normal and mutant fibroblasts we have suggested a close relationship between 64000-M_r mature β-galactosidase, its 32000-M_r 'protective' protein and lysosomal neuraminidase [5, 6, 10].

In the present study we demonstrate that it is possible to generate and stabilize considerable neuraminidase activity which enabled us the partial purification of neuraminidase from human placenta. Furthermore the relationship of human lysosomal neuraminidase with β-galactosidase and its 32000-M_r 'protective' protein could be investigated.

EXPERIMENTAL PROCEDURES

Partial purification

All steps were carried out at 4 °C, unless otherwise noted. Frozen human placenta was obtained from a local hospital. About 5 kg placenta was homogenized in 7 l distilled water in a Waring Blendor for 3 min. The homogenate was centrifuged at 25000 × *g* for 30 min. The clear supernatant was collected, set to pH 6.3 with 1 M sodium phosphate buffer and mixed with 200 ml packed Concanavalin-A–Sepharose (Pharmacia). The mixture was stirred overnight. The ConA-Sepharose beads were collected on a glass filter and washed at room temperature with 2 l sodium phosphate buffer pH 6.3 containing 100 mM NaCl. Afterwards the beads were washed with 2 l of the last buffer containing 1 M NaCl. Glycoproteins

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Abbreviations. PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; ConA, concanavalin A.

Enzymes. Neuraminidase (EC 3.2.1.18); β-galactosidase (EC 3.2.1.23).

were eluted with 2 l of buffer containing 1 M NaCl and 1 M methyl α-D-glucopyranoside. The eluate was concentrated and dialysed on an Amicon Hollow Fiber Concentrator. Dialysis was against 20 mM sodium phosphate buffer containing 100 mM NaCl, pH 5.2. The final volume was about 160 ml.

Activation and stabilization of partly purified neuraminidase

The glycoprotein preparation was further concentrated in an Amicon Ultrafiltration Cell, using a PM 10 filter. Concentration was continued to a protein concentration of about 60 mg/ml. This preparation was incubated at 37°C for 90 min to stabilize neuraminidase activity (see Results). Afterwards the preparation was frozen and stored at −70°C.

Affinity purification

Affinity chromatography using *p*-aminophenylthio-galactoside-CH-Sepharose was performed as described earlier [10]. Experiments were performed with activated and stabilized glycoprotein preparations derived from about 5 kg placenta.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of the affinity purified enzyme preparation was performed in the presence of sodium dodecyl sulphate (SDS-PAGE) as described by Laemmli [11]. About 30 μg of protein was applied to the slot.

Immunotitration using antibodies against β-galactosidase

A sample of the ConA-Sepharose eluate, activated and stabilized for neuraminidase, was diluted tenfold in 20 mM sodium phosphate buffer pH 6, containing 100 mM NaCl. 20 μl from this dilution, containing about 0.1 mU neuraminidase and 4 mU β-galactosidase, was mixed with 10 μl of the same buffer containing 2 mg/ml bovine serum albumin, and 0–20 μl of an IgG preparation (prepared from an antiserum raised against purified human placental β-galactosidase, using protein-A–Sepharose [5]) was added. The final volume was made 50 μl by the addition of 20 mM sodium phosphate buffer pH 6, containing 100 mM NaCl. After 2 h incubation at 4°C, 10 μl protein-A–Sepharose suspension (1:1) in the last buffer was added. After 2 h gentle rotation all tubes were centrifuged at 3000 × *g* for 1 min. The supernatants were assayed for both neuraminidase and β-galactosidase activity.

Immunotitration using monospecific antibodies

After sucrose density gradient centrifugation of an activated and stabilized glycoprotein preparation the top fractions from peak I and II were diluted with 40 mM sodium phosphate buffer pH 6, containing 200 mM NaCl and 1 mg/ml bovine serum albumin. About 0.25 mU β-galactosidase was used for each immunoprecipitation. The immunoprecipitation was performed as described above.

Immunoprecipitation of neuraminidase activity was performed using an activated and stabilized glycoprotein preparation. This was diluted appropriately as described for peak I and II. In these experiments about 0.01 mU neuraminidase and about 0.3 mU β-galactosidase were used.

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was performed as described by Martin and Ames [12]. Linear gradients of 20–40% (w/v) sucrose in 20 mM sodium acetate buffer pH 5.2, containing 100 mM NaCl were prepared in a total volume of 5 ml. 100 μl sample was applied. Centrifugation was carried out at 40000 rpm for 15 h at 4°C in a Beckman L5-65 ultracentrifuge with a SW-50 rotor. Fractions of 200 μl were collected and assays were performed on 5-μl samples.

Nitrocellulose blot affinity purification of antibodies

Specific antibodies were purified from a conventional β-galactosidase antiserum, using a slightly modified method of Smith et al. [13]. 30–50 μg of the affinity purified preparation containing β-galactosidase, neuraminidase and 'protective' protein was applied to each slot of a 10% slab gel. After SDS-PAGE the protein was transferred to a nitrocellulose filter by the electroblotting method of Towbin et al. [14]. A strip of the nitrocellulose was cut and stained for protein using 0.1% amido black in 45% methanol and 10% acetic acid. Using the stained strip as comparison, unstained regions of the nitrocellulose containing the 32000-M_r and 64000-M_r protein bands were cut, transferred to separate tubes and incubated for 1 h with P_i/NaCl containing 100 mg/ml bovine serum albumin. Thereafter the tubes were drained and conventional antiserum diluted in P_i/NaCl containing 0.5% Tween 20 was added to each of the tubes. The tubes were rotated at room temperature for 1–3 h. The antiserum was removed and the nitrocellulose pieces were washed 3 times 15 min with P_i/NaCl containing 0.5% Tween 20. Monospecific antibodies were eluted from the appropriate nitrocellulose piece by washing 3 times 30 s with 100 μl 5 mM glycine-HCl pH 2.3, containing 0.5% Tween 20 and 100 μg/ml bovine serum albumin. The eluates were neutralized immediately with 20 μl 1 M sodium phosphate buffer pH 6.

Assays

Protein was measured by the method described by Lowry [15]. Neuraminidase and β-galactosidase activities were measured with the appropriate 4-methylumbelliferyl substrates as reported earlier [16]. (4-Methylumbelliferyl-*N*-acetyl-neuraminic acid was a generous gift of Prof. R. Brossmer, Dept. of Biochemistry, University of Heidelberg, FRG). One unit of activity is defined as the amount of enzyme releasing 1 μmol of 4-methylumbelliferone/min at 37°C.

RESULTS

Activation of partly purified neuraminidase by concentration

When frozen human placenta is homogenized in water and the homogenate is centrifuged at 25000 × *g*, β-galactosidase activity is present in the supernatant, but neuraminidase activity is below detection (Table 1). When the glycoproteins from the supernatant were purified using ConA-Sepharose affinity chromatography, the specific activity of β-galactosidase had increased, but still hardly any neuraminidase activity could be measured. Neuraminidase activity can, however, be generated through concentration of the glycoprotein fraction (Fig. 1A). For every twofold concentration, the neuraminidase activity increases about fourfold, whereas the increase of β-galactosidase activity is linear with concentration as expected. The process of neuraminidase activation

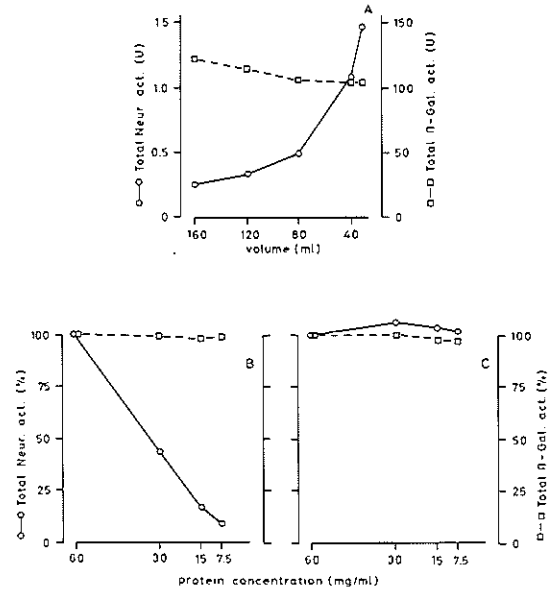

Fig. 1. *Activation and stabilization of neuraminidase in a partly purified glycoprotein preparation from human placenta.* (A) Effect of concentration of the preparation on neuraminidase (Neur.) and β-galactosidase (β-Gal.) activity. (B) Effect of dilution after concentration. (C) The same, but before dilution the preparation was incubated at 37 °C and pH 5.2 for 90 min. For experimental details, see text

Table 1. *Partial purification of β-galactosidase and neuraminidase*
5 kg frozen human placenta was homogenized and centrifuged at 25000 × *g*. The glycoproteins from the supernatant were purified using Concanavalin-A–Sepharose. The concentration and stabilization treatments were performed as described in the text. All data shown are derived from a representative experiment. n.d. = not detectable. Neur., neuraminidase; β-Gal., β-galactosidase. Neuraminidase recovery cannot be calculated because of lack of initial activity

Preparation	Total activity		Specific activity		Recovery β-Gal.
	Neur.	β-Gal.	Neur.	β-Gal.	
	U		mU/mg protein		%
25000 × *g* supernatant	n.d.	293	n.d.	0.5	100
Glycoprotein preparation	0.26	127	0.13	63.3	43
Glycoprotein preparation after concentration	1.6	109	0.83	55.0	37
The same but after stabilization of neuraminidase activity	3.0	115	1.50	58.3	39

seems to be reversible, since dilution of the concentrate leads to a loss of the neuraminidase activity, while that of β-galactosidase remains constant (Fig. 1 B).

Stabilization of the generated neuraminidase activity can be achieved by incubation of the concentrated glycoprotein fraction at 37 °C and pH 5.2. During this stabilization a further increase in activity is observed, and after this treatment the neuraminidase activity is no longer lost upon dilution (Fig. 1 C). The activity thus obtained appears to be very stable. The preparation can even be sonicated for long periods and can be frozen without any loss of activity. The activities during the various steps described above are summarized in Table 1.

The relation of neuraminidase and β-galactosidase

To investigate the relationship between this neuraminidase and β-galactosidase the concentrated and stabilized glycoprotein preparation was chromatographed on a *p*-aminophenylthiogalactoside-CH-Sepharose affinity column. This experiment is summarized in Table 2. In the preparation

Table 2. *Copurification of β-galactosidase and neuraminidase during β-galactosidase specific affinity chromatography*
A glycoprotein preparation purified from human placenta was treated as described in the text, to activate and stabilize neuraminidase activity. Afterwards the preparation was chromatographed on a *p*-aminophenylthiogalactoside-CH-Sepharose column, which is an affinity column specific for β-galactosidase. Neur., neuraminidase; β-Gal., β-galactosidase

Preparation	Total activity		Specific activity		Recovery	
	Neur.	β-Gal.	Neur.	β-Gal.	Neur.	β-Gal.
	U		mU/mg protein		%	
Before affinity chromatography	4.35	70.4	3.0	48.3	100	100
After affinity chromatography	1.00	40.7	555	22600	23	58

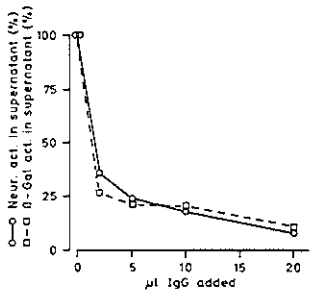

Fig. 2. *Immunotitration of activated and stabilized human placental neuraminidase (Neur.) and β-galactosidase (β-Gal.).* A glycoprotein preparation from human placenta was concentrated to a protein concentration of about 60 mg/ml, and was incubated at 37°C pH 5.2 for 90 min. This fully neuraminidase-activated and stabilized preparation was titrated with anti (β-galactosidase) IgG. The antibodies were raised against a β-galactosidase preparation that was purified under such conditions that neuraminidase did not copurify

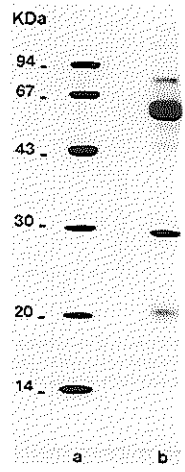

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of the affinity purified β-galactosidase/'protective' protein/neuraminidase preparation.* Before affinity chromatography the glycoprotein preparation was treated as described in the text to activate and stabilize neuraminidase. Lane a: marker proteins. Lane b: purified preparation

obtained after this β-galactosidase specific affinity chromatography the specific activities of both β-galactosidase and neuraminidase had increased dramatically. Furthermore no neuraminidase activity could be eluted prior to the specific elution together with β-galactosidase. This points to copurification of both enzymes, presumably because they are present as a complex.

