Multiple lysosomal enzyme deficiency in man

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J. SPERNA WEILAND
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 28 APRIL 1982 DES NAMIDDAGS
TE 3.45 UUR.

DOOR

Alessandra d'Azzo
GEBOREN TE PAVIA

Promotor : Prof. Dr. H. Galjaard

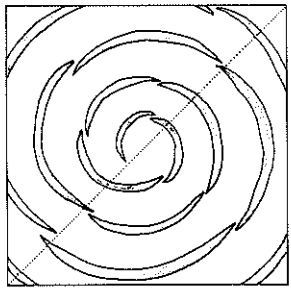
Co-referenten : Prof. D. Robinson
Prof. Dr. J.F. Koster

Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam.

PERCHÉ NON PRENDERE LA VITA
COME UN ATTO DI FEDE!
PERCHÉ NON VOTARSI
A QUELLA VOCE DI NATURA
SEPPELLITA NELLA COSCIENZA!
PERCHÉ NON CONSACRARSI
A QUESTA CONFORTANTE VITTORIA!
CREDERE E' L'ARIETE
CHE BATTE IN BRECCIA.

MARIO D'AZZO

CONTENTS



CHAPTER I GENERAL INTRODUCTION

1.1 LYSOSOMAL STORAGE DISEASES - RELATION BETWEEN CLINICAL AND BIOCHEMICAL FINDINGS

The dynamic state of the living cell rests on a balance between biosynthesis and breakdown of numerous substances. These processes are carried out at normal body temperature, low ionic strength, and narrow range of pH, by enzymes specifically located in the different cellular compartments. Knowledge of the localization, grouping and function of enzymes within the cell is therefore essential for the understanding of the total cellular machinery.

The major site of digestion of intra- and extracellular macromolecules is the lysosomal apparatus, a morphologically heterogeneous population of cytoplasmic organelles present in all animal cells with the exception of erythrocytes (De Duve et al. 1955; De Duve 1964). Lysosomes are characterized by their content of hydrolytic enzymes with an acidic pH optimum, and by their single-unit limiting membrane (De Duve & Wattiaux 1966; Novikoff 1973-1976; Holtzman 1976; Schellens et al. 1977). The latter protects the cellular milieu from random degradation and favours the generation of the somewhat acid conditions needed for the maximal function of the lysosomal hydrolases. Activation of the lysosomal system occurs under certain physiologic and pathologic stimuli. The result is multiform and displays the role of lysosomes in such fundamental mechanisms as metabolic regulation, nutrition, differentiation and cell defence (De Duve & Wattiaux 1966; Holtzman 1976).

Early studies of Novikoff et al. (1964) and De Duve and Wattiaux (1966) suggested that lysosomes form as vesicles which bud off the Golgi apparatus or GERL, a

smooth tubular membrane system closely associated with the Golgi apparatus and the endoplasmic reticulum (Novikoff 1976). More recently, this idea was expanded to include receptor-mediated transport of lysosomal enzymes in these budding vesicles or "primary" lysosomes (Sly & Stahl 1978; Fischer et al. 1980a). The newly formed "primary" lysosomes may fuse with pinocytotic, phagocytotic or autophagic vacuoles containing diverse intracellular and extracellular materials which have been sequestered for digestion. Hence "secondary" lysosomes are those vesicles which are or have been sites of digestive activity. After the catabolism of endogenous and exogenous material in the secondary lysosomes, the diffusible low molecular weight hydrolysis products can return to the cytoplasm and undigested residues are either discarded by the process of exocytosis or are retained in the cells as residual bodies.

At least 100 different hydrolases have been demonstrated and their heterogeneity allows the degradation of a wide range of cellular constituents. Proteins, glycoproteins, polysaccharides, nucleic acids and glyco-, phospho- and neutral lipids are their natural substrates (for reviews, see Vaes 1973; Barrett and Heath 1977). The majority of these hydrolases have been characterized as glycoproteins (Barrett 1972; Touster 1973, 1978; for review, see Strawser & Touster 1980). They express maximal activity at acid pHs, ranging from about 3.5 to 5.5 (Barrett & Dean 1976) and they are specific with respect to the chemical linkage and structure of the monomeric unit that can be hydrolyzed. This specificity is reflected in the wide range of glycosidases that have been demonstrated in lysosomes and which represent, together with the proteases, the largest class of lysosomal enzymes (Strawser & Touster 1980). While the proteases are primarily endohydrolases breaking down proteins into smaller

fragments, the glycosidases are, with few notable exceptions (Neufeld et al. 1975) exohydrolases, i.e. they bring about the catabolism of their specific macromolecular substrates in a step-wise manner. Since these substrates with the exception of glycogen, are mainly heteropolymers consisting of a defined sequence of different sugars, a number of glycosidases are required to act in concert in order that efficient and complete degradation can occur. Each reaction is controlled by a specific hydrolase and the product of one reaction forms the substrate for the next one in the pathway. If any one of these enzymes is functionally deficient, then the process of degradation is arrested at the level of the missing enzyme. This block leads first to the progressive intralysosomal accumulation of partially degraded macromolecules with a characteristic non-reducing terminal residue and subsequently to the specific biochemical and clinical symptoms associated with a lysosomal storage disease (for reviews, see Hers & Van Hoof 1973; Stanbury et al. (eds.) 1978; Galjaard 1980).

Following the discovery of Hers (1963) that the accumulation of glycogen in Pompe's disease was due to a deficiency of acid α-1,4 glucosidase, a large number of different rare inherited metabolic disorders have been identified, each due to the deficit of a single or several lysosomal hydrolases. Although a single or multiple enzyme deficiency has been demonstrated in most of these conditions, the underlying nature of the defect is understood in very few.

Most lysosomal storage diseases are inherited as an autosomal recessive trait and some are X-linked. At the genetic level various types of mutations might result in a defective enzyme activity. These include mutations in the structural gene, in genes controlling post-translational modifications of the gene product and in regula-

tory genes controlling the synthesis of the active enzyme. These different possibilities of genetic regulation of lysosomal enzymes have been extensively studied in animal models (for reviews, see Paigen 1979, 1981).

A single base substitution (point mutation) in the structural region of a gene coding for a certain enzyme, might alter its structure in such a way as to affect one or more of its functional properties. Protein synthesis might be terminated prematurely so that the resultant polypeptide never realizes its catalytic activity and/or its antigenic properties (cross-reactive material negative). When protein synthesis is occurring at normal rates, then the mutation might have modified the kinetic characteristics of the enzyme. These kinetic mutants can have partially or totally altered catalytic activity and be cross-reactive material positive or negative depending on whether the antigenic site is also affected. Furthermore, even if the components of the active site are normal, the mutation might alter the enzyme's configuration rendering it excessively susceptible to degradation by endogenous proteases, or might influence the enzyme's ability to interact and aggregate with stabilizing "factors" and subunits respectively. Alternatively, a mutation which alters the gene(s) responsible for post-translational modifications of the enzyme might also lead to dramatic loss of its catalytic activity. Such disturbances might interfere with processes like glycosylation, phosphorylation or peptide cleavage and consequently alter tissue or subcellular localization of the enzyme even though a normal rate of synthesis of the enzyme polypeptide is occurring (for reviews, see Paigen 1979, 1981; Neufeld 1981).

At the metabolic level, as was previously mentioned, a single or multiple lysosomal enzyme deficiency results in a progressive accumulation of undegraded metabolites

in the lysosomes. Since these organelles are present in almost all cell types and they exhibit a nearly constant enzyme spectrum, the enzyme deficiency is, as a rule, observed in most tissues of the body, giving rise to a generalized disorder. The clinical manifestations of the disease are, however, a function of the quantity, nature and localization of the storage products. The latter may vary considerably from one tissue to another according to the autophagic and heterophagic potential of the cells, to their capacity to extrude digested material via the process of exocytosis and according to the distribution and rates of synthesis and breakdown of the different substrates in different cells. Hence, among the lysosomal storage diseases there are conditions with primary neural manifestations (e.g. gangliosidoses), visceral abnormalities (e.g. Fabry's disease), or systemic features (mucopolysaccharidoses) (for reviews, see Neufeld et al. 1975; Stanbury et al. (eds.) 1978; Galjaard 1980).

Furthermore, the rate of accumulation depends on the degree and character of the enzyme defect. Lysosomal glyco-hydrolases are in general specific for a particular type of linkage rather than for a particular substrate. Since the same monosaccharide unit may be present in glycolipids, glycoproteins and glycosaminoglycans, it follows that the storage material is likely to be heterogeneous. Nevertheless the various storage diseases that have been described, are primarily characterized by the accumulation of one specific potential substrate which predominates and causes specific clinical symptoms. In the classical type of G_{M1}-gangliosidosis (O'Brien 1972, 1978), for instance, an isolated β-galactosidase deficiency leads to a massive accumulation of G_{M1}-ganglioside resulting in the progressive neurological degeneration. The enzyme is, however, also capable of degrading galactose residues present in the acid mucopolysaccharide,

keratan sulphate. Consequently an associated storage of this compound is found in cells of the reticuloendothelial system (Suzuki 1968; Suzuki et al. 1969).

An opposite situation was recently observed in Morquio disease type B (Groebe et al. 1980), clinically regarded as a mucopolysaccharidosis, but biochemically related to a G_{M1}-gangliosidosis, since a severe β-galactosidase deficiency is the only defect so far detected. In this case the catabolism of the keratan sulphate is primarily altered, while the gangliosides are apparently normally degraded. It is apparent from these examples that clinically different disorders due to the preferential accumulation of one of the substrates normally degraded by the same hydrolase, may result from a change in the relative specificity or affinity of the mutant enzyme for the substrates in different tissues.

In other cases, however, a deficiency of the "same" enzyme and accumulation of the "same" storage substance or substances are found in association with clinically distinct syndromes. For a number of lysosomal storage disorders, patients with severe or mild clinical manifestations of the same disease, have been described (Galjaard 1980). A particularly striking example of this general phenomenon is found in Hurler's syndrome and Scheie's syndrome. In both cases a gross deficiency of α-iduronidase (Wiesmann and Neufeld 1970; Bach et al. 1972) is the biochemical cause of the disease, but the corresponding clinical features are so different that the two disorders were originally regarded as totally distinct mucopolysaccharidoses (McKusick 1972). An analogous situation is observed in Glycogenosis type II (Pompe's disease), where the accumulation of glycogen as the result of the deficiency of acid α-glucosidase is considered to cause the severe malfunction of muscles (Hers & De Barsy 1973). Nevertheless in patients with the

infantile form of the disease, both skeletal and cardiac muscles are pathologically and clinically involved, whereas in adult patients the cardiac symptoms are virtually absent (Engel et al. 1973; Reuser 1977; Loonen 1979). Similarly the infantile, juvenile and adult forms of G_{M1}-gangliosidosis illustrate considerable differences in the rate of development of clinical abnormalities (O'Brien 1972, 1978; Galjaard & Reuser 1977; Reuser 1977; Suzuki et al. 1977). In all conditions a marked deficiency of β-galactosidase results in accumulation of G_{M1}-ganglioside in the brain and of partially degraded keratan sulfate in the viscera.

The occurrence of two or more clinically distinct disorders associated with a deficiency of one and the same enzyme, might be explained by differences in the level and character of the residual enzyme activity. In these cases, complementation studies may reveal underlying genetic heterogeneity.

Another difficulty in elucidating the relationship between clinical and biochemical heterogeneity is that more than one locus may be involved in determining the molecular structure of a particular enzyme. In those instances a mutation at one of the loci may result in a deficiency of some isozymes but not of others. Furthermore if tissues or organs differ in their isozyme pattern, this will be reflected in the biochemical and clinical manifestations of a particular mutant. This point is well illustrated by the extensive studies on the isozymes of N-acetyl-β-D-hexosaminidase which have been carried out in relation to the two major variants of G_{M2}-gangliosidosis, Tay-Sachs disease and Sandhoff's disease (for reviews, see O'Brien 1978; Hoeksema 1979; Sandhoff & Christomanou 1979). In human tissue, three lysosomal hexosaminidases can be distinguished: the main isoenzymes, A and B (Robinson and Stirling 1968; Sandhoff 1968) and

likely that a number of other lysosomal storage diseases will also be due to genetic defects involving post-translational processing. We will therefore describe the current knowledge about the complex life history of lysosomal enzymes in more detail.

1.2 LIFE HISTORY OF LYSOSOMAL ENZYMES

a. Translation and signal sequence

The lysosomes can be regarded as specialized forms of secretory vacuoles and the nature of lysosomal enzymes has many common features with those of secretory proteins. Nevertheless, lysosomal hydrolases are segregated from the bulk of secretory products and routed to their final intracellular location via a series of specific co- and post-translational events (Fig. 1) (for review, see Neufeld 1981).

There is good evidence that some lysosomal enzymes, if not all, are synthesized as high molecular weight precursors (Skudlarek & Swank 1979, 1980; Hasilik & Neufeld 1980a,b) on membrane-bound ribosomes. They presumably gain access to the cisternal spaces of the endoplasmic reticulum by a conventional "signal sequence" (signal-peptide) of some 15-30 aminoacids along which hydrophobic residues, such as leucine, are predominant (Palade 1975; Blobel & Dobberstein 1975; Blobel et al. 1979). The signal sequences on the initial protein precursors are designated "pre" to distinguish them from the earlier discovered "pro" forms of intermediate protein precursors, such as proinsulin and proalbumin (for review, see Leader 1979). The first example of a pre-proform for a lysosomal enzyme has been recently described by Blobel and coworkers (Erickson et al. 1981). They demonstrated

that the primary translation product of cathepsin D, a pre-procathepsin D, synthesized in vitro from porcine spleen mRNA, is not yet glycosylated and carries a "pre" sequence of 20 aminoacid residues closely resembling the signal sequence of some presecretory proteins.

Such sequences are likely to occur in all newly synthesized lysosomal enzymes since they seem to be essential for translocation of nascent protein chains across the membrane into the intracisternal space of the endoplasmic reticulum. The signal peptide is thought to be recognized by a specific integral membrane protein that effects translocation of the polypeptide chain (Blobel 1980; Walter & Blobel 1980). The mode is probably co-translational, i.e. strictly coupled to translation, as earlier described for secretory proteins (Blobel & Dobberstein 1975; Campbell & Blobel 1976). The signal peptide has a transient existence and is detached from the growing chain by a membrane-bound peptidase, referred to as "signalase", before polypeptide synthesis is complete (Blobel & Dobberstein 1975; Campbell & Blobel 1976; Jackson & Blobel 1977; Walter et al. 1979; Erickson et al. 1981). Blobel and coworkers (Erickson et al. 1981) showed that addition of microsomal vesicles to the in vitro translation system resulted in the cleavage of the "pre" sequence from the pre-proform of cathepsin D and yielded segregated and glycosylated procathepsin D.

b. Glycosylation

The newly formed polypeptide is now subjected to glycosylation under the influence of a number of glycotransferases present in the rough and smooth endoplasmic reticulum. This process probably begins as a co-translational event (Rothman & Lodish 1977; Rothman et al. 1978) and can occur either during or after cleavage of

the signal peptide (for reviews, see Hubbard & Ivatt 1981; Schachter 1981). The process involves a complex interaction between the acceptor protein, that is being extruded through a membrane, a precursor lipid-linked oligosaccharide and a membrane-bound oligosaccharide transferring enzyme. The discovery of a glucose containing lipid oligosaccharide, the identification of enzymes such as endo-H, which release some oligosaccharides from glycoproteins, and the use of virus infected cells contributed significantly to the understanding of the various steps of the glycosylation process (see for review Hubbard & Ivatt 1981).

It is now established that a prerequisite for glycosylation is the occurrence on the nascent protein of certain susceptible asparagine (Asn) residues identified by their particular neighbouring aminoacids. The acceptor peptide must be unfolded and must contain the sequence -Asn-X-Thr (or Ser)-, where X can be almost any aminoacid (for reviews, see Struck & Lennarz 1980; Hubbard & Ivatt 1981; Schachter 1981). The Asn residue is subjected to glycosylation via transfer of a large high mannose precursor oligosaccharide. The latter is not assembled on the protein but is synthesized while linked to a lipid carrier, dolichol (Dol) (for reviews, see Elbein 1979; Struck & Lennarz 1980). For a variety of viral, plasma membrane and secretory glycoproteins the lipid linked precursor oligosaccharide has the composition: $(Glc)_3(Man)_9(GlcNAc)_2$-P-P-Dol., and its molecular structure in chinese hamster ovary cells has been determined (Li et al. 1978; Hubbard & Robbins 1979, 1980; for reviews, see Hubbard & Ivatt 1981). This high molecular weight precursor oligosaccharide is transferred "en bloc" from the lipid carrier to acceptor proteins (Czichi & Lennarz 1977; Hart et al. 1979; Struck et al. 1978; Struck & Lennarz 1980), under the action of specific

membrane-bound transferases. Membrane fractions with oligosaccharide transferring activity have been prepared from a wide variety of eukaryotic sources (for reviews, see Parodi & Leloir 1979; Struck & Lennarz 1980); and recently Das & Heath (1980) succeeded in purifying an oligosaccharide transferase from hen oviduct. These enzymes seem to have an absolute requirement for the sugar structure carrying three glucoses (Turco & Robbins 1979).

Good evidence that lipid-linked oligosaccharides are the source of Asn-linked glycans has come from studies with inhibitors and mutant cell lines. Drugs that inhibit lipid-linked oligosaccharide assembly have invariably been found to block glycosylation of Asn residues as well (for reviews, see Elbein 1979; Struck & Lennarz 1980). Tunicamycin, for instance, which blocks the formation of N-acetylglucosaminylpyrophosphoryldolichol, prevents the synthesis of any of the oligosaccharides normally transferred to asparagine residues (Schwarz & Datema 1980). The compound causes the production of unglycosylated enzymes which are consequently not targeted to lysosomes (von Figura et al. 1979). Its effect on the processing of cathepsin D, leads to a non-glycosylated procathepsin D, which is not proteolytically processed and whose secretion is inhibited (Erickson et al. 1981). These results suggest that the oligosaccharide chains of glycoproteins may contribute to stabilizing protein structure and are necessary for the correct delivery of proenzymes to their subcellular compartment.

Immediately after transfer to acceptor proteins the initially homogeneous population of precursor oligosaccharides begins to undergo a series of modifications that will eventually produce the diverse N-linked glycans of mature glycoproteins. While this is taking place, the glycoprotein is being transported through different

subcellular compartments. In several systems recently described (Hubbard & Robbins 1979; Kornfeld et al. 1978; Hunt 1979, 1980) the first phase of the trimming process entails the removal of glucose residues from the precursor oligosaccharide (for review, see Hubbard & Ivatt 1981).