Additional evidence for the existence of such a complex was derived from immunotitration experiments, using antibodies raised against purified β-galactosidase. The activities of β-galactosidase and neuraminidase were found to precipitate simultaneously (Fig. 2).

Polyacrylamide gel electrophoresis of the affinity purified preparation in the presence of sodium dodecyl sulphate showed three major protein bands (Fig. 3): a protein with a M_r of about 64000, a protein with a M_r around 30000 and a protein with a M_r around 20000. Some minor bands are visible.

Since β-galactosidase is known to exist in different molecular forms, we used sucrose density gradient centrifugation to study the exact relationship between β-galactosidase and neuraminidase. In the partially purified glycoprotein preparation β-galactosidase activity is mainly present in the 64000-M_r monomeric form, and neuraminidase activity was undetectable (Fig. 4a). In the same preparation after activation and stabilization of neuraminidase, β-galactosidase activity was found both in the monomeric form (about 30%), and in a high molecular mass multimeric form (about 60%). In addition β-galactosidase activity is present in a second multimeric form of still higher density. All neuraminidase activity was found to be present in this latter multimeric peak (Fig. 4b). When a concentrated preparation was not stabilized, β-galactosidase activity was present in two peaks of activity, one monomeric and one multimeric. The higher density multimeric form was not present under these conditions and neuraminidase activity was absent (Fig. 4c). Since without stabilization treatment neuraminidase activity is lost upon dilution (Fig. 1b) and sucrose density gradient centrifugation implies dilution, the loss of neuraminidase activity can be explained.

From these experiments we conclude that the presence of lysosomal neuraminidase in a complex with the high density multimeric form of β-galactosidase is essential for its stability and catalytic activity, at least *in vitro*.

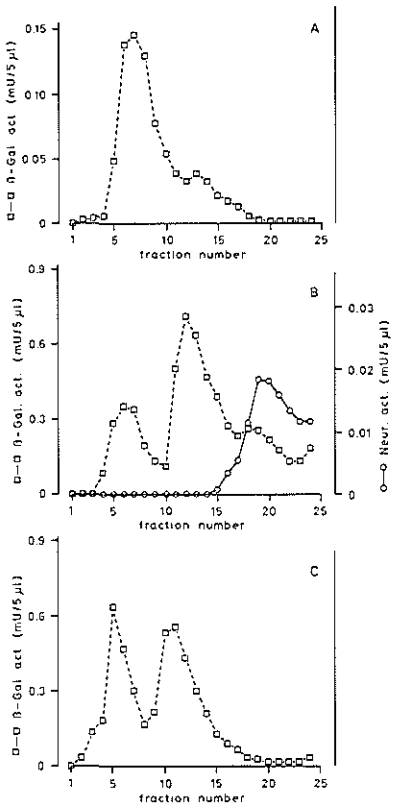

Fig. 4. *Sucrose density gradient centrifugation of the placental glycoprotein preparation.* (A) Before concentration; (B) after concentration and stabilization; (C) after concentration only. For experimental details, see text. Neur., neuraminidase; β-Gal., β-galactosidase

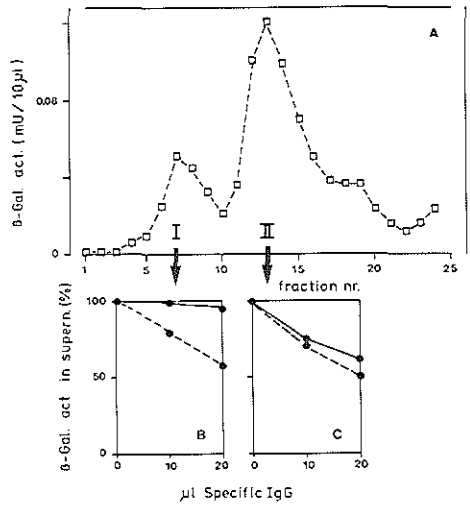

Fig. 5. *Immunoprecipitation of different molecular forms of β-galactosidase (β-Gal.) using monospecific antibodies.* Sucrose density gradient centrifugation of β-galactosidase (A), was followed by immunotitration of the β-galactosidase monomeric form I (B) and of the β-galactosidase multimeric form II (C), with anti- (β-galactosidase) specific IgG (●-----●) and with anti-('protective' protein) specific IgG (●——●). These specific IgG preparations were extracted from an conventional anti-(β-galactosidase) antiserum containing a mixture of antibodies against β-galactosidase and 'protective' protein, using a nitrocellulose-blot affinity-purification procedure, as described in the text

The relationship between neuraminidase and the 32000-M_r*, 'protective' protein*

For the multimerization of β-galactosidase a 32000-M_r 'protective' protein is required, as was demonstrated for human fibroblasts [6]. Fig. 3 shows that this protein is also present in the affinity column eluate when the placental glycoprotein preparation is applied to the column after induction of complex formation. The apparent M_r of this protein seems to be slightly different from the M_r of the protein in human fibroblasts. However, since this protein is a glycoprotein, the observed difference is not significant. Therefore, to avoid confusion with the previous literature [5, 6], we maintain the established terminology as a 32000-M_r protein.

To investigate whether the 32000-M_r protein was also present in the complex of the high density multimer of β-galactosidase and neuraminidase, immunoprecipitation studies were carried out. For this purpose, specific antibodies against the 32000-M_r protein and against the 64000-M_r protein were extracted from a rabbit antiserum containing both types of antibodies, as described under Experimental Procedure. With immunoblotting it was verified that the anti-(32000-M_r protein) antibodies did not cross-react with the 64000-M_r protein band, and vice versa (results not shown).

Fig. 5 illustrates the reactivity of both types of monospecific antibodies with peak I and peak II of β-galactosidase activity. Antibodies against the 64000-M_r protein band are able to precipitate both the monomeric (peak I) and the multimeric (peak II) form of β-galactosidase, whereas anti-(32000-M_r protein) antibodies only precipitate the high molecular mass form of β-galactosidase (peak II).

As is shown in Fig. 6, neuraminidase and β-galactosidase activity coprecipitate with both the anti-(32000-M_r protein) and the anti-(64000-M_r protein) antibodies. No precipitation was observed with monospecific antibodies against a 50000-M_r protein contaminant which were extracted from the same rabbit antiserum. This demonstrates the specificity of the immunological method used, and it shows the stability of neuraminidase during the immunoprecipitation.

From these results we conclude that the 32000-M_r 'protective' protein is not only closely associated with the β-galactosidase multimer, but also with the neuraminidase associated high density multimer of β-galactosidase.

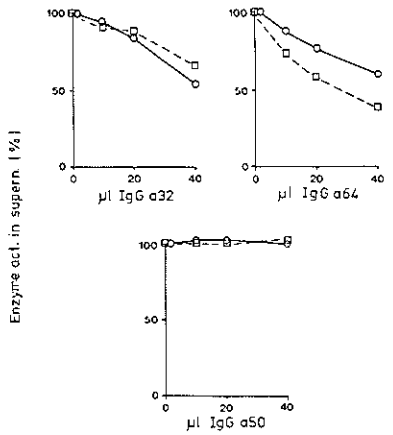

Fig. 6. *Immunotitration of placental neuraminidase and β-galactosidase with different nitrocellulose-blot affinity-purified antibodies.* An activated and stabilized glycoprotein fraction from human placenta containing neuraminidase activity (○——○) and β-galactosidase activity (□----□) was treated with increasing amounts of nitrocellulose-blot affinity-purified monospecific IgG preparations against the 32000-M_r protein band, the 64000-M_r protein band and a 50000-M_r protein band contaminant. After incubation as described in the text the activities remaining in the supernatants were measured

DISCUSSION

Although lysosomal neuraminidase is known as a membrane-bound enzyme [3, 7–9, 17], our data show that neuraminidase is also present in the supernatant of human placental tissue though in an inactive form. Its activity can be generated by concentration. The activity increases quadratically with concentration, which suggests that the aggregation of at least two subunits is essential for neuraminidase activity. The stabilization of the generated activity by incubation at 37°C and acid pH probably shifts the existing equilibrium between inactive, not aggregated subunits and active aggregated subunits towards the latter. This equilibrium apparently shifts easily by freezing and homogenization to the inactive not aggregated subunits, which explains the well-known lability of neuraminidase and the absence of activity in frozen and homogenized preparations [3, 8, 17, 18].

Using affinity chromatography and immunotitration we provide evidence that the existence of neuraminidase in a complex with β-galactosidase is quite a general phenomenon, since it was found in human placenta (Fig. 4 and Table 2) in addition to bovine testis [10], human fibroblasts (unpublished results) and human leucocytes [19].

The 32000-M_r protein, which is present in the final preparation of β-galactosidase and neuraminidase, is a prerequisite for the formation of the high molecular mass enzyme complexes. In fibroblasts from patients with a genetically determined deficiency of the 32000-M_r protein (galactosidosis), β-galactosidase remains in the monomeric form which is sensitive to degradation by lysosomal proteases [6]. The deficiency of neuraminidase in galactosialidosis points to a further relation between this enzyme and the 32000-M_r 'protective' protein. In the present studies we have demonstrated, *in vitro*, the association of lysosomal neuraminidase with a high density multimeric β-galactosidase.

Using monospecific anti-(32000-M_r protein) antibodies it was shown that the 32000-M_r 'protective' protein is also associated with this high density multimeric β-galactosidase together with neuraminidase. The simultaneous disappearance of this high density β-galactosidase multimer and neuraminidase activity, as observed in Fig. 4c, supports the hypothesis that the 32000-M_r protein is a common subunit of lysosomal β-galactosidase and neuraminidase. It prevents intralysosomal degradation of β-galactosidase, but at the same time it is essential for the expression of neuraminidase activity.

Since cells from patients with G_{M1}-gangliosidosis have a normal neuraminidase activity despite the absence of β-galactosidase molecules [20], the complex of neuraminidase with β-galactosidase is not essential for its activity, at least *in vivo*.

In addition to the 32000-M_r subunit, another yet unknown subunit seems to be required for neuraminidase activity. The 20000-M_r protein, which is present in the purified enzyme preparation, is not likely to be the unknown subunit of neuraminidase. This cleavage product formed during proteolytic processing of the 'protective' protein [6] is present with the 32000-M_r protein in the multimeric form of β-galactosidase (peak II), where no neuraminidase activity was found. With the identification of the unknown subunit it must be taken into account that the present studies have been performed on soluble neuraminidase, whereas most of this enzyme is membrane-bound.