The reaction is controlled by at least two specific neutral glucosidases (Grinna & Robbins 1979, 1980; Michael & Kornfeld 1980; Elting et al. 1980). These enzymes isolated from rat liver are integral membrane proteins that appear to be localized on the cisternal surface of the rough (and smooth) endoplasmic reticulum (Grinna & Robbins 1979) and are thus ideally situated for the processing of newly synthesized glycoproteins. After the loss of its glucose residues, the protein-linked oligosaccharide contains only mannose and N-acetylglucosamine. In a variety of cells this mannose rich intermediate has the composition: $(Man)_9(GlcNAc)_2$ (for review, see Hubbard & Ivatt 1981). The next stage of the trimming sequence entails the hydrolysis of some of the mannose residues, a process that is likely to occur in the Golgi apparatus, since specific mannose removing enzymes isolated from rat liver and tested in in vitro systems were found to be associated with Golgi fractions (Tulsiani et al. 1977; Opheim & Touster 1978; Tabas & Kornfeld 1979).

The protein-linked glycans that result may at this point undergo two subsequent fates:

a. - The cleavage of Man residues leaves a core structure consisting of $(Man)_3(GlcNAc)_2$, which is further processed by stepwise addition of "outer sugars", GlcNAc, galactose, sialic acid and fucose, carried out directly from nucleotide sugars by specific glycosyl transferases (see for reviews, Schachter & Roseman 1980; Schachter 1981). The mature glycoprotein, which arises from this process, carries the so-called "complex" type of branched oligosaccharide

that usually indicates that a protein is destined for export in a secretory vacuole.

b. - The incomplete processing of the mannose-rich oligosaccharide precursor gives rise to smaller high mannose glycans, with a variable number of residues. These are susceptible to endo-N-acetylglucosaminidases (i.e. endo-H), a characteristic peculiar to high mannose oligosaccharides (Tarentino & Maley 1974; Tarentino et al. 1978). Endo-H is widely used to discriminate between "complex" and "high mannose" carbohydrate moieties of glycoproteins (for reviews, see Schachter 1981; Hubbard & Ivatt 1981). Although a clear distinction has been made so far between "high-mannose" and "complex" types of oligosaccharides, we must allow for the existence of hybrid structures which combine in a single moiety features usually associated exclusively with either of the two types of oligosaccharides (Tai et al. 1977; Yamashita et al. 1978; Harpaz & Schachter 1980).

c. Phosphorylation and recognition

These initial steps of the biosynthetic pathway of cellular glycoproteins are probably shared by both secretory proteins and proteins, like the lysosomal hydrolases, destined to a defined subcellular compartment. It is now established that the carbohydrate moieties of lysosomal enzymes consist of Asn-linked "complex" and "high mannose" oligosaccharides. This is indicated by sugar composition (Strawser & Touster 1980) binding to certain lectins (van Elsen & Leroy 1979; Fiddler et al. 1979), partial sensitivity to endo-H (von Figura & Klein 1979; Natowicz et al. 1979) and inhibition of glycosylation by tunicamycin (von Figura et al. 1979). Nevertheless very little is known of the controlling factors that determine whether

the "complex" or "high mannose" type structure should predominate on any given protein. One factor may be the tertiary structure of the protein which could limit the extent to which an oligosaccharide is accessible to the modifying enzymes.

In this respect Hasilik and von Figura (1981) have recently made the important observation that in cathepsin D and β-hexosaminidase both forms of post-translational glycosylation can take place on the same enzyme molecule, leading to heterogeneity with regard to their carbohydrate content. The glycosylated enzyme precursors must, however, be packaged at a specialized region of the Golgi or Golgi related endoplasmic reticulum. This event can only occur if phosphorylation of the carbohydrate chain of the enzyme has taken place, to enable its segregation from the bulk of secretory proteins and its correct delivery to the lysosomes. This unique form of post-translational processing of the lysosomal enzymes has been extensively studied in cultured human fibroblasts (for review, see Sly & Stahl 1978; Sly et al. 1981; Neufeld 1981). The process involves the acquisition by the enzymes of a phosphorylated mannosyloligosaccharide, which acts as recognition marker for highly specific membrane receptors.

The basic work which led to the discovery and characterization of a recognition marker on lysosomal hydrolases came from Neufeld and coworkers in the course of experiments with "corrective factors" taken up via adsorptive pinocytosis by enzyme deficient fibroblasts (Neufeld & Cantz 1971; see for review Neufeld et al. 1975). These "corrective factors" proved to be lysosomal enzymes secreted by normal cultured fibroblasts. The uptake of these enzymes was found to be specific and saturable (von Figura & Kresse 1973). Subsequent studies with hydrolytic enzymes purified from various tissues and

body fluids (Brot et al. 1974; Nicol et al. 1974; for reviews, see Neufeld et al. 1977; Halley 1980) revealed the existence of "high" and "low uptake" forms. These findings suggested the presence of a "recognition marker" on the enzymes distinct from their catalytic site, which was needed for their uptake via specific receptors on the cell surface of fibroblasts (Neufeld et al. 1977).

Hickman & Neufeld (1972) postulated that most lysosomal enzymes share a similar or common "recognition marker" on the basis of the biochemical findings in "I-cell" disease. The multiple enzyme deficiency in mesenchymal cells of patients with this disease is accompanied by high levels of extracellular activity of the deficient enzymes. It was shown that these extracellular enzymes could not be taken up by cultured fibroblasts and hence were supposed to be "recognition defective" (Hickman & Neufeld 1972; for review, see Halley 1980). The basic molecular defect in "I-cell" disease has recently been elucidated (Hasilik et al. 1981; Reitman et al. 1981) and will be discussed later.

The first chemical analysis of the recognition markers of lysosomal enzymes had been performed with indirect methods since adequate quantities of high uptake enzymes for chemical analysis were not at that time available. The observation that the high uptake form of β-hexosaminidase was converted to a low uptake form by oxidation with periodate, suggested that the recognition marker might be a carbohydrate group (Hickman et al. 1974). Subsequently Hieber et al. (1976) reported that high concentrations of mannose inhibited pinocytosis of bovine testis β-galactosidase by fibroblasts and that the uptake marker on this enzyme was sensitive to α-mannosidase. Although these findings proved to be misleading, they helped to focus attention on mannose residues. Kaplan et al. (1977) demonstrated that polymeric mannans

inhibited the uptake of β-glucuronidase. Among these compounds the most potent inhibitors were found to be those carrying mannose residues phosphorylated in the 6 position. This observation led to the suggestion that Man-6-phosphate was a structural analogue of the recognition marker. Further more, alkaline phosphatase treatment of "high uptake" forms of β-glucuronidase and α-iduronidase converted the enzymes to less acidic, low uptake forms, (Kaplan et al. 1977; Sando & Neufeld 1977). These findings have been extended to a number of other acid hydrolases (Kaplan et al. 1977; Ullrich et al. 1978; for reviews, see Neufeld 1981; Sly et al. 1981), and their generality strongly implicated Man-6-P as the common recognition marker.

Direct evidence came from results obtained in different laboratories (Natowicz et al. 1979; Distler et al. 1979; von Figura and Klein 1979; Hasilik & Neufeld 1980a,b). Man-6-P was shown to be present on "high uptake" forms of acid hydrolases, recovered from extracellular sources whereas this marker is absent in mature intracellular forms of the same enzymes (for reviews, see Neufeld & Ashwell 1980; Neufeld 1981; Sly et al. 1981).

How does the phosphorylation of the carbohydrate side chain of the enzymes occur and what is the key role of the phosphorylated sugars in determining the fate of newly synthesized lysosomal hydrolases? It has been established that mannose-6-phosphate residues are located on oligosaccharides that can be cleaved by endoglycosidase H, i.e. of the high mannose type (Natowicz et al. 1979; von Figura & Klein 1979; Hasilik & Neufeld 1980b). Those mannose residues that are phosphorylated on the 6 position are probably linked α-1,2 to the remainder of the oligosaccharide (Distler et al. 1979). The surprising findings of Tabas and Kornfeld (1980) that the phosphate groups are not initially susceptible to alkaline phospha-

tase unless the oligosaccharide is first treated with mild acid, suggested the presence of a phosphodiester linkage and inferred that the phosphate groups might be transferred to the oligosaccharide in a "blocked" form. The blocking sugar present in the oligosaccharide moieties of several lysosomal enzymes (precursors) of human and murine origin, proved to be α-N-acetylglucosamine (Tabas & Kornfeld 1980; Hasilik et al. 1980). The major phosphorylated oligosaccharides contain one, two or possibly three phosphate groups each buried in a diester with N-acetylglucosamine (Hasilik et al. 1980). These findings implied that the synthesis of phosphorylated recognition markers of lysosomal glycoproteins requires the consecutive action of two processing enzymes: first a phosphotransferase capable of transferring N-acetylglucosamine-phosphate from a phosphate donor to the 6 position of mannose, and secondly a diesterase which cleaves the phosphodiester bond to expose Man-6-P groups on the oligosaccharides.

The first processing step has recently been characterized by Reitman and Kornfeld (1981a). They demonstrated the existence of a membrane-bound UDP-GlcNAc: glycoprotein-N-acetylglucosamine-1-phosphotransferase capable of the transfer "en bloc" of α-linked N-acetylglucosamine-1-phosphate to the 6 hydroxyl of mannose in high mannose oligosaccharides of glycoproteins. The same authors were also able to partially purify the transferase and examine its substrate specificity (Reitman & Kornfeld 1981b). It exhibits a striking preference for acid hydrolases over other potential glycoprotein acceptors. This high specificity suggests a possible influence of the polypeptide backbone of the lysosomal glycoproteins on the action of the processing enzyme. Although no evidence to support this proposal is yet available, the reported data strongly infer that this phosphotrans-

ferase is the initial and determining enzyme for the pathway which eventually results in the segregation of acid hydrolases into lysosomes.

The next post-translational modification step involves the action of a specific α-N-acetylglucosaminyl phosphodiesterase capable of removing the outer "blocking" α-N-acetylglucosamine residues. It has recently been identified in microsomal fractions isolated from rat liver (Varki & Kornfeld 1980) and subsequently purified and characterized (Varki & Kornfeld 1981). It is apparent that both post-translational processes described above are occurring in the Golgi or Golgi related regions since subcellular localization studies showed that the transferase and diesterase activities fractionate along with Golgi marker enzymes (Varki & Kornfeld 1980; Waheed et al. 1981). The precursor lysosomal hydrolases are at this stage fully equipped for their interaction with specific membrane receptors, which will ensure their eventual location in the lysosomes. Their subsequent fate is thus directly related to this step of the processing pathway. The receptor protein has recently been partly characterized by Sahagian and coworkers (1981), who have also demonstrated its ability to bind high uptake forms of β-galactosidase.

There remains, however, the question concerning the route the enzymes follow to reach their final subcellular location. The fact that exogenous enzymes are taken up by fibroblasts via Man-P receptor-mediated pinocytosis and that endogenous enzymes are to a certain extent released in the extracellular fluid, along with the observation that a small pool of lysosomal hydrolases is always present at the cell surface, raised various hypotheses regarding the mechanism of delivery of newly synthesized enzymes to the lysosomes (for reviews, see Hasilik 1980; Halley 1980; Neufeld 1981; Sly et al. 1981). The currently

favoured opinion is that the receptor/mannose-6-phosphate system that operates in the uptake of exogenous enzymes primarily serves to deliver newly synthesized endogenous enzymes to the lysosomes by a direct intracellular route (Sly et al. 1981). Studies of enzyme binding to membranes from broken fibroblasts (Fischer et al. 1980b) revealed that over 80% of the specific receptors are on intracellular membranes and only 20% are present on the cell surface. The internal pool of receptors probably plays an essential role in the intracellular traffic of newly made lysosomal enzymes. Receptor bound hydrolases collect into vesicles which bud off the Golgi or GERL to become primary lysosomes. Before or when primary lysosomes become secondary lysosomes, a decrease in pH allows a pH-dependent release of enzyme molecules from the receptors and recycling of the receptor to the Golgi apparatus by a route that is not yet clear. The assumption of recycling is mainly based on a calculation of the number of receptors that is needed to explain the observed rate of pinocytosis of exogenous enzymes by fibroblasts (Sly et al. 1981).

Thus the recognition marker and the receptor can be considered as key components which allow lysosomal enzymes to segregate from other glycoproteins in the Golgi that are destined for secretion. This is illustrated by the fact that the lack of the recognition marker, as is the case in "I-cell" disease, prevents lysosomal enzymes from being segregated and results in their abnormal release in the extracellular space. Further evidence comes from the results obtained after treatment of cultured fibroblasts with lysosomotropic agents like NH_4^+ and chloroquine (Gonzalez-Noriega et al. 1980). These compounds interfere with the normal intracellular pathway of lysosomal enzymes which results in the secretion of their precursor forms into the extracellular medium (Hasilik & Neufeld

1980a). The mechanism by which this occurs was reported by Gonzalez-Noriega et al. (1980; for review, see Neufeld 1981). The amines are capable of diffusing through cell membranes and become protonated within the lysosome (De Duve et al. 1974). The lysosomal membrane is impermeable to the protonated amines which become entrapped, with consequent increase of the intralysosomal pH (Okhuma & Poole 1978). The rise in pH is thought to inhibit the release of receptor-bound enzyme so that eventually all receptors are saturated. At that point no further enzyme can be transported to lysosomes, and newly synthesized precursor forms of lysosomal enzymes are secreted and rapidly accumulate extracellularly.

d. Intralysosomal processing

When the lysosomal hydrolases are eventually sequestered within the lysosomes, a further modification of their polypeptide chain occurs, which enables their conversion into mature and fully active forms. The process of limited proteolysis closely resembles the "zymogen activation", involved in a great variety of biological processes, such as blood coagulation, complement activation, hormone production etc. (see for review Neurath & Walsh 1976), in which an inactive precursor protein is converted to an active form at the appropriate site of action. The final maturation step of the lysosomal enzymes thus requires the action of specific processing proteases which carry out a selective cleavage of susceptible peptide bonds. This limited proteolysis reduces the high molecular weight precursor molecules, which may already exhibit some enzymic activity, to the ultimate size and conformation of their purified tissue counterparts (see for reviews Hasilik 1980; Neufeld 1981).

This event has been shown to occur for a number of

lysosomal hydrolases recovered from different sources: β-galactosidase of mouse peritoneal macrophages (Skudlarek & Swank 1979, 1980); β-hexosaminidase, α-glucosidase, cathepsin D and α-L-iduronidase of cultured human fibroblasts (Hasilik & Neufeld 1980a; Myerowitz & Neufeld 1981); cathepsin D of cultured porcine kidney cells (Erickson & Blobel 1979; Erickson et al. 1981); β-hexosaminidase and α-L-iduronidase of CHO cells (Robbins & Myerowitz 1981). In all but one case the enzymes are found to be synthesized as larger precursor forms and trimmed to their eventual size over the course of several hours or, in some cases, days (Hasilik & Neufeld 1980a,b). This process probably occurs as a late event after the hydrolases become packaged within the lysosomes. Consistent with this assumption is that the newly synthesized lysosomal enzymes of cultured fibroblasts, artifically diverted extracellularly with lysosomotropic agents (NH_4Cl) are the high molecular weight precursor forms. Nevertheless they show all the prerequisites for their final subcellular localization and even exhibit in some instances a hydrolytic activity (Sly et al. 1981; Neufeld 1981).

Further evidence is derived from the observation that some precursor forms of lysosomal enzymes can undergo shortening of their polypeptide chain in a cell-free system (Frisch & Neufeld 1981). The nature of the proteolytic processing varies from one enzyme to another. It may consist of a simple removal of a short peptide, presumably allowing a more efficient conformation of the enzyme to be attained. It may also take several intermediate steps and results in a multipeptide structure being generated from a single polypeptide chain. Such a reaction is seen in the β-hexosaminidases in which the β-subunit is synthesized as a single chain but subsequently appears in the mature enzyme as two forms with a differ-

ent molecular weight (Hasilik & Neufeld 1980a). Similarly in cathepsin D both a single peptide and a form with two peptide chains have been observed (Erickson et al. 1981). In the latter case the two products, generated by cleavage of the precursor form of the enzyme, represent a light and heavy chain that remain non-covalently bound.

Within the lysosomes the mature forms of the enzymes appear to be relatively resistent to proteolytic attack, since they can exist in the presence of high concentrations of cathepsins and other lysosomal hydrolases. The turnover time is, on average, relatively long (see for review Touster 1978). We do not yet know the molecular mechanisms that play a role in the protection of enzymes in the hostile environment within the lysosome. It may be that a particular configuration of the enzyme or the presence of carbohydrate side chains or other components with a protective effect play a role in maintaining a long half life of the lysosomal enzymes. Eventually the enzymes are denatured and degraded presumably while they exist within the lysosomes, although cytosolic degradation after the rupture of old lysosomes or of residual bodies may not be impossible.

From the description of the life history of the lysosomal enzymes it will be clear that the many events that must occur with some precision between the initial translation of a lysosomal enzyme protein and its final correct sequestering and full activation within the lysosomes, offer numerous opportunities for a genetic lesion that could result in faulty expression of the enzyme activity.

CHAPTER II THE EXPERIMENTAL WORK
INTRODUCTION - DESCRIPTION - DISCUSSION

2.1 INTRODUCTION

So far two inherited lysosomal storage diseases have been reported, which are associated with a multiple lysosomal enzyme deficiency. They are mucolipidosis II ("I-cell" disease) and combined β-galactosidase and neuraminidase deficiency and share the common feature of resulting from a post-translational modification error.

This thesis presents a series of studies on these two genetic disorders, carried out using a variety of experimental approaches, with the purpose of investigating the molecular and genetic nature of these diseases.

Complementation studies after somatic cell hybridization have been the most direct means for clarifying whether clinical heterogeneity was based on different gene mutations. After fusion of cultured fibroblasts from patients with the same enzyme deficiency, the analysis of multinuclear heterokaryons carrying the genetic information of both mutant cell strains, may in some instances reveal the restoration of the metabolic defect (for reviews, see Ringertz & Savage 1976; Bootsma & Galjaard 1979; Galjaard 1980). Since I-cell fibroblasts are deficient for a number of lysosomal hydrolases, they are a useful model in complementation studies. Thus we have performed a series of fusions between "I-cells" and other mutant fibroblasts with a single or multiple enzyme deficiency and followed the occurrence of complementation (Publications I-II-III).