The method which we developed to generate neuraminidase activity *in vitro* will be useful for the further purification and characterization of the different neuraminidase subunits. Information about the subunit structure of neuraminidase is essential for the understanding of the molecular nature of human disorders associated with neuraminidase deficiency.

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PURIFICATION AND PARTIAL CHARACTERIZATION OF LYSOSOMAL NEURAMINIDASE FROM HUMAN PLACENTA

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SUMMARY

Lysosomal neuraminidase and β-galactosidase are present in a complex together with a 32 kDa protective protein. This complex has been purified and the different components have been dissociated using potassium isothiocyanate (KSCN) treatment. β-Galactosidase remains catalytically active, but neuraminidase loses its activity upon dissociation. The inactive dissociated neuraminidase was purified by removing the remaining non-dissociated β-galactosidase/protective protein complex using β-galactosidase specific affinity chromatography. The dissociated neuraminidase material shows two major polypeptides on SDS-PAGE with an apparent molecular mass of 76000 and 66000. Subsequently the 32 kDa protective protein was dissociated from the β-galactosidase/protective protein complex, and purified.

Antibodies raised against the dissociated inactive neuraminidase preparation specifically immunoprecipitate the active neuraminidase present in the complex with β-galactosidase and protective protein.

By immunoblotting evidence is provided that the 76 kDa protein is a subunit of neuraminidase which, in association with the 32 kDa protective protein, is essential for neuraminidase activity.

INTRODUCTION

Studies on neuraminidase (EC 3.2.1.18) from bacterial, viral and eukaryotic sources have provided information on the substrate specificity of this enzyme. Substrates may be either sialylated glycoproteins and oligosaccharides or gangliosides. Furthermore evidence has been presented for the existence of different neuraminidases, with a different substrate specificity and subcellular localization (1-4).

Interest in human lysosomal neuraminidase has been stimulated by the discovery that this enzyme is involved in different inherited lysosomal storage disorders (5-8).

We have recently demonstrated that in various cell types lysosomal neuraminidase occurs in a complex with the lysosomal enzyme β-galactosidase (EC 3.2.1.23) and a 32000 molecular mass glycoprotein (9,10). This 32 kDa protein is known to be essential for the aggregation of β-galactosidase monomers (11), and is required for the expression of neuraminidase activity (10). The molecular nature of neuraminidase, however, is still unknown.

Since human placental neuraminidase can be activated, stabilized and subsequently copurified with β-galactosidase by β-galactosidase specific affinity chromatography, we have developed a purification procedure for neuraminidase subunits, which is based on the complex formation with β-galactosidase. This paper deals with this purification procedure, the preparation of specific antibodies and the characterization of the purified neuraminidase associated polypeptides.

EXPERIMENTAL PROCEDURES

Dissociation of the β-galactosidase/ protective protein/neuraminidase complex

A by concanavalin A chromatography obtained glycoprotein fraction from human placenta, was excessively concentrated and subsequently incubated at 37°C for 90 min., to obtain active and stable neuraminidase activity (10).

A 70 µl sample of this glycoprotein fraction was diluted 1 to 1 with either water, or a 1.6 M or 3 M KSCN solution to study the association and dissociation of the neuraminidase/β-galactosidase complex. The analysis was performed by sucrose density gradient centrifugation as described (12) . Linear gradients of 20-40 % (w/v) sucrose in 20 mM sodium acetate buffer containing 100 mM NaCl pH 5.2 were prepared in a total volume of 5 ml. After 15 h. centrifugation (40000 rpm, Beckman L5-65 centrifuge, SW-50 rotor) 200 µl fractions were collected and enzyme assays were performed.

Purification of inactive neuraminidase polypeptides

Neuraminidase was copurified with β-galactosidase by affinity chromatography on p-aminophenylthiogalactoside-CH- Sepharose (PAPS-Gal-Seph.) as described previously (9,10). The purified β-galactosidase/ protective protein/neuraminidase complex was then mixed with an ice-cold 3M KSCN solution to a final concentration of 0.8 M KSCN, which dissociates neuraminidase from the β-galactosidase / protective protein complex.

Subsequently the KSCN concentration was lowered ten-fold by dilution with 20 mM sodium acetate buffer containing 100 mM NaCl pH 5.2 and the preparation was applied again on a PAPS-Gal-Seph. column (3 ml bedvolume) at a flowrate of 15 ml/h. The unretained material of this column containing dissociated inactive neuraminidase was collected, dialysed and concentrated in an Amicon Ultrafiltration Cell, using a PM 10 filter.

The column was washed with 20 mM sodium acetate buffer containing 0.5 M NaCl, pH 5.2. and the ß-galactosidase/ protective protein complex was eluted with the same buffer containing 100 mM γ-galactonolactone. After concentration and dialysis this complex was dissociated by dropwise addition of a cold solution of 3M KSCN to a final concentration of 1.5 M. The KSCN concentration was lowered ten-fold by dilution with 20 mM sodium acetate buffer containing 100 mM NaCl, pH 5.2 before the preparation was chromatographed again on a PAPS-Gal-Seph. column. The unretained material was checked for the presence of dissociated protective protein subunits and the eluate for ß-galactosidase polypeptides. All preparations were frozen and stored at -70°C.

Polyacrylamide gel electrophoresis

Electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed on 10% slab gels according to Laemmli (13).

Immunological methods

The purified inactive dissociated neuraminidase preparation (20-50 µg) was diluted to 1 ml with phosphate buffered saline pH 7.4 and mixed with 1 ml incomplete Freund's adjuvant. Rabbits were injected subcutaneously. After three weeks a first booster was given and ten days later a second one. Serum was collected eight days later.

A glycoprotein fraction from human placenta containing about 4 mU ß-galactosidase and about 0.1 mU activated and stabilized neuraminidase was immunotitrated with an IgG preparation, which was prepared from the antiserum raised against the purified inactive neuraminidase preparation, using protein A-Sepharose.

The experiment was performed as described earlier for antibodies against ß-galactosidase (10); for the precipitation of immunecomplexes 30 µl of a 1:1 suspension of protein A-Seph. in phosphate buffered saline was added.

The different aggregation forms of β-galactosidase/ protective protein/neuraminidase were immunotitrated as follows. After sucrose density gradient centrifugation of an activated and stabilized glycoprotein preparation the topfractions from peak I, II and III were diluted with 40 mM sodium phosphate buffer containing 200 mM NaCl and 1 mg/ml bovine serum albumin, pH 6.0.

Samples containing about 4 mU β-galactosidase and 0.1 mU neuraminidase (only in peak III) were used for each precipitation. Immunotitration was performed as described (10).

Immunoblotting was performed as follows. After SDS-PAGE the proteins (20-50 µg) were transferred to a nitrocellulose filter by electroblotting as described by Towbin et al. (14). Strips of nitrocellulose containing the transferred proteins were incubated 1 h at room temperature in phosphate buffered saline containing 100 mg/ml bovine serum albumin and subsequently overnight at 4°C in the appropriate antiserum dilution in phosphate buffered saline containing 0.5 % Tween 20, pH 7.4.

Bands were visualized by 2 h incubation at room temperature with an alkaline phosphatase goat-(anti-rabbit IgG) conjugate (Tago) (2 µl in 5ml PBS containing 0.5 % Tween 20). Then strips were incubated in an alkaline phosphatase substrate solution (1 mg Naphtol AS-MX phosphate disodium salt, Sigma, and 3 mg 4-aminodiphenylamine diazonium sulphate, Sigma, per ml 0.2 M Tris-HCl containing 10 mM $MgCl_2$, pH 9.1). After 1 to 5 min blue-coloured spots developed and the reaction was terminated by washing with water.

Assays

Protein was measured as described by Lowry et al (15). Neuraminidase and β-galactosidase activities were measured with the appropriate 4-methylumbelliferyl substrates as reported earlier (16). One Unit of activity is defined as the amount of enzyme releasing 1 µmol 4-methylumbelliferone/min at 37°C.

RESULTS

Dissociation of the β-galactosidase/protective protein/neuraminidase complex

Lysosomal neuraminidase was activated and stabilized in a glycoprotein fraction from human placenta by excessive concentration and subsequent incubation at 37°C.

Sucrose density gradient centrifugation shows that the high density molecular mass complex between β-galactosidase, the 32 kDa protective protein and neuraminidase can be dissociated by treatment with 0.8 M KSCN (Fig.1A and B). This dissociation is accompanied by a total loss of neuraminidase activity. When the preparation is treated with KSCN, and not separated on sucrose density gradients, but dialysed rapidly to remove KSCN, the neuraminidase activity can be partially restored (data not shown). The middle peak, representing an aggregation form of β-galactosidase and protective protein, without neuraminidase, dissociates only after treatment with a higher concentration (1.5 M) of the chaotropic agent KSCN (Fig.1C).

Purification of the different molecular components of the complex

The observations described above have led to a purification procedure for the different neuraminidase associated polypeptides. A summary of the procedure is shown by the flow chart in Fig.2.

Firstly, neuraminidase is activated and stabilized in a glycoprotein fraction from human placenta and copurified with β-galactosidase by PAPS-Gal-Seph.chromatography. Neuraminidase activity can still be measured in the purified complex as is shown in Table 1.

To separate the different components of this complex KSCN was first added to a concentration of 0.8 M, under which conditions the very high density complex containing neuraminidase activity is lost, whereas the β-galactosidase-32 kDa protein

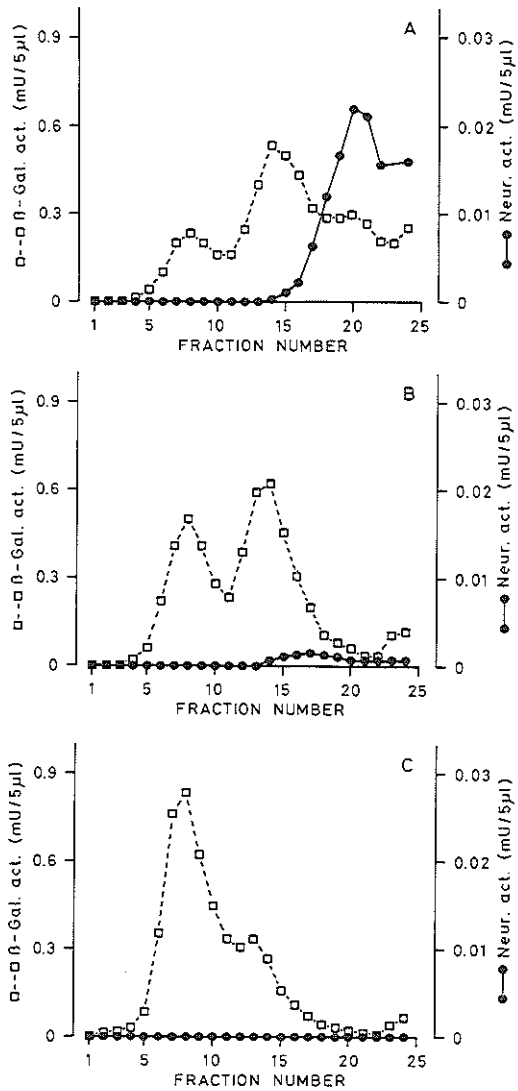

Fig.1 Dissociation of the ß-galactosidase/ protective protein/neuraminidase complex by KSCN treatment. A glycoprotein fraction containing ß-galactosidase and activated and stabilized neuraminidase activity was treated with different concentrations of KSCN and subsequently applied on sucrose density gradients to study the dissociation of the different aggregation forms. a) no treatment b) 0.8 M KSCN treatment c) 1.5 M KSCN treatment.