An advance in this type of investigations has been achieved using "cybridization" techniques. The term "cybrid" was introduced (Bunn et al. 1974) to indicate a fusion between a whole cell and an enucleated cell (cyto-

plast). This approach could be applied in our complementation studies, in order to investigate the possible influence of cytoplasmic factors, for which a de novo synthesis is apparently not mandatory, for the restoration of the hydrolytic activity of the lysosomal enzyme deficient in the two different mutant cell strains (Ringertz & Savage 1976; Jongkind et al. 1981). In the course of our experimental work cybridization analyses have been carried out after fusion of I-cell fibroblasts with enucleated cells with an isolated lysosomal enzyme deficiency due to a structural mutation (Publication II).

In parallel with the series of experiments where somatic cell hybridization was performed, the two mutant cell strains involved were co-cultivated in mixed population. The latter method, extensively used in studies on the uptake and release of lysosomal hydrolases by normal and mutant fibroblasts (Halley 1980), has usually been included in our experimental procedures to avoid any misleading interpretation of the complementation data. In some instances, however, the unexpected finding of an increased enzyme activity (neuraminidase) after co-cultivation of two fibroblast strains both deficient for the same enzyme (publication III), favoured a different interpretation of the experimental results and required other approaches for supporting a new theory.

Lysosomotropic agents such as NH_4Cl or chloroquine have been used widely to enrich the population of lysosomal hydrolases secreted by cultured normal and mutant fibroblasts. These enzymes recovered in the medium are high uptake forms and, provided that they are stable in that environment, they can be readily taken up by fibroblasts since they carry the Man-P recognition marker (Sly et al. 1981; see also chapter I). In our uptake studies, we have used NH_4Cl-induced secretions, collected from normal and mutant cell cultures, as the enzymic source,

and β-galactosidase/neuraminidase deficient fibroblasts as the recipient cells (Publication IV).

In view of the results so far obtained, our latest work mainly concerned the characterization of the molecular and biochemical properties of β-galactosidase using immunological approaches (Publication V). Immunoprecipitation of radiolabelled lysosomal glycoproteins by means of specific antisera raised against their purified placental couterparts, has contributed a great deal of knowledge on the post-translational processing and fate of these hydrolases (Neufeld 1981).

2.2 MUCOLIPIDOSIS II ("I-CELL" DISEASE)

a. Clinical-pathological-biochemical manifestations

This neurodegenerative lysosomal storage disease which is inherited as an autosomal-recessive trait, was first described by Leroy & De Mars (1967). Clinical and radiologic features are present from birth on, such as dislocation of the hips, thoracic deformities, hernia and hypotonia. Patients show coarsening of facial features, gingival hyperplasia, severe growth deceleration, recurrent respiratory infections and progressive psychomotor retardation. The disease has a slowly progressing course and patients usually die between 5 and 6 years of age because of pneumonia or congestive heart failure (Leroy et al. 1971; for reviews, see McKusick et al. 1978; Galjaard 1980). Although patients with "I-cell" disease may in some ways resemble those with Hurler's disease (Mucopolysaccharidosis I-H) they can be distinguished by the absence of corneal opacity, absent or only slight hepatosplenomegaly and normal level of glycosaminoglycans in the urine.

The histopathological characterists of this disorder

is the presence in cultured fibroblasts and a few other cell types of numerous cytoplasmic inclusions visible by phase microscopy, hence the name "I-cell" disease (Leroy & De Mars 1967). These inclusions have been identified as very large lysosomes filled with heterogeneous undegraded material (Tondeur et al. 1971; Leroy et al. 1972; for review, see Martin et al. 1975). It is so far unclear why the pathological and biochemical abnormalities in this disease are limited to cells of mesenchymal origin.

Cultured fibroblasts derived from patients with "I-cell" disease demonstrate a total or partial deficiency of most lysosomal hydrolases which are recovered in excess in the culture medium (Leroy et al. 1972; Wiesmann & Herschkowitz 1974). In patients an increased extracellular activity of lysosomal hydrolases has been found in plasma, cerebrospinal fluid, tears and urine (Wiesmann et al. 1971b; Den Tandt et al. 1974; Pittman et al. 1979; for reviews, see McKusick et al. 1978; Galjaard 1980). The intracellular multiple enzyme deficiency which accounts for the intralysosomal storage of glycosaminoglycans and glycolipids, seems to be confined to cells of mesenchymal origin. Normal enzyme activities have been found in liver, spleen and brain, with the exception of β-galactosidase, neuraminidase and acid lipase (Miller et al. 1979; Minami et al. 1979; Pittman et al. 1979). Even in fibroblasts, β-glucosidase and acid phosphatase have enzymic activities within the range of control values (Neufeld et al. 1977), although their binding capacity to lectins shows the same aberrant behaviour as the deficient hydrolases (Rousson et al. 1979). Furthermore the intra- and extracellular "I-cell" hydrolases show abnormal electrophoretic mobility (Champion & Shows 1977; Vladutiu & Ratazzi 1979; Honey et al. 1981) as a result of their high sialic acid content, which may in turn be due to a severe deficiency of the lysosomal α-N-acetylneuraminidase

(Thomas et al. 1976; Strecker et al. 1976). The enzymes secreted by "I-cell" fibroblasts, in contrast to their normal counterparts, fail to function as corrective factors, as a result of being poorly taken up by fibroblasts of another genotype (Hickman & Neufeld 1972).

During the last decade a variety of hypotheses have been put forward to explain the genetic defect responsible for the numerous pleiotropic effects observed in "I-cell" disease. The idea that the multiple lysosomal enzyme deficiency was caused by "cellular leakage" (Wiesmann et al. 1971a) has been experimentally proven to be incorrect (Hickman & Neufeld 1972). On the basis of their biochemical findings, Hickman and Neufeld (1972) proposed that many lysosomal hydrolases share a common type of recognition marker for their uptake which is missing or masked on the enzymes secreted by "I-cells". The authors also thought that acid hydrolases synthesized by normal fibroblasts are first secreted into the medium and then recaptured via receptor-mediated endocytosis, to be packaged in the lysosomes (Hickman & Neufeld 1972; Neufeld et al. 1977). The former proposal has subsequently been supported by various studies implicating Man-6-P as the essential component of the recognition marker of the lysosomal acid hydrolases (Kaplan et al. 1977; Sly & Stahl 1978; Natowicz et al. 1979). Hasilik and Neufeld (1980b) have recently demonstrated that "I-cells", in contrast to normal fibroblasts, fail to incorporate (^{32}P) phosphate into newly synthesized β-hexosaminidase, cathepsin D and α-glucosidase. These observations and those of Bach et al. (1979) have supported the hypothesis that the defect in "I-cell" disease is in the biosynthesis of the phosphomannosyl-signal responsible for the correct sequestering of newly synthesized acid hydrolases within the lysosomes (the various steps involved in this biosynthetic pathway have been described in the first

chapter).

The basic defect in "I-cell" disease has now been elucidated as a deficiency of a membrane bound UDP-GlcNAc: glycoprotein-N-acetylglucosamine-1-phosphotransferase (Hasilik et al. 1981; Reitman et al. 1981). This enzyme located in the Golgi apparatus normally transfers α-linked N-acetylglucosamine-1-phosphate to the 6-hydroxyl of mannose in high mannose oligosaccharides of glycoproteins. Both in "I-cell" disease and in a clinically milder variant of this disease, Mucolipidosis III, the defective phosphorylation of lysosomal hydrolases results in the abnormal secretion of incorrectly processed precursor glycoproteins. The report that "I-cell" secreted enzymes not only lack the Man-6-P recognition marker but also bear complex type oligosaccharide chains (Hasilik & von Figura 1981) supports the hypothesis that the presence of the mannose-6-phosphate residue may normally prevent further processing to the complex type (for review, see Sly et al. 1981).

Sly lately reported (personal communication) about two variants of ML III disease. In one type of this disorder the mutation seems to affect the catalytic site of the phosphotransferase, whereas in another type it appears to be confined to the binding site of the transferase to the glycoprotein.

b. Discussion of the experimental work

In Publication I fibroblasts from two patients with "I-cell" disease were hybridized with fibroblasts from patients with G_{M1}-gangliosidosis (β-galactosidase deficient), G_{M2}-gangliosidosis type Sandhoff (β-N-acetylglucosaminidase A and B deficient) and a patient with a combined β-galactosidase and neuraminidase deficiency ($\beta\text{-gal}^-/\text{neur}^-$). In all instances the heterokaryon populations

showed a marked increase (3 to 10 fold) of the enzyme activity tested. The β-galactosidase deficiency in G_{M1}-gangliosidosis had been demonstrated to be due to a structural defect of the 64,000-dalton monomeric form of the enzyme polypeptide because of a mutation on chromosome 3 (Norden et al. 1974; Hoeksema et al. 1980). The restoration of β-galactosidase after fusion of these mutant fibroblasts with "I-cells" must thus be due to a correction of the post-translational modification defect which is responsible for the multiple lysosomal enzyme deficiency in "I-cell" disease. The same is true for the complementation observed for hexosaminidase A and B after fusions of "I-cells" with Sandhoff fibroblasts since the hexosaminidase deficiency in the latter cells are also due to a structural mutation (Beutler & Kuhl 1975; Hoeksema 1979). Normal levels of β-hexosaminidase and α-mannosidase activity could be detected in the culture medium above "I-cells" fused with Sandhoff fibroblasts, and "I-cells" fused with mannosidosis fibroblasts respectively. This observation (publication I) specifically points to a correction of the molecular defect in "I-cell" disease, since the abnormal release of lysosomal hydrolases into the extracellular space is one of the characteristics of this disorder. Concomitantly a normalization of the altered electrophoretic pattern of "I-cell" hexosaminidase isoenzymes was observed after fusion of "I-cells" with Sandhoff fibroblasts. The abnormal electrophoretic mobilities of most of the "I-cell" lysosomal hydrolases is due to the increased sialic acid content of the enzymes, related to the gross deficiency of neuraminidase. Thus the correction after cell hybridization probably results from restoration of the neuraminidase activ ity in the heterokaryons. Experiments carried out by us and others (Spritz et al. 1979) failed, however, to demonstrate that addition of exogenous neuraminidase to

"I-cell" fibroblasts could diminish the abnormal secretion of the lysosomal hydrolases.