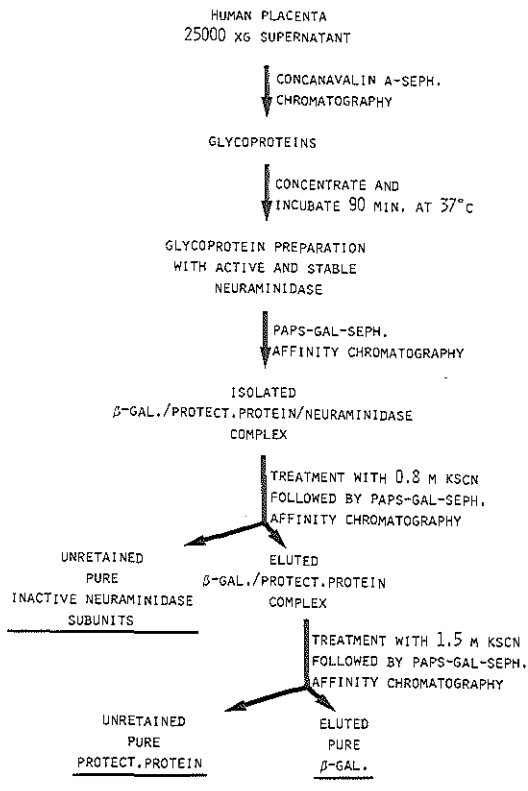

Fig.2 Flow chart of the purification procedure developed for the purification of the two different neuraminidase subunits (for details see text).

complex remains intact. The proteins that are no longer associated with the β-galactosidase-32 kDa protein complex are recovered from the run through from a second PAPS-Gal-Seph. column, whereas the remainig β-gal./32 kDa complex is specifically eluted with γ-galactonolactone. This complex is then dissociated with a higher concentration of KSCN (1.6 M), and its components are separated by affinity chromatography on PAPS-Gal-Seph. The neuraminidase activity, β-galactosidase activity and protein concentrations of all fractions during purification are listed in Table 1.

Table 1

Purification of the different β-galactosidase and neuraminidase subunits from 3 kg human placenta. n.d. = not detectable, Neur. = neuraminidase, β-Gal. = β-galactosidase

purification step		protein	total activity Neur.	total activity β-Gal.	specific activity Neur.	specific activity β-Gal.	recovery β-Gal.
		mg	U		mU/mg protein		%
supernatant after homogenization and centrifugation (25000 x g. 30 min.)		180000	n.d.	94.0	-	0.5	100
Con-A-Seph.chromatogr. followed by activation and stabilization treatment		1968	1.6	63.0	0.81	32.0	67
PAPS-gal.-Seph. chromatogr.		1.41	0.29	26.3	206	18650	28
0.8M KSCN treatment followed by PAPS-gal.-Seph. chromatogr.	eluted	0.52	n.d.	9.5	-	18460	10
	unretained	0.22	n.d.	n.d.	-	-	-
1.5M KSCN treatment followed by PAPS-gal.-Seph. chromatogr.	eluted	0.20	n.d.	6.0	-	30000	6
	unretained	0.10	n.d.	n.d.	-	-	-

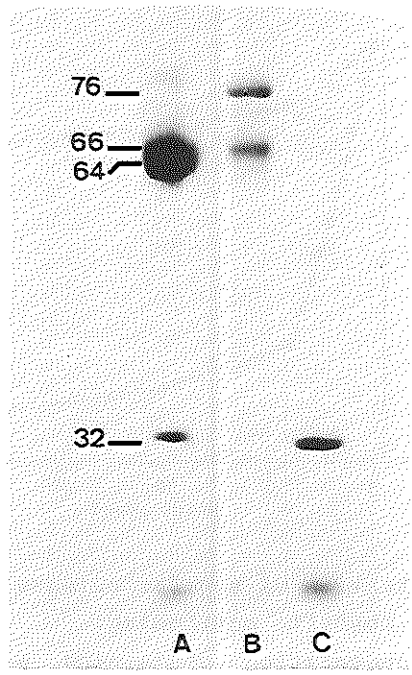

Fig.3 SDS-PAGE pattern of the different purified polypeptides. Lane A : purified β-gal./prot.protein/neur.complex.
Lane B : purified inactive neuraminidase preparation.
Lane C : purified protective protein.
Numbers show molecular mass in kDal.

The SDS-polyacrylamide gel electrophoresis pattern of the preparations during the different stages of purification is shown in Fig.3.

Lane A shows that the purified intact complex consists of two major protein bands. The band with a relative molecular mass of 64 kDa is known to be the mature form of β-galactosidase and the 32 kDa band represents the earlier described protective protein. Lane B shows that the 0.8 M KSCN run through of the second PAPS-Gal-Seph. column contains two major bands with molecular masses of 76 kDa and 66 kDa, which could thus represent dissociated subunits of neuraminidase. The purified 32 kDa protective protein after the third PAPS-Gal-Seph. column is shown in lane C.

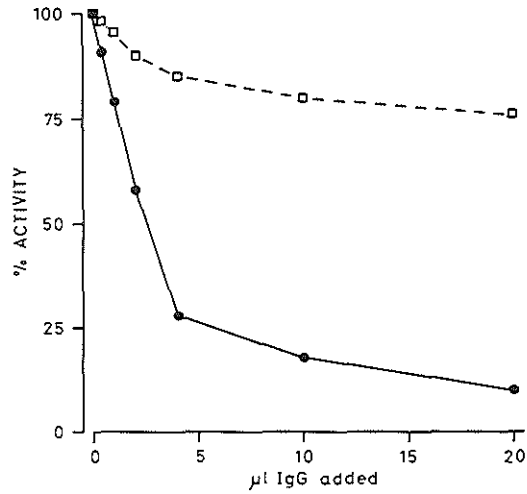

Fig.4 Immunotitration of ß-galactosidase and neuraminidase with neuraminidase specific antibodies.
A glycoprotein preparation containing ß-galactosidase (□---□) and activated and stabilized neuraminidase(●——●) was titrated with an IgG preparation prepared from antiserum against the purified inactive neuraminidase preparation.

Immunological studies of the neuraminidase associated poly peptides

Antibodies were raised in rabbits against the purified inactive neuraminidase polypeptides illustrated in Fig.3 lane B. The antibodies were tested with a total glycoprotein preparation from human placenta containing both ß-galactosidase and neuraminidase (Fig.4). The antibodies precipitated 20% of the ß-galactosidase activity and all neuraminidase activity.

The specificity of the antibodies was further investigated by immunoprecipitation of the different aggregation forms of ß-galactosidase as observed after sucrose density gradient centrifugation (Fig.5 middle and bottom).

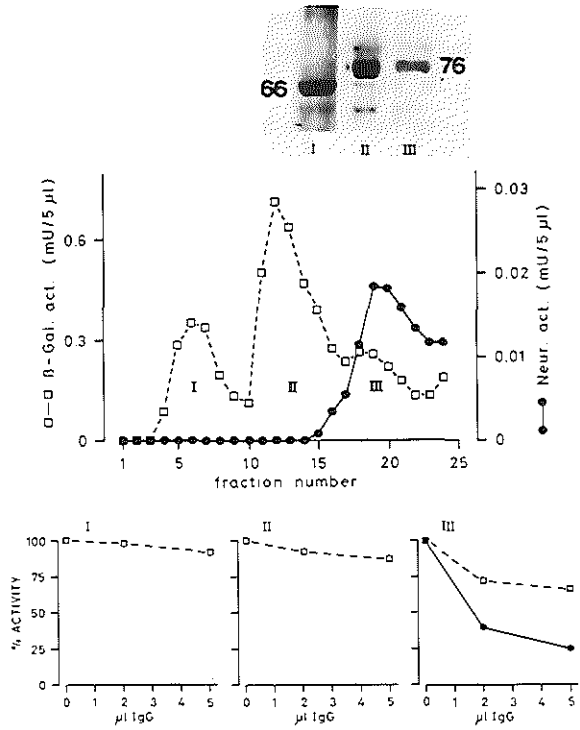

Fig.5 Immunotitration and immunoblotting of the different molecular forms of β-galactosidase/ protective protein/neuraminidase using sucrose density gradient centrifugation of a placental glycoprotein preparation.
Fractions from peaks I, II, and III (middle) were immunotitrated with an IgG preparation prepared from the neuraminidase specific antiserum (bottom).

□--□ ,β-galactosidase; ●—● ,neuraminidase

The same fractions were also subjected to SDS-PAGE followed by immunoblotting with the same IgG preparation (top, inserted photograph). Numbers are shown in kDal.

The antibodies precipitated neuraminidase activity and β-galactosidase activity present in the high density multimeric complex of the two enzymes (peak III). The other aggregation forms of β-galactosidase (peak I and II) could not be precipitated by this antiserum.

To further specify the nature of the immune recognition and to identify the 76 kDa and 66 kDa molecular components of the neuraminidase complex immunoblotting experiments were performed on a total glycoprotein preparation. The results for the different aggregation forms after activation and stabilization of neuraminidase are shown in Fig. 5 (top inserted photograph). In peak III, where all neuraminidase activity is present, only the 76 kDa polypeptide is reacting with the antibodies raised against inactive dissociated neuraminidase.

This polypeptide is also present in peak II, probably representing inactive not fully associated neuraminidase. The 66 kDa polypeptide could be visualized only in peak I, which is known to contain monomeric β-galactosidase.

Similar results were obtained when a purified β-galactosidase/protective protein/neuraminidase complex was subjected to SDS-PAGE followed by immunoblotting.

Immunoblotting of the purified inactive neuraminidase preparation revealed that the antibodies were mainly directed against the 76 kDa component.

DISCUSSION

Recently we have demonstrated that lysosomal neuraminidase from human placenta consists of at least two different polypeptides which upon aggregation yield active neuraminidase (10).

One of these polypeptides was already known as the 32 kDa β-galactosidase protective protein, which is necessary for the aggregation of β-galactosidase molecules. This high molecular mass multimer protects β-galactosidase against rapid intralysosomal proteolytic degradation (11,17).

This 32 kDa polypeptide is also essential for the catalytic activity of lysosomal neuraminidase (10). Its genetic deficiency occuring in the human lysosomal storage disorder galactosialidosis consequently results in a combined deficiency of β-galactosidase and neuraminidase (5,18). So far other neuraminidase specific polypeptides had not been identified.

In the present paper we describe the purification and partial characterization of different neuraminidase polypeptides.

The purified inactive neuraminidase preparation contains a 76 kDa and 66 kDa polypeptide.

Immunoblotting studies showed that the 66 kDa component is not associated with neuraminidase activity. At sucrose density gradient centrifugation this polypeptide is found in the same position as monomeric β-galactosidase. The 66 kDa polypeptide could either be a breakdown product or a contaminant, which perhaps represents some β-galactosidase which remained associated with neuraminidase.

The 76 kDa protein on the other hand is associated with neuraminidase activity and is present together with the high density multimeric form of β-galactosidase. These characteristics suggest that the 76 kDa polypeptide is a subunit of neuraminidase essential for its catalytic activity.