The results of cell hybridization between "I-cells" and cells with a combined β-galactosidase/neuraminidase deficiency (β-gal$^-$/neur$^-$) are of particular interest. The multiple lysosomal enzyme deficiency in "I-cell" disease is known to involve an early step in the post-translational processing of lysosomal hydrolases and there were several arguments to favour the hypothesis that β-gal$^-$/neur$^-$ is also caused by a modification error. The observed restoration of β-galactosidase activity (Publication I) in the heterokaryon populations implies that at least two different genes are responsible for the defective β-galactosidase activity in the two diseases.

In Publication II we have further investigated the nature of the correction of the "I-cell" defect. Whole fibroblasts from patients with "I-cell" disease were fused with enucleated fibroblasts with a single lysosomal enzyme deficiency (hexosaminidase A+B, β-galactosidase, α-mannosidase). To produce a high percentage of such "cybrids" we have applied fluorescence activated cell sorting (FACS) in order to obtain a pure population of cytoplasts (Schaap et al. 1979; for review, see Jongkind et al. 1981). The fibroblasts with a single lysosomal enzyme deficiency were labelled with green fluorescent latex spheres before the enucleation was performed; this enabled further identification of the fusion products (cytoplasts x nucleated "I-cells"). In all instances these "cybrids" showed partial restoration of lysosomal enzyme activity, whereas no increase was seen when "I-cell" cytoplasts were fused with whole mutant fibroblasts. The results indicate that the "I-cell" defect can be corrected by a longlasting "factor" present in enucleated cells of other genotypes. This "factor" can apparently act without the need of de novo synthesis although

the absence of correction in in vitro experiments implies that cellular integrity is required.

Publication III deals with co-cultivation and hybridization of fibroblasts with a multiple lysosomal enzyme deficiency and with cells from patients with a single β-galactosidase deficiency (different clinical G_{M1}-gangliosidosis variants) or a single neuraminidase deficiency (different variants of mucolipidosis I or sialidosis). These studies became only possible after a sensitive fluorescent assay for neuraminidase had been developed (Warner & O'Brien 1979). For the experiments reported in Publication III Dr. J.S. O'Brien (Dept. of Neurosciences, La Jolla, Calif., U.S.A.) kindly provided the 4-methylumbelliferyl-α-2-N-acetylneuraminic acid and in later studies (Publications IV and V) a similar substrate was synthesized for us by Prof. Dr. R. Brossmer (Institut für Biochemie, University of Heidelberg). Fusions of "I-cells" with single $neur^-$ fibroblasts and with $\beta\text{-}gal^-/neur^-$ cells resulted in a clear increase of neuraminidase activity in the heterokaryon populations. The occurrence of complementation indicates that at least three different gene mutations must be responsible for the neuraminidase deficiency in "I-cell" disease, sialidosis and combined β-galactosidase/neuraminidase deficiency.

Since the basic molecular defect of "I-cell" disease has now been discovered, it is apparent that the restoration of lysosomal enzyme activities after fusion of "I-cells" with a variety of other mutant fibroblasts (described in Publications I-III) is the result of the presence in the latter of the phosphotransferase missing in "I-cells", and the presence in "I-cells" of the structural gene for a particular enzyme. Our studies on cybrids (publication II) showed that the phosphotransferase also acts after enucleation of the complementing cells and

that it is stable in the cytoplasm for at least three days. An interesting finding was that β-gal$^-$/neur$^-$ cells could be partially corrected when co-cultivated with other mutant fibroblasts (publication III) whereas "I-cells" failed to exhibit this corrective activity. This suggested that the "corrective factor" for combined β-gal$^-$/neur$^-$ is probably a lysosomal glycoprotein which is affected in "I-cells" by the same phosphorylation defect responsible for the multiple lysosomal enzyme deficiency.

2.3 COMBINED DEFICIENCY OF β-GALACTOSIDASE AND NEURAMINIDASE

a. Clinical-pathological-biochemical manifestations

Since the discovery by Okada and O'Brien (1968) that β-galactosidase deficiency is the responsible molecular defect in G_{M1}-gangliosidosis a variety of patients with the infantile, progressive form (type 1), later onset juvenile form (type 2) and milder adult forms (Suzuki et al. 1977) have been described (for reviews, see Galjaard & Reuser 1977; O'Brien 1978; Galjaard 1980). In addition, a number of atypical patients have been reported who showed clinical manifestations resembling those in mucopolysaccharidosis, gangliosidosis and mucolipidosis (Goldberg et al. 1971; Orii et al. 1972; Loonen et al. 1974; Fukunaga et al. 1976; Suzuki et al. 1977). Since one of these atypical patients was discovered by our own group (Loonen et al. 1974; Koster et al. 1976) the genetic and molecular background of the β-galactosidase deficiency could be studied in more detail (for reviews, see Galjaard & Reuser 1977; Reuser 1977; O'Brien 1978; Hoeksema 1979; Galjaard et al. 1981).

The patient described by Loonen et al. (1974) had a

relatively normal development until young adulthood and then developed myoclonus, cerebellar ataxia, mental retardation, angiokeratoma corporis diffusum, macular cherry-red spot and mild bone deformities. Cultured skin fibroblasts from this patient were hybridized with β-galactosidase deficient fibroblasts from patients with infantile and juvenile forms of G_{M1}-gangliosidosis and a restoration of β-galactosidase activity was found in single heterokaryons (Galjaard et al. 1974). This complementation demonstrated that two different gene mutations were involved. Norden et al. (1974) had, however, purified normal human liver β-galactosidase and found that this enzyme consisted of a single polypeptide with a molecular weight of about 70,000 and a multimeric form of the same polypeptide of 700,000 daltons. Since intragenic complementation had never been demonstrated in human cells, the results of the complementation studies suggested that the β-galactosidase deficiency in the atypical patient described by Loonen et al. (1974) was due to a defect other than a structural abnormality of the β-galactosidase polypeptide. Nevertheless the patient was classified as a type 4 G_{M1}-gangliosidosis since another, younger atypical patient with β-galactosidase deficiency had been designated as type 3 G_{M1}-gangliosidosis by Pinsky et al. (1974). Fusion of fibroblasts from these two atypical patients did not result in complementation (Galjaard et al. 1975), which would be consistent with the assumption that they are due to a similar type of gene mutation.

Biochemical analysis of the residual β-galactosidase activity in the atypical patients revealed normal kinetic properties (Galjaard & Reuser 1977) and immunological studies (O'Brien & Norden 1977) indicated the presence of normal amounts of cross-reactive material, as is the case in the infantile and juvenile forms of G_{M1}-gangliosidosis (Meisler & Ratazzi 1974; O'Brien 1975, 1978). Gel filtra-

tion analysis subsequently showed that in cells from atypical patients the residual β-galactosidase activity was due to the 70,000-dalton monomeric form, whereas the high molecular weight form was totally absent (Hoeksema et al. 1979). This pointed to a mutation affecting the aggregation of β-galactosidase polypeptides.

Gene localization studies using Chinese hamster x human hybrids showed that in normal cells two genes, on chromosome 3 and 22, are involved in the expression of β-galactosidase activity and that the infantile and juvenile forms of G_{M1}-gangliosidosis are due to a mutation on chromosome 3 leading to an abnormal β-galactosidase polypeptide (for review, see Hoeksema 1979).

In cybridization studies (de Wit-Verbeek et al. 1978) cytoplasts of infantile type G_{M1}-gangliosidosis cells were found to be able to restore the β-galactosidase activity in fibroblasts from atypical patients described by Pinsky et al. (1974) and Loonen et al. (1974). Cytoplasts of the latter fibroblasts could not, however, complement classical G_{M1}-gangliosidosis cells. These results suggested that a relatively stable cytoplasmic factor present even in β-galactosidase deficient fibroblasts was able to correct the defect in the atypical patients.

In 1978 Wenger et al. made an important contribution by their observation that β-galactosidase deficient fibroblasts from one of their atypical patients showed a coexistent deficiency of neuraminidase using neuraminlactose, N-acetyl-neuraminic acid and fetuin as substrates. This finding has led to a reexamination of several patients that previously had been classified as atypical forms of G_{M1}-gangliosidosis. So far the combined β-galactosidase/neuraminidase deficiency has been confirmed in cells from patients described by Loonen et al. (1974); Pinsky et al. (1974); Justice et al. (1977);

Suzuki et al. (1977); Andria et al. (1978, 1981). O'Brien (1981); Lowden et al. (1981)). Also our group has made the first prenatal diagnosis of this disease in a pregnancy at risk, where two previous babies had died with fetal hydrops (Kleijer et al. 1979).