The 76 kDa subunit is also present in the low density molecular mass complex consisting of β-galactosidase and

protective protein without neuraminidase activity (Fig.5 peak II) . Extensive concentration of dialysed fractions from this complex did however generate some higher molecular mass form with neuraminidase activity.

In conclusion it appears that neuraminidase is composed of two different subunits with molecular masses of 32 kDa and 76 kDa, which are both essential for the expression of the enzyme activity. In glycoprotein fractions of human placenta, bovine testis and some other tissues neuraminidase activity can be generated upon association of these two subunits with each other and with lysosomal β-galactosidase. It is still unclear how these observations relate to the in vivo situation and further studies on the stoichiometry of the complex are required.

Several neuraminidases with different substrate specificity and different subcellular localization have been described (1), but only a few reports deal with purified preparations of lysosomal neuraminidase. Partial purification of a lysosomal neuraminidase from rat liver has been described (19). This enzyme was reported to be a monomer of 60 kDa and aggregation with β-galactosidase or any other polypeptide was not required for its catalytic activity. The enzyme presented cleavage specificity for oligosaccharides and glycopeptides. Also a neuraminidase from human leucocytes was partially purified. This enzyme has an average molecular mass of 48 kDa and specificity for oligosaccharides and glycoproteins (20). Since human leucocytes contain at least two different neuraminidases (4), it is not clear which enzyme has been purified.

In the studies mentioned above (19,20) no relationship between neuraminidase, a 32 kDa polypeptide and β-galactosidase was demonstrated, which makes it unlikely that the enzymes concerned are the same as the one involved in the human genetic disorders sialidosis and galactosialidosis.

One report deals with neuraminidase in rough tissue homogenates from human placenta and the kinetic characteristics of this enzyme are similar to those of our

activated and stabilized form (21).

Our previous work (10) indicated the presence of two different subunits in human placental neuraminidase and in the present paper this work is extended by their purification. The antibodies raised against the inactive neuraminidase preparation will enable us to further study the stoichiometry of the ß-galactosidase/neuraminidase complex and to elucidate the nature of the molecular defect in different variants of the autosomal recessive lysosomal disorder sialidosis.

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TWO GENETICALLY DIFFERENT MU-NANA* NEURAMINIDASES IN HUMAN LEUCOCYTES

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Human leucocytes contain two different MU-NANA neuraminidases, which can be distinguished by Concanavalin A binding. The Con A binding form is predominant in lymphocytes (more than 80%) and the non-binding form predominates in granulocytes. The pH optima of both these neuraminidases as well as their subcellular localization as determined by Percoll gradient centrifugation suggest that they are both lysosomal. Immunological studies indicate that the Con A binding form is present in a complex with β-galactosidase whereas the non-binding form is not. Leucocytes from patients with sialidosis or galactosialidosis are deficient in the Con A binding neuraminidase, whereas the non-binding form is normal. In sialolipidosis both forms are normal. These results demonstrate that leucocytes contain at least two genetically different MU-NANA neuraminidases. Thus, the use of leucocytes should be avoided for the diagnosis of sialidosis and galactosialidosis, and isolated lymphocytes should be used to obtain reliable results.

In man several inherited disorders are known with a deficiency of neuraminidase activity (1). With the present biochemical knowledge these disorders can be separated in three distinct groups; within each group clinical heterogeneity is observed. Firstly, there is a group of patients, classified as sialidosis, characterized by a deficiency of a lysosomal neuraminidase specific for glycoproteins and oligosaccharides with terminal sialic acid (2). This group includes disorders previously described as mucolipidosis I and cherry-red spot myoclonus syndrome (1-7). The second group, galactosialidosis, is characterized by a combined deficiency of β-galactosidase and neuraminidase (8,9). The primary defect lies in a glycoprotein which normally prevents degradation of β-galactosidase (9-12). Neuraminidase is deficient as well, which may find its origin in the fact that β-galactosidase and neuraminidase occur as enzyme complex (13). The third group has recently been classified as sialolipidosis (i.e. mucolipi-

*MU-NANA, 4-methylumbelliferyl-α-D-N-acetylneuraminic acid

dosis IV), in which the enzyme deficiency is a ganglioside specific neuraminidase (14,15).

For the diagnosis of sialidosis and galactosialidosis hydrophilic substrates were employed: glycoproteins or oligosaccharides and 4-methylumbelliferyl α-D-N-acetylneuraminicacid (MU-NANA). Sialolipidosis has been diagnosed using labelled ganglioside substrates. Nearly complete enzyme deficiencies have been detected in cultured fibroblasts from patients with these different disorders (1-9,15). However, using leucocytes as the source of neuraminidase, considerable residual activities (sometimes close to normal) were found in sialidosis and galactosialidosis (16-20).

Recently it was proposed that leucocytes contain two MU-NANA neuraminidases distinguished by slight physical differences (17,20,21). In contrast to these reports, we demonstrate the presence of two neuraminidases with qualitative biochemical differences and have extended the studies to all three disorders. Our results indicate that both MU-NANA neuraminidases are of different genetic origin. The implications for diagnosis of sialidosis and galactosialidosis are discussed.

MATERIALS AND METHODS

Blood cell isolation

Isolation of leucocytes was performed by sedimentation of freshly collected heparinized blood samples in 3% Dextran (T-500, Pharmacia) in 0.9% NaCl, whereafter lymphocytes and granulocytes were separated on Ficoll Paque (Pharmacia). Remaining erythrocytes in leucocytes and granulocytes were lysed during 10 minutes in 50 mM NaCl at 4°C, after which the cells were spun down at 300 x g for 10 min.

Preparation of cell homogenates

Cell homogenates for specific activity determination were made by homogenizing the cells in 60 µl 0.1% sodium taurocholate (Sigma, synthetic) using a glass-teflon Potter homogenizer. Leucocytes derived from 2-3 ml blood samples were used, and lymphocytes and granulocytes from 4-7 ml.

Assay of protein and enzyme activity

Protein was measured by the method of Lowry (22). Neuraminidase was measured with 4-methylumbelliferyl N-acetylneuraminic acid (MU-NANA) (a generous gift of Prof. R. Brossmer, Heidelberg). 20 µl Cell homogenate was added to 10 µl 2 mM substrate solution in 0.25 M sodium acetate buffer pH 4.3. Incubation was at 37°C for one hour. The reaction was terminated by addition of 500 µl, 0.5 M sodium carbonate buffer pH 10.7. β-Galactosidase was assayed in a thirtyfold diluted homogenate as described earlier (23). Fluorescence was measured with a Perkin Elmer fluorimeter.

Solubilization of neuraminidase and β-galactosidase

Cells derived from 10-25 ml blood were homogenized in 50 µl 20 mM sodium phosphate buffer containing 100 mM NaCl and 1% Zwittergent 3.12 (Calbiochem.) pH 6.0 using a glass-teflon Potter homogenizer. The homogenates were centrifuged at 10000 x g for 15 minutes at 4°C. The supernatant containing solubilized neuraminidase and β-galactosidase was used in the precipitation experiments with Con A-Sepharose and anti placental β-galactosidase IgG.

Con A-Sepharose precipitation

5 µl Supernatant was added to 5 µl 20 mM sodium phosphate buffer pH 6.0 containing 100 mM NaCl, 1% Zwittergent 3.12 and various amounts of Con A-Sepharose-4B (Pharmacia). The tubes were gently rotated at 4°C. After one hour all tubes were centrifuged at 10000 x g for 20 seconds. Supernatants were assayed

for both neuraminidase and β-galactosidase. The incubation volume was reduced to 3 µl and the reaction was terminated with only 200 µl sodium carbonate buffer pH 10.7.

Immunotitration

3 µl Supernatant was added to 2 µl of a serial dilution of IgG (13) in 20 mM sodium phosphate buffer containing 100 mM sodium chloride pH 6.0. After two hours incubation at 4°C, 5 µl of a 1:1 Protein A Sepharose-4B (Pharmacia) suspension in the same buffer was added. After one hour gently rotating at 4°C, all tubes were centrifuged at 10000 x g for 20 seconds. Supernatants were assayed for both neuraminidase and β-galactosidase activity as described under Con A-Sepharose precipitation.

Subcellular fractionation in Percoll gradients

Lymphocytes or granulocytes isolated from 200 ml respectively 100 ml freshly collected blood from healthy donors, were suspended in 0.5 ml 0.25 M sucrose, 10 mM sodium phosphate buffer pH 6.8 and disrupted by 25 strokes in a 2 ml glass-teflon Potter homogenizer. Nuclei and intact cells were removed by centrifugation for 10 min. at 600 x g. About 90% of granulocyte β-hexosaminidase (lysosomal latency >80%) and about 80% of lymphocyte β-hexosaminidase (lysosomal latency >60%) was recovered in the postnuclear supernatant. Percoll (Pharmacia) was made iso-osmotic with 0.25 M sucrose and this solution (100% Percoll) was diluted to a final Percoll concentration of 40% with 0.25 M sucrose, 10 mM sodium phosphate buffer pH 6.8. 0.3 ml Postnuclear supernatant was applied on top of 8 ml of 40% Percoll solution underlayed with 0.5 ml 2.5 M sucrose. The samples were centrifuged at 25000 rpm (~48000 x g) for 45 minutes at 4°C in a Beckman type 50 fixed angle rotor. Fractions of 300 µl were collected with a density-gradient removing apparatus (Auto densi-flow II, Buchler instruments). For neuraminidase assay 15 µl gradient fraction was mixed with 15 µl H_2O and incubated with 10 µl substrate containing 0.4% taurocholate and 4% Zwittergent 3.12. Recovery of neuraminidase activity after the Percoll centrifugation was >70%. Alkaline phosphatase activity was measured with 1.5 mM methylumbelliferyl phosphate (Koch-Light) in 1 mM $MgCl_2$ and 75 mM 2-amino-2 methyl-1-propanol/HCl pH 10.1 in the presence of 0.1% triton X-100 (24).

RESULTS

Total leucocytes and isolated lymphocytes and granulocytes from patients with sialidosis (patient of Dr. H.D. Bakker, (9)), galactosialidosis (patient of Dr. M.C.B. Loonen, (9,25)) and sialolipidosis (15), were tested for neuraminidase and β-galactosidase activity (Table 1). Leucocyte neuraminidase activities of sialidosis and galactosialidosis patients were found to be close to the lowest control value (up to 39% of the normal mean value). Leucocytes from a patient with sialolipidosis had neuraminidase activities in the control range. However, isolated lymphocytes from sialidosis and galactosialidosis patients were clearly deficient in neuraminidase activity (<15% of the mean control value), whereas lymphocytes from the patient with sialolipidosis had normal activity. In contrast granulocytes from all different types of patients showed normal neuraminidase activities. As expected, β-galactosidase activities were normal in all cell types of the sialidosis and sialolipidosis patients and clearly deficient in all cell types of the galactosialidosis patient. The fact that neuraminidase activity in both sialidosis and galactosialidosis, which are two genetically distinct diseases, is deficient in lymphocytes, but normal in granulocytes, suggests that there are at least two genetically different neuraminidases.