The onset of clinical manifestations and their nature vary among different patients with combined β-galactosidase/neuraminidase deficiency (for reviews, see Lowden & O'Brien 1979; Galjaard 1980). Most patients have been normal at birth and subsequent development has been undisturbed for a period varying from a few years up to nearly two decades. The most common manifestations are skeletal abnormalities, coarse facial features, myoclonus, psychomotor degenerative changes and macular cherry-red spot at around ten years of age. Seizures, ataxia, loss of hearing and vision, hepatosplenomegaly and corneal clouding may also occur. Mental retardation has become apparent at different ages among various patients but seems to be a common feature in the disease.

Thus far very few pathological investigations have been reported (Suzuki et al. 1977; Kleijer et al. 1979; Lowden et al. 1981). Cytoplasmic inclusions with positive histochemical staining for glycogen, glycosaminoglycans and lipids have been found in connective tissue, macrophages, in spleen, liver and bone marrow, glomerular epithelium, hepatocytes, and some endocrine organs. In addition, vacuolization was seen in ganglion cells of dorsal root ganglia, the living cells of the choroid plexus, and moderate lysosomal storage was present in the neurons in the basal ganglia, spinal cord and cortex. Electron microscopy shows membrane bound inclusions, filling most of the cytoplasm. In the neurons in addition to many empty vacuoles or lysosomes filled with granular material there is accumulation of membranous and vesicular structures similar to those seen in gangliosidoses.

Biochemical analyses of stored products at autopsy (Suzuki et al. 1977; Kleijer et al. 1979; Svennerholm (personal communication), Lowden et al. 1981) revealed an increased content of sialyloligosaccharides in visceral organs and in some instances also in brain tissue though less marked. There is no change in the quantity and types of gangliosides. Kleijer et al. (1979) did not find any residual neuraminidase activity in the organs of an affected fetus and like other investigators (Suzuki et al. 1977; Lowden et al. 1981) they found a residual β-galactosidase activity of 10-15%. Previous studies (Galjaard et al. 1975) had shown that this residual β-galactosidase activity is similar for different natural and artificial substrates (for reviews, see also O'Brien 1978; Galjaard 1980).

The patients with combined β-galactosidase/neuraminidase deficiency excrete high amounts of sialyloligosaccharides in their urine and in their leucocytes or cultured fibroblasts hardly any residual neuraminidase activity can be demonstrated with either natural substrates or 4-methylumbelliferyl-α-2-N-acetylneuraminic acid. Studies on fibroblasts from heterozygous parents of $\beta\text{-gal}^-/\text{neur}^-$ patients revealed β-galactosidase activities in the low control range whereas neuraminidase activities were about half the control values (Wenger et al. 1978; Hoogeveen et al. 1980; O'Brien 1981; Lowden et al. 1981).

These observations led some investigators to believe that in these patients neuraminidase deficiency is the primary defect and that the deficiency of β-galactosidase is a secondary effect caused by inhibition by intracellular storage of sialylated products (O'Brien 1979). Unfortunately this assumption has led to a premature classification of the patients with combined β-galactosidase/neuraminidase among the sialidoses (Lowden & O'Brien 1979; O'Brien 1981; Lowden et al. 1981). This is

especially confusing since the sialidoses have been subdivided in two groups based on the clinical manifestations without taking into account the precise nature of the genetic and molecular defect underlying the disease.

The name sialidosis was first used by Durand et al. (1977) to designate the syndrome of two siblings of 22 and 13 years of age who developed visual impairment and mild neurological manifestations in late childhood. Cultured fibroblasts and leucocytes from these patients showed a neuraminidase deficiency. Up to that period a single neuraminidase deficiency had been found in patients with classical mucolipidosis I (Spranger 1975). This disease, which is inherited as an autosomal-recessive trait, has a progressive course and patients show from early after birth on, skeletal dysplasia, myoclonus, mental deterioration, coarse facial features, ataxia and macular cherry-red spot (for reviews, see Lowden & O'Brien 1979; Galjaard 1980). Cantz et al. (1977) were first to find that a neuraminidase deficiency is the primary defect in mucolipidosis I.

During the last few years a number of patients with a single neuraminidase deficiency but milder clinical manifestations have been reported (Durand et al. 1977; O'Brien 1977; Rapin et al. 1978; Thomas et al. 1978; for reviews, see Lowden & O'Brien 1979; O'Brien 1981; Galjaard et al. 1981). The main features in these patients have been macular cherry-red spot, myoclonus, visual impairment and sialyloligosaccharides in the urine. Differences with the classical form of mucolipidosis I are the normal intelligence, absence of skeletal and visceral abnormalities and a longer survival. In all these patients with a single neuraminidase deficiency the β-galactosidase activity in various cell types is normal, which implies that a neuraminidase deficiency per sé does not result in a β-galactosidase deficiency. The term "sialidosis"

should be reserved for patients with a single neuraminidase deficiency, irrespective of their clinical manifestations. The designation G_{M1}-gangliosidosis must be restricted to patients with a single β-galactosidase deficiency and accumulation of G_{M1}-gangliosides, again irrespective of the time of onset and severity of the clinical manifestations. Patients with a combined β-galactosidase/neuraminidase deficiency can only be properly classified when the responsible molecular defect has been elucidated. Within this syndrome there is also a wide spectrum of clinical features, ranging from fetal hydrops resulting in intrauterine death (Kleijer et al. 1979) to patients who had a nearly normal development up to late childhood or young adulthood (Goldberg et al. 1971; Orii et al. 1972; Yamamoto et al. 1974; Loonen et al. 1974; Suzuki et al. 1977; Wenger et al. 1978; for reviews, see Galjaard & Reuser 1977; Lowden & O'Brien 1979).

b. Discussion of the experimental work

The experimental work described in publications III, IV and V was aimed at the elucidation of the genetic and molecular background of combined β-galactosidase/neuraminidase deficiency in man.

In publication III the results are reported of cell hybridization and co-cultivation studies using different human cell strains with a neuraminidase deficiency. Fusion of $neur^-$ cells from patients with classical mucolipidosis I (sialidosis) and from one of the patients with a mild form of sialidosis described by Durand et al. (1977) did not result in a restoration of neuraminidase activity. This may indicate that these two clincial variants are due to allelic mutations, each of which leads to a structural abnormality of neuraminidase. Using methylumbelliferyl substrate we could barely demonstrate

any neuraminidase activity in fibroblasts from the patient with the severe infantile form of sialidosis, whereas the cells from the patient with a mild clinical manifestation showed 5% residual activity and a somewhat lower neuraminic acid content. No relationship between the residual neuraminidase activity and the course and severity of the clinical manifestations in sialidosis was ever reported.

An interesting observation in the experiments described in publication III was the restoration of neuraminidase activity after hybridization of different types of β-gal$^-$/neur$^-$ fibroblasts with cells with a single neuraminidase deficiency. The occurrence of complementation implies that at least two different gene mutations are involved in sialidosis and combined β-galactosidase/neuraminidase deficiency. There is not yet sufficient knowledge about the structure of neuraminidase to interpret the complementation in molecular terms. It is well documented that N-acetylneuraminic acid is a common component of glycoproteins, certain glycosaminoglycans and gangliosides (Rosenberg & Schengrund 1976; Ledeen 1978; for review, see Svennerholm 1980). In sialidosis and combined β-galactosidase/neuraminidase deficiency the neuraminidase activity appears to be deficient towards glycoprotein oligosaccharides and artificial methylumbelliferyl-neuramic acid substrates, but normal towards gangliosides (Wenger et al. 1978; Hoogeveen & Verheijen, personal communication).

An important observation, also reported in publication III, was the partial restoration of neuraminidase activity after co-cultivation of β-gal$^-$/neur$^-$ fibroblasts with cells from infantile and mild forms of sialidosis (neur$^-$). The particular contribution of the two cell types in this correction was clarified by co-cultivating sialidosis fibroblasts and β-gal$^-$/neur$^-$ cells in close

contact. Prior to co-cultivation, one of the two cell strains was labelled with green fluorescent latex particles to allow their subsequent separation with a cell sorter (FACS). It was shown that the partial restoration of neuraminidase activity only occurred in β-gal$^-$/neur$^-$ fibroblasts and not in sialidosis cells. It thus seemed that sialidosis fibroblasts secrete an as yet unidentified "corrective factor". In the previous section it has already been mentioned that co-cultivation with "I-cells" did not correct the β-gal$^-$/neur$^-$ fibroblasts. This suggested that the "corrective factor" is a glycoprotein that normally is localized in lysosomes, but can be secreted into the medium.

Further studies on the correction of combined β-galactosidase/neuraminidase deficiency in human fibroblasts are described in publication IV. To achieve a more efficient secretion of the "corrective factor", ammonium chloride was added to the culture medium of normal and mutant human fibroblasts. The conditioned medium, concentrated and dialyzed, was administered to various types of neuraminidase and/or β-galactosidase deficient cell cultures. It appeared that the addition of NH_4Cl-induced secretions from β-gal$^-$ fibroblasts or neur$^-$ fibroblasts, resulted in a marked increase of the β-galactosidase activity (5-7 fold) and of the neuraminidase activity (15-20 fold) in β-gal$^-$/neur$^-$ cells. When the same conditioned medium was given to mutant fibroblasts with a single β-galactosidase deficiency or neuraminidase deficiency, no change in their residual enzyme activity occurred. These results ruled out an early proposal (O'Brien 1979; Lowden et al. 1981) that the combined deficiency was due to a primary defect of neuraminidase and they implied the existence of a third gene product underlying the deficiencies of β-galactosidase and neuraminidase in this disease. Further studies, described in

publications III and IV indicated that the "corrective factor" is a macromolecular glycoprotein, labile above 60°C and the uptake of which is competitively inhibited by mannose-6-phosphate. We also found that the "factor" once taken up by β-gal⁻/neur⁻ fibroblasts, continues to exert its corrective effect over a period of at least three days. It probably exhibits its activity within the lysosomes, since endocytosed exogenous macromolecules become eventually entrapped in the lysosomes (Willingham & Pastan 1980).