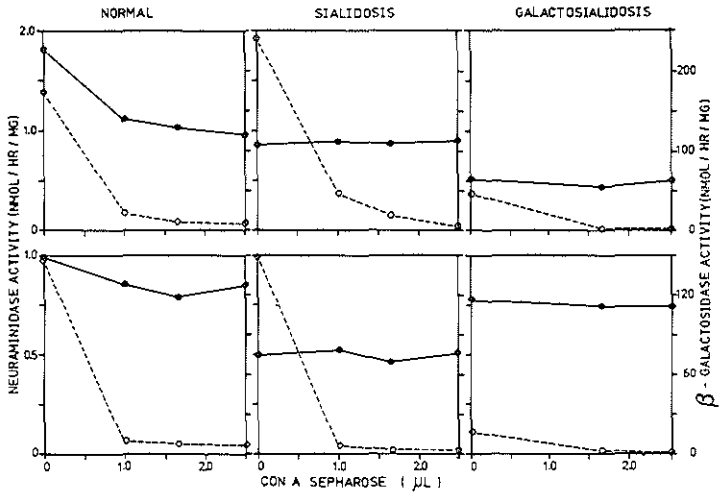

Fig. 1. Con A-sepharose precipitation of neuraminidase and β-galactosidase from normal, sialidosis and galactosialidosis cells.
Upper graphs: lymphocytes
Lower graphs: granulocytes
● Neuraminidase
○ β-Galactosidase

To pursue this hypothesis the neuraminidases were further characterized. Neuraminidase, which is insoluble under our standard assay conditions using sodium taurocholate, was solubilized with a zwitterionic detergent. In normal lymphocytes 40 to 70% of the total activity could be solubilized, whereas in granulocytes the solubilization was 85 to 100%. From the solubilized neuraminidase activity of normal human lymphocytes only 40 to 60% could be precipitated with Con A (Fig. 1). Granulocytes contain only minor amounts of Con A precipitable neuraminidase activity. β-Galactosidase activity was precipitated completely. In sialidosis and galactosialidosis the residual neuraminidase activity of lymphocytes and the normal activity of granulocytes could not be precipitated, whereas β-galactosidase activity could be precipitated normally. Apparently two different neuraminidases are present in leucocytes: a Con A binding form, deficient in sialidosis and galactosialidosis, and a non-binding form, normal in sialidosis and galactosialidosis.

It has been demonstrated that acid neuraminidase and β-galactosidase form an enzyme complex in bovine testis (13). To investigate whether the neuraminidases from human leucocytes are also complexed with β-galactosidase, immunotitration using an antiserum raised against purified β-galactosidase was performed (fig. 2). In normal lymphocytes 40 to 60% of the solubilized neuraminidase activity coprecipitated with β-galactosidase. In granulocytes only minor amounts could be coprecipitated. The residual neuraminidase activity of neither

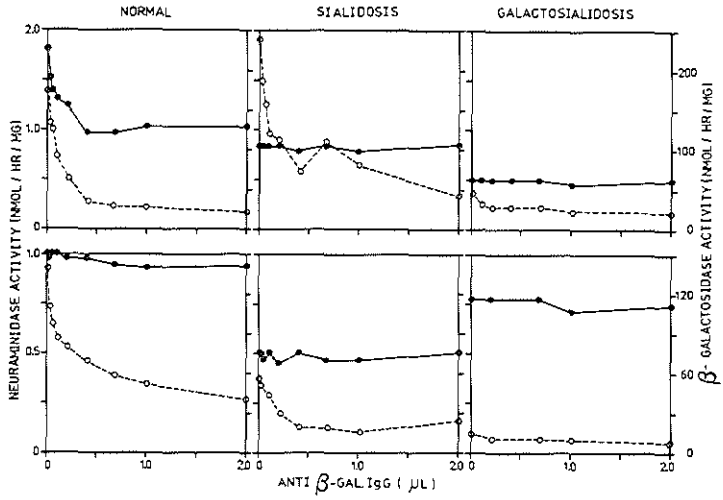

Fig. 2. Immunotitration of neuraminidase and ß-galactosidase from normal, sialidosis and galactosialidosis cells.
Upper graphs: lymphocytes
Lower graphs: granulocytes
● Neuraminidase
○ ß-Galactosidase

lymphocytes nor granulocytes from the patients coprecipitated with ß-galactosidase. Apparently the Con A binding form of neuraminidase is complexed with ß-galactosidase while the non-binding form is not.

We investigated whether both forms of neuraminidase are lysosomal or in contrast might be plasma membrane associated. Fig. 3 shows the results of Percoll gradient centrifugation studies and it is evident that lysosomes and plasma membranes are clearly separated. In both lymphocytes and granulocytes neuraminidase sedimented in a single band, coinciding with the lysosomal marker.

Finally a number of biochemical properties of neuraminidase from lymphocytes and granulocytes were analysed (Table 2). No differences were observed in pH optimum and K_m for the MU-NANA substrate. In lymphocytes neuraminidase was somewhat more labile than in granulocytes during sonication, freeze-thawing and heat denaturation. Cu^{2+}, which is reported to be an inhibitor of ganglioside neuraminidase (26), inhibited both lymphocyte and granulocyte neuraminidase to the same extent. Similar results were obtained with 2,3-dehydro-2-deoxyNANA, a possible inhibitor of the lysosomal glycoprotein and oligosaccharide specific neuraminidase (26).

DISCUSSION

Our results demonstrate the presence of two different neuraminidases in human leucocytes: a Con A binding form, complexed with ß-galactosidase and a

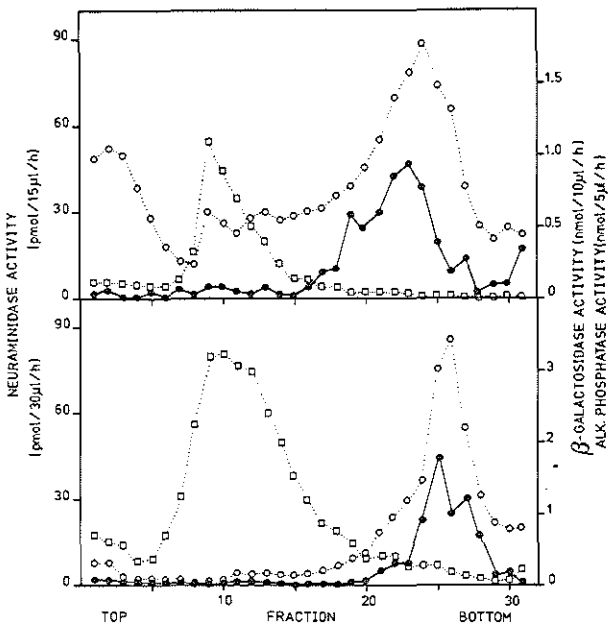

Fig. 3. Percoll density gradient centrifugation of subcellular components from normal lymphocytes (upper figure) and normal granulocytes (lower figure).
● Neuraminidase
□ Alkaline phosphatase (Plasma membrane marker)
○ β-Galactosidase (Lysosomal marker)

non-binding form which is not complexed. Only the binding form is deficient in sialidosis and galactosialidosis. The residual activities of neuraminidase in cell homogenates of white blood cells from patients (Table 1) indicate that normal lymphocytes contain predominantly (>80%) the binding form, whereas normal granulocytes contain mainly the non-binding form. It should be noted that studies with solubilized neuraminidase (Con A binding and immunotitration experiments) are not informative for the relative amounts of the two neuraminidases present in leucocytes, since different degrees of solubilization were observed for both forms.

Diagnoses of sialidosis and galactosialidosis, using total leucocytes, have been reported and often very high residual neuraminidase activities were found (1,16-20) similar to the activities found in our cases (Table 1). This is caused by the normal activity of the non-binding neuraminidase in these patients. For this reason granulocytes and hence total leucocytes should not be used for diagnosis. On the other hand we show that lymphocytes can be used satisfactorily.

Multiple forms of neuraminidase have been described previously. Nguyen Hong et al. (27) reported on two neuraminidases in human leucocytes, a heat stabile

TABLE I

ENZYME ACTIVITIES IN VARIOUS WHITE BLOOD CELLS IN SIALIDOSIS, GALACTOSIALIDOSIS AND SIALOLIPIDOSIS

	NEURAMINIDASE*				β-GALACTOSIDASE*				
	Normal (n=19)		Sial.	Gal.sial.	Sialolip.	Normal (n=19)		Sial.	Gal.sial.	Sialolip.
	range	mean				range	mean			
Leucocytes	1.4-4.3	2.8	1.1	0.74	2.3	95-200	133	129	9.2	98
Lymphocytes	3.9-11	7.4	1.1	0.84	4.3	135-280	203	132	13	191
Granulocytes	0.46-1.2	0.94	1.3	0.59	1.2	65-150	102	284	6.5	148

* enzyme activity in nmol/h/mg protein

and labile form, both deficient in sialidosis patients. This indicates that both forms are related to our binding form. Tsuji et al. (20) and Suzuki et al. (17) distinguished two neuraminidase activities in leucocytes with different sonication and freeze-thaw stability. Only the more labile form was deficient in their galactosialidosis patients. Our binding and non-binding neuraminidase exhibit comparable freeze-thaw and sonication stability and are probably the same enzymes as those described (17,20).

TABLE 2

PROPERTIES OF NEURAMINIDASE IN NORMAL LYMPHOCYTES AND GRANULOCYTES

	LYMPHOCYTES	GRANULOCYTES
pH optimum	4.1	4.0
K_M for 4MU-NANA (mM)	0.18	0.26
Inhibition by 5mM Cu^{2+}	46%	51%
Inhibition by 0.05 mM 2,3 dehydro-2-deoxy-NANA	69%	76%
Sonication stability ($t_{\frac{1}{2}}$)	15 sec	58 sec
Freeze/thaw stability*	32%	55%
Heat stability pH7/37°C ($t_{\frac{1}{2}}$)	13 min	50 min

* residual enzyme activity after 10 freeze/thaw cycles.

Subcellular fractionation showed that both neuraminidase activity of lymphocytes (>80% Con A binding form) and neuraminidase activity of granulocytes (mainly non-binding form) sedimented in a peak coinciding with the lysosomes clearly separate from the plasma membranes. Together with the acid pH optima, this suggests lysosomal localization of both forms. This deviates from the report by Yamada et al. (21) proposing a plasma membrane localization of their analogue of our non-binding neuraminidase.

Since in sialidosis as well as galactosialidosis only the Con A binding neuraminidase is deficient we conclude that the binding and non-binding neuraminidases are of different genetic origin. The physiological significance of the lysosomal Con A binding neuraminidase and the ganglioside neuraminidase is emphasized by the existence of lysosomal storage disorders caused by a deficiency of one of these neuraminidases. The function of the non-binding neuraminidase is still unknown and future studies on the substrate specificity should help to reveal its physiological importance. It is unlikely that this enzyme represents ganglioside neuraminidase since a patient with sialolipidosis with a proven deficiency of ganglioside neuraminidase (15) had a normal non-binding neuraminidase activity. The present results may lead to the discovery of a lysosomal storage disorder which is associated with an abnormal non-binding neuraminidase.

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Galactosialidosis: Molecular Heterogeneity among Distinct Clinical Phenotypes

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SUMMARY

The lysosomal storage disorder galactosialidosis has been recognized as a distinct genetic and biochemical entity, associated with a combined β-galactosidase and neuraminidase deficiency that is due to the lack of a 32-kilodalton (kDa) glycoprotein. The molecular basis of different clinical variants of galactosialidosis has been investigated.

In the early-infantile form, the synthesis of the 52-kDa precursor of the 32-kDa "protective protein" is markedly reduced and the absence of the latter protein explains the severe neuraminidase deficiency. In the juvenile-adult form, there is relatively more 52-kDa precursor but no 32-kDa protein can be detected.

Cells from the late-infantile form have in comparison with controls, besides a small amount of the 32-kDa glycoprotein, an accumulation of the 52-kDa precursor. Apparently, this protein is genetically altered in such a way that its further processing is impaired. Furthermore, in this mutant, the residual neuraminidase activity is stimulated four- to sixfold upon leupeptin treatment together with an increase of the 32-kDa glycoprotein.

INTRODUCTION

During the last few years, about 30 patients with galactosialidosis have been described (for reviews, see [1–3]). Somatic cell hybridization studies have shown that this autosomal recessive disease, associated with a combined defi-

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ciency of lysosomal β-galactosidase and neuraminidase, is a genetic entity distinct from G_{M1} -gangliosidosis and sialidosis [4].

D'Azzo et al. [5] elucidated the responsible molecular defect in galactosialidosis that appears to be a lack of a 32-kDa glycoprotein, subsequently shown to be required for the aggregation of β-galactosidase monomers [6]. This conformation was found to be essential to protect the 64-kDa β-galactosidase monomers against intralysosomal proteolytic degradation. Also, the 32-kDa protective protein is essential for the activation of neuraminidase that forms a complex with β-galactosidase [7, 8].

As in most other lysosomal storage diseases, different clinical phenotypes of galactosialidosis have been recognized. Patients with an early-infantile form, showing severe edema, ascites, skeletal dysplasia, and ocular abnormalities, die shortly after birth [9, 10]. In the late-infantile form, patients present symptoms after 6–12 months and the main features are dysmorphism, dysostosis, and skeletal dysplasia, visceromegaly, macular cherry-red spot, and mild mental retardation [3, 11, 12]. The largest number of patients, mainly of Japanese origin, are those with the juvenile/adult form [2, 13, 14], where symptoms have appeared between infancy and adulthood. The major features are skeletal dysplasia, dysmorphism, corneal clouding, cherry-red spot, angiokeratoma, neurological manifestations, and mental retardation.

In our present studies on the molecular background of these different clinical phenotypes, we found marked differences in the biosynthesis of the 32-kDa "protective protein." These findings also explain the discrepancy in earlier studies on the effect of leupeptin on the residual neuraminidase activity in galactosialidosis [12, 14, 15].

MATERIALS AND METHODS

Cell Culture

Fibroblasts from normal individuals and patients with an early-infantile form [9] and a juvenile/adult form of galactosialidosis [16] were derived from the Rotterdam Cell Repository (Prof. M. F. Niermeijer). Cells from patients with the late-infantile form were provided by Dr. G. Andria [3] and Dr. L. Pinsky [11]. Early passages from all cell strains were used and were maintained in Ham's F-10 medium (Flow Laboratories, McLean, Va.) supplemented with antibiotics and 10% fetal calf serum.

Radioactive Labeling, Immunoprecipitation, and Electrophoresis

One week prior to radioactive labeling, the medium was replaced by Dulbecco's modification of Eagle's medium (Flow Laboratories) supplemented with 10% fetal calf serum and antibiotics and cells were grown in 25-cm^2 falcon flasks. One hour prior to labeling, this medium was replaced by Dulbecco's medium free of leucine, fetal calf serum, and antibiotics.

Biosynthetic labeling was performed using the same medium plus 2% of dialyzed fetal calf serum and 0.15 ml of L [4-5^{3}H]leucine (0.15 mCi; 135 Ci mmol; Amersham Radiochemical Centre, England). After 48 hrs, cells were harvested and comparable amounts of cell material, based on the protein content, were immunoprecipitated. A polyclonal antiserum raised against β-galactosidase purified from human placenta was used [5]. Since the enzyme exists in a complex with its 32-kDa "protective protein," the antiserum reacts with β-galactosidase, its protective protein, and their precursor forms

[6]. Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate, was performed according to Laemmli [17] with minor modifications. The radioactive bands were visualized by fluorography and exposure for 1 week at −70°C using X-Omat R film (Eastman Kodak, Rochester, N.Y.). The [^{14}C]methyl-labeled protein molecular weight standards used were: phosphorylase B, 92,500; bovine serum albumine, 69,000; ovalbumin, 46,000; and carbonic anhydrase, 30,000 (Amersham Radiochemical).

To study the effect of inhibition of lysosomal proteases, leupeptin, at a final concentration of 0.02 mM, was added to the medium. In addition to immunoprecipitation studies, as described above, the activities of β-galactosidase and neuraminidase were measured 4–5 days after leupeptin administration using 4-methylumbelliferyl substrates according to Galjaard [18].

For the pulse-chase study, five 25-cm^2 flasks were prepared for every strain, and after labeling for 30 min, the cells were subjected to chase with fresh unlabeled Dulbecco's modification of Eagle's medium for 0, 15, 30, 60, and 120 min, respectively.

Analysis in the Medium of NH_4Cl-induced Secretions

The cells were prepared as described above, but at the moment of labeling, the medium was supplemented with 10 mM NH_4Cl for 48 hrs. Then, the medium was collected and prepared for immunoprecipitation as in previous studies [5, 19]. For the study of glycoprotein phosphorylation, 200 μCi $^{32}P_i$-carrier free was added to a 25-cm^2 Falcon flask, in the presence of NH_4Cl, and the medium was prepared as described [20].

Uptake studies. Confluent fibroblasts from the late-infantile galactosialidosis form were maintained in 75-cm^2 Falcon flasks for 48 hrs in Dulbecco's modification of Eagle's medium containing [^{3}H]leucine and NH_4Cl. The medium from two falcon flasks was collected, and the (precursor) glycoproteins secreted by the cells were precipitated with $(NH_4)_2\ SO_4$. The pellet was dissolved in 400 μl water and desalted on a small column of Sephadex G_{50} that was equilibrated with phosphate-buffered saline [21]. Subsequently, the sample was added to 5 ml Ham's F-10 medium, which was then added to control fibroblasts in a 25-cm^2 falcon flask and incubated for 48 hrs. After harvesting, immunoprecipitation studies were performed. Reversely, the same procedure was carried out with medium containing secretions from control cells, which was added to mutant cells.

RESULTS

Biosynthesis in Normal and Mutant Cells

Immunoprecipitation studies were performed with conventional antiserum reacting with β-galactosidase, protective protein, and their precursor forms. The results for normal fibroblasts and cells derived from the three clinical phenotypes of galactosialidosis are shown in figure 1. In all cell lines, the 85-kDa precursor of β-galactosidase is synthesized, but in the three mutant lines, the amount of mature 64-kDa β-galactosidase is decreased compared with the control (fig. 1*A*). In cells from both patients with the late-infantile form of galactosialidosis (fig. 1*B* and *E*), there is a large amount of a 52-kDa protein and a band at the 32-kDa position. In the two other mutant cell types, no 32-kDa band is visible and the amount of 52-kDa protein is less than in the control or in the late-infantile form. The juvenile/adult form (fig. 1*D*) shows more 52-kDa protein than the early-infantile form (fig. 1*C*).

The pulse-chase experiments, illustrated in figure 2, indicate that the 52-kDa protein is the precursor of the 32-kDa protective protein. In control fibroblasts, this precursor is visible after 30-min labeling and the 32-kDa band appears after

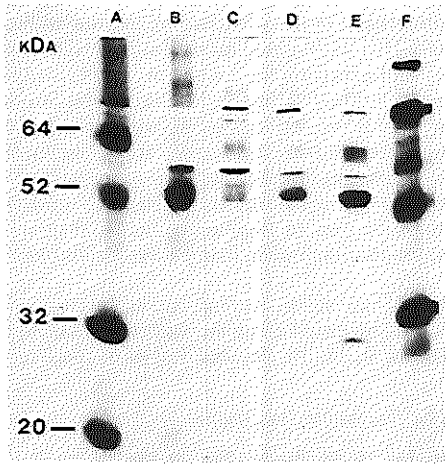


FIG. 1.—Immunoprecipitation of β-galactosidase and 32-kDa protective protein and their precursors in normal and mutant fibroblasts, after [^{3}H]leucine labeling for 48 hrs followed by SDS-PAGE. *Lane A:* control; *lane B:* late-infantile galactosialidosis (Andria et al. [3]); *lane C:* early-infantile form; *lane D:* juvenile/adult form; *lane E:* late-infantile form (Pinsky et al. [11]); *lane F:* standards.

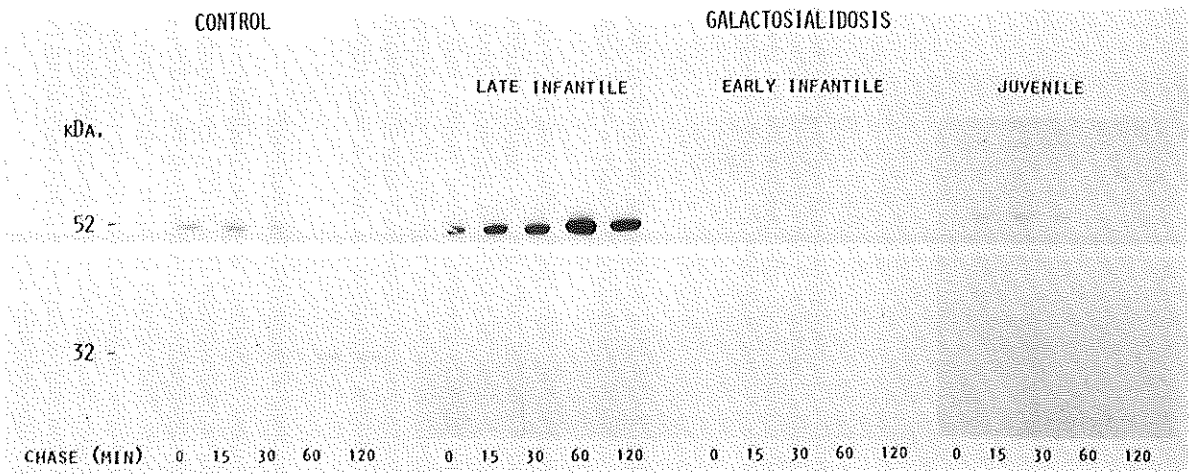
15-min chase; after 60-min chase, the 52-kDa band has disappeared whereas the 32-kDa protective protein remains visible.

In cells from the late-infantile form of galactosialidosis, the 52-kDa precursor remains during the whole chase period and no 32-kDa band appears. These results suggest that the processing of 52-kDa precursor into 32-kDa protective protein is impaired. In the early-infantile form, hardly any 52-kDa precursor is detectable, and in the juvenile/adult form, the amount of precursor is somewhat higher. The 32-kDa protective protein is detectable in neither of these two mutants.

Secretion and Uptake of Precursor

Immunoprecipitation studies on medium above [^{3}H]leucine-labeled cells after NH_4Cl stimulation show equal amounts of the secreted 88-kDa precursor of β-galactosidase in normal and mutant cells (fig. 3*A–D*). Medium above control cells also contains the 54-kDa precursor of the protective protein (fig. 3*A*). This precursor can also be demonstrated in the medium above late-infantile galactosialidosis cells (fig. 3*B*), although much less than in controls. No 54-kDa protein band is seen in the medium above the two other mutant cell types (fig. 3*C* and *D*). Labeling with $^{32}P_i$-carrier free showed that the secreted precursors in the medium are all phosphorylated (results not shown).