At the same time that the experiments described above were carried out, van Diggelen et al. (1980, 1981, 1982) used a specific irreversible inhibitor of β-galactosidase, developed by Sinnott and Smith (1976, 1978) to determine the half life of β-galactosidase in normal and mutant human fibroblasts. They found that in normal fibroblasts and in G_{M1}-gangliosidosis cells the turnover time of the enzyme is about 10 days whereas in β-gal⁻/neur⁻ fibroblasts it is reduced to less than 24 hours. Subsequent studies (van Diggelen et al. 1981) showed that the rate of synthesis of β-galactosidase in β-gal⁻/neur⁻ cells is normal (0.4-0.5 pmol/day per mg of cellular protein) and the reduced level of protein is due to an increased rate of degradation of the enzyme. When purified bovine testicular β-galactosidase was added to the culture medium above β-gal⁻/neur⁻ fibroblasts, it was readily taken up by the mutant cells but, once ingested, it was degraded within hours. In contrast, the same enzyme remained stable for a long period when internalized by G_{M1}-gangliosidosis fibroblasts (Galjaard et al. 1981; van Diggelen et al. 1982). These observations suggested that β-gal⁻/neur⁻ fibroblasts lack a factor that normally is required to protect both endogenous and exogenous "high-uptake" forms of β-galactosidase against excessive degradation.

The experiments described in publication V were carried out to identify the exact nature of this "corrective" (and "protective") factor. The hypothesis that the β-galactosidase deficiency in β-gal$^-$/neur$^-$ fibroblasts is due to an abnormally high rate of degradation was proven by experiments with leupeptin, an inhibitor of thiol proteases (Seglen et al. 1979). Addition of this compound to β-gal$^-$/neur$^-$ fibroblast cultures resulted in a 7-8 fold increase in β-galactosidase activity whereas no effect was seen in G_{M1}-gangliosidosis cells. The effect on neuraminidase was less clear since only one of the β-gal$^-$/neur$^-$ cell strains showed a slight increase in activity.

To investigate whether precursor forms of β-galactosidase were normally synthesized in β-gal$^-$/neur$^-$ fibroblasts, the cells were cultured in the presence of NH_4Cl. The result was that two different β-gal$^-$/neur$^-$ cell strains secreted as much β-galactosidase activity as controls. Ammonium chloride treatment of different types of G_{M1}-gangliosidosis did not lead to a measurable amount of extracellular β-galactosidase activity. These results indicated that the ability to synthesize β-galactosidase precursor is not impaired in β-gal$^-$/neur$^-$ cells. Further studies on the properties of precursor β-galactosidase in normal and β-gal$^-$/neur$^-$ fibroblasts showed no differences in kinetic and molecular properties. The precursor form in both cell strains has an apparent molecular weight of about 85,000 dalton, and a K_m of 1.4 mM towards methylumbelliferyl substrate. The K_m is thereby about 6 times higher than that of the intracellular mature enzyme, which has a molecular weight of 64,000 dalton and a K_m of 0.25 mM.

When precursor β-galactosidase was purified from the medium of β-gal$^-$/neur$^-$ cells it was readily taken up by G_{M1}-gangliosidosis fibroblasts and the ingested enzyme

remained stable for at least two days, as did precursor enzyme prepared from control cells. This implies that the enhanced degradation of β-galactosidase in β-gal$^-$/neur$^-$ fibroblasts is not caused by any obvious mutant properties of the β-galactosidase precursor.

The ultimate elucidation of the molecular defect in combined β-galactosidase/neuraminidase deficiency came from immunoprecipitation studies of radiolabelled β-galactosidase (also described in publication V). In normal human fibroblasts 4 radiolabelled immunoprecipitable components were found to be present: 85,000-dalton precursor of β-galactosidase, 64,000 mature β-galactosidase, both with hydrolytic activity, and two components with molecular weights of about 50,000 and 32,000 daltons. In NH_4Cl-induced secretions from control fibroblasts only the 85,000- and 54,000-dalton components were evident; the former being the precursor of β-galactosidase and the latter probably the precursor of the intracellular 32,000-dalton component. Pulse-chase experiments confirmed this assumption.

In β-gal$^-$/neur$^-$ fibroblasts the main components had molecular weights of 85,000 and 51,000. Mature 64,000-dalton β-galactosidase was hardly demonstrable and the 32,000 dalton component was always absent. Extracellularly the only detectable constituent was 85,000-dalton precursor β-galactosidase. Pulse-chase experiments with the mutant fibroblasts indicated that the newly synthesized 85,000-dalton precursor β-galactosidase was normally processed to a 66,000-dalton form which appeared after about 5 hours chase. The mature 64,000-dalton β-galactosidase never accumulated to any significant extent.

Inhibition of thiol proteases by leupeptin resulted in an accumulation of 85,000-dalton precursor β-galactosidase and of a partially processed 66,000-dalton form both in control fibroblasts and β-gal$^-$/neur$^-$ cells. This

indicated that these precursor forms are normally degraded to some extent by intralysosomal proteases.

When "corrective factor", purified from medium after NH_4Cl stimulation of G_{M1}-gangliosidosis fibroblasts was added to β-gal$^-$/neur$^-$ fibroblasts a complete normalization of the β-galactosidase labelling pattern occurred. There was no longer accumulation of precursor β-galactosidase but instead, like in control cells, the precursor was rapidly converted to mature 64,000-dalton β-galactosidase which apparently was resistent to excessive intralysosomal proteolysis.

In principle it would be possible that the molecular defect responsible for the combined β-galactosidase/neuraminidase deficiency is a block in the final maturation step, converting 66,000-dalton β-galactosidase to the 64,000-dalton mature form. The accumulation of the 66,000-dalton form after leupeptin treatment both in control and in β-gal$^-$/neur$^-$ fibroblasts is most likely due to inhibition of this proteolytic step by leupeptin. A similar observation has been made in in vitro experiments on the processing of hexosaminidase (Frisch & Neufeld 1981). We have, however, observed that leupeptin does not inhibit the "corrective factor" and it is therefore unlikely that the molecular defect responsible for the combined β-galactosidase/neuraminidase deficiency is a block in the last maturation step. Instead, it seems that the "corrective factor" provides protection of mature 64,000-dalton β-galactosidase against excessive intralysosomal proteolytic degradation.

On the basis of all these findings we propose that the defect in combined β-galactosidase/neuraminidase deficiency is a mutation leading to loss of function of 32,000-dalton lysosomal glycoprotein that may be the protective factor for β-galactosidase.

Although we have not yet performed similar pulse-

chase studies on neuraminidase, the results described in publication V show that addition of the "corrective factor" leads also to a restoration of neuraminidase and therefore is common to both enzymes.

In contrast to β-galactosidase, precursor forms of neuraminidase have not been demonstrated nor was there any residual neuraminidase activity in β-gal$^-$/neur$^-$ fibroblasts. It may well be that the missing factor is normally required to activate neuraminidase, and to unite β-galactosidase monomers and neuraminidase in a complex, at the same time protecting them towards excessive proteolytic degradation. The absence of high molecular weight aggregates of β-galactosidase monomers, previously observed in β-gal$^-$/neur$^-$ fibroblasts (Hoeksema et al. 1979) could be explained in this way.

Previous gene localization studies on Chinese hamster x human hybrids have shown that two gene loci on chromosomes 3 and 22 are involved in the expression of β-galactosidase activity (Shows et al. 1979; de Wit et al. 1979; Hoeksema et al. 1980). A gene on chromosome 3 was found to code for the β-galactosidase polypeptide but the role of a gene on chromosome 22 was not understood (Hoeksema 1979; Hoeksema et al. 1980). It may well be that this latter gene codes for the (precursor of) 32,000-dalton glycoprotein that is defective in combined β-galactosidase/neuraminidase deficiency. Recently we have shown with radiolabelled immunoprecipitation studies that the 54,000-dalton component, which is present in control fibroblasts and in the medium after NH_4Cl stimulation, is indeed the precursor of the 32,000-dalton glycoprotein. This explains its absence in the medium of β-gal$^-$/neur$^-$ fibroblasts after NH_4Cl stimulation. Further studies on Chinese hamster x human hybrids will provide the definite answer whether or not a gene on chromosome 22 codes for the 54,000-dalton component.

The experiments described in this section have not only led to the identification of the molecular species that is defective in the combined β-galactosidase/neuraminidase deficiency, they may also provide a start for a better understanding of the cell biology of lysosomes and of the way lysosomal enzymes are protected against the aggression of neighbouring lysosomal enzymes.

REFERENCES

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

Andria, G., Strisciuglio, P., Pontarelli, G., Sly, W.S. & Dodson, W.E. (1981) Infantile neuraminidase