To investigate whether the 54-kDa precursor secreted by the late-infantile galactosialidosis cells can be processed into 32-kDa protective protein, the following experiment was carried out. Medium above [^{3}H]leucine-labeled mutant cells was collected after 48 hrs treatment with NH_4Cl (see MATERIALS AND METHODS) and added to control fibroblasts. Immunoprecipitation studies of these latter cells were performed after 48 hrs, and the results are shown in


CONTROL
GALACTOSIALIDOSIS
LATE INFANTILE
EARLY INFANTILE
JUVENILE
kDa.
52 -
32 -
CHASE (MIN) 0 15 30 60 120 0 15 30 60 120 0 15 30 60 120 0 15 30 60 120

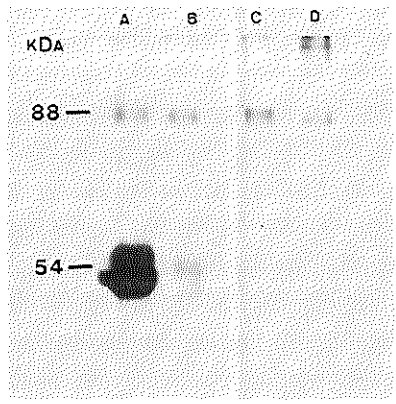


FIG. 3.—Immunoprecipitation of secreted precursor glycoproteins in the medium above normal and mutant fibroblasts after NH_4Cl treatment in the presence of [^{3}H]leucine. *Lane A:* medium above control cells; *lane B:* medium above cells from late-infantile galactosialidosis; *lane C:* medium above cells from early-infantile form; *lane D:* medium above cells from juvenile/adult galactosialidosis.

figure 4A. It is clear that the 54-kDa precursor secreted by the mutant cells has been taken up by the control fibroblasts but only a very faint 32-kDa band is visible.

In the reverse experiment (fig. 4*B*), the 54-kDa precursor secreted by normal cells is taken up by the mutant cells and is rapidly processed into 32-kDa protective protein; also, a clear 20-kDa band appears, which is derived either from the 54-kDa or the 32-kDa protein. These results indicate that the cells from late-infantile galactosialidosis are capable of processing normal 54-kDa precursor. The uptake experiments also show that the mutation in this clinical phenotype alters the 54-kDa precursor in such a way that its processing is impaired.

Inhibition of Intralysosomal Proteolytic Degradation

Since the β-galactosidase deficiency in galactosialidosis is due to enhanced intralysosomal proteolytic degradation [5, 14, 15], we investigated the effect of inhibition of lysosomal cathepsins by leupeptin in the different clinical phenotypes of galactosialidosis. The results in table 1 show that the activity of β-galactosidase increases three- to sevenfold in the three mutant cell types after 4 days treatment with leupeptin. The effect on neuraminidase activity, however, is different among the mutants. Leupeptin leads only to an increase of neuraminidase activity in the late-infantile form.

To study the molecular background of this effect, we performed immunoprecipitation studies after leupeptin treatment (fig. 5). In the late-infantile form of galactosialidosis, leupeptin treatment results in an increased amount of the 85-kDa precursor of β-galactosidase (as in controls) and to an increased amount

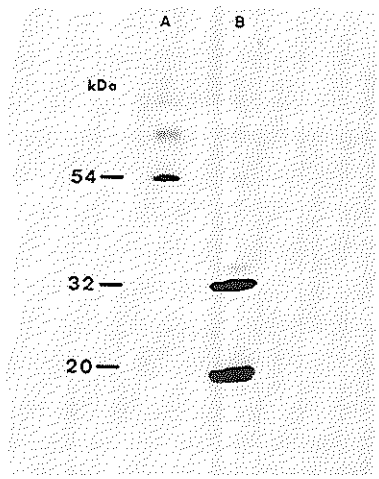


FIG. 4.—Uptake of radiolabeled glycoprotein precursors secreted into the medium. *Lane A:* uptake and processing of secreted precursors from late-infantile mutant fibroblasts by control cells; *lane B:* uptake from control fibroblasts by late-infantile galactosialidosis cells.

of the 32-kDa protective protein. The latter effect was not seen in the two other mutant cell lines (results not shown).

DISCUSSION

The accumulation of knowledge about the biosynthesis of human β-galactosidase [5], its different forms [6, 22, 23], and its relationship with lysosomal neuraminidase [7, 8, 24] made our present study on the molecular background of different clinical variants of galactosialidosis feasible.

The results of immunoprecipitation studies with conventional antiserum reacting with β-galactosidase and its 32-kDa protective protein show that in all three types of galactosialidoses there is little 64-kDa β-galactosidase monomer. This is reflected in the 10% residual enzyme activity found in a variety of cells in these patients [1–3, 23, 25]. Previous studies [5] indicated that the reduced amount of 64-kDa β-galactosidase is due to enhanced degradation of the monomeric form of the enzyme. In the lysosomes of normal cells, β-galactosidase exists for at least 80% as a high molecular weight aggregate [6, 22, 23], and, more recently, it was demonstrated that the 32-kDa "protective protein" is needed for this aggregation process [6].

The results of our present study show that the intracellular 52-kDa protein is the precursor form of this 32-kDa "protective protein" and a 20-kD protein that is present in minor amounts in the high molecular weight aggregate and that probably is a degradation product. The amount of the 52-kDa precursor varies markedly among the different clinical phenotypes (figs. 1 and 2). In the late-infantile form of galactosialidosis [3, 11], the 52-kDa band is even stronger than in control cells but the amount of 32-kDa "protective protein" is much smaller. In the early-infantile form [9], there is very little 52-kDa precursor, and in the

TABLE 1

EFFECT OF LEUPEPTIN ON THE ACTIVITY OF β-GALACTOSIDASE AND NEURAMINIDASE IN CONTROL CELLS AND IN DIFFERENT TYPES OF GALACTOSIALIDOSIS

TYPE OF CELLS	β-GALACTOSIDASE ACTIVITY		NEURAMINIDASE ACTIVITY	
	$^{-}$Leupeptin	$^{+}$Leupeptin	$^{-}$Leupeptin	$^{+}$Leupeptin
Early-infantile galactosialidosis [9]	33.3	207	0.4	0.6
Late-infantile galactosialidosis [3]	27.0	181	2.6	9.0
Late-infantile galactosialidosis [11]	73.0	357	1.2	7.1
Juvenile/adult galactosialidosis [16]	38.6	132	0.5	0.8
Control	706 (Range 350–1,050)	675	106 (Range 40–130)	149

NOTE: Enzymatic activities are expressed in nmol/hr/mg protein. Each value is the mean of three or four separate experiments.

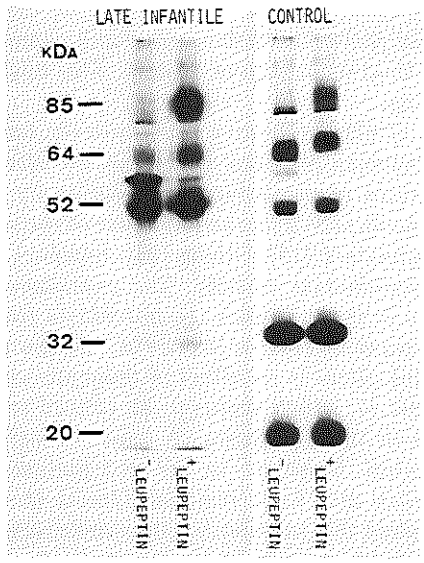


FIG. 5.—Immunoprecipitation of β-galactosidase and 32-kDa protective protein in fibroblasts from controls and from a patient with the late-infantile form of galactosialidosis; [^{3}H]leucine labeled for 4 days with and without leupeptin.

juvenile/adult form [13], only some more is present. No 32-kDa protein can be detected in both forms.

In the pulse-chase experiment, the labeling period is too short to demonstrate the 85-kDa precursor of β-galactosidase and the 32-kDa protective protein in the late-infantile form of galactosialidosis, but these bands are visible after 48 hrs labeling. Precursor forms secreted into the medium after NH_4Cl stimulation show a 2,000–3,000 higher molecular mass than the intracellular forms, an observation made earlier for other lysosomal proteins [19].

The immunoprecipitation studies of the medium after NH_4Cl stimulation indicate equal amounts of secreted 88-kDa precursor β-galactosidase in normal and mutant cells, which points to a normal synthesis of this protein in all forms of galactosialidosis. The secreted 54-kDa precursor of the protective protein is seen only in medium above controls and above cells from the late-infantile form. The discrepancy between the large amount of 52-kDa precursor intracellularly in this mutant and the smaller amount (compared with control) of the secreted 54-kDa precursor suggests that the mutation interferes with normal intracellular routing from the Golgi to the lysosome so that NH_4Cl does not effectively stimulate secretion.

The uptake studies (fig. 4) prove that 54-kDa precursor secreted by normal cells can be processed into 32-kDa "protective protein" by cells from the late-infantile form of galactosialidosis. Consequently, the mechanism needed for this processing is not affected in the mutant. On the other hand, the 54-kDa precursor secreted by the mutant cells is hardly processed after uptake by normal cells. This indicates that the gene mutation in this clinical phenotype

affects the 54-kDa precursor protein in such a way that it cannot be effectively processed into functional 32-kDa protective protein. This might be related to aberrant intracellular routing.

The residual amount of 32-kDa protective protein in the late-infantile form explains the discrepancy between previous studies on the effect of leupeptin. After addition of this inhibitor of proteolytic degradation by cathepsins, our group found in galactosialidosis cells a marked increase of the activities of both β-galactosidase and neuraminidase [15]. Others [14], however, did not find an effect of leupeptin on neuraminidase activity. The explanation is the use of different mutant cell types in these studies. In fibroblasts from the juvenile/adult form, studied by Suzuki et al. [14], or in cells of a similar patient reported earlier by Loonen et al. [16], there is no effect of leupeptin on the neuraminidase activity (see also table 1), which seems to be related to the absence of 32-kDa protective protein in these patients. A four- to sixfold increase of neuraminidase activity occurs, however, in the two patients with the late-infantile form, both of which also have residual 32-kDa protective protein. Recent experiments [8] explain this relationship on a molecular basis, because the 32-kDa protective protein appears to be an essential subunit for lysosomal neuraminidase activity.

Our present study provides a molecular basis for some differences among patients with galactosialidosis. The three main clinical forms have in common a 10%–15% residual β-galactosidase activity that is based on the monomeric form of the enzyme. The differences between the clinical phenotypes must be due to differences in lysosomal neuraminidase activity and in the 32-kDa protective protein and its precursor. In the late-infantile form, there is a normal synthesis of 52-kDa precursor of the protective protein but its processing is impaired. The small amount of 32-kDa protein is sufficient for some 2% residual neuraminidase activity, which might be sufficient to explain the relatively mild clinical course [3].

In the early-infantile form, the mutation results in a marked reduction of the amount of 52-kDa precursor and no 32-kDa protein, essential for neuraminidase activity, can be detected. The pulse-chase experiments suggest a reduced rate of synthesis rather than an enhanced degradation of the precursor.

In the juvenile/adult form, there is relatively more 52-kDa precursor than in the early-infantile form, but the amount of 32-kDa protective protein is below detection level even after 48 hrs labeling. We do not yet know whether the 52-kDa precursor may play any functional role, nor can we as yet relate the molecular observations and the clinical features in the juvenile/adult form. It should, however, be mentioned that these molecular studies are limited to fibroblasts and defective processing might have fewer consequences in other cells and organs. Also, the neuraminidase activities have been measured with artificial substrates under in vitro conditions. It may well be that the differences between the early-infantile form and the juvenile/adult forms are based on small differences in residual neuraminidase activity toward their natural substrates under in vivo conditions [26].

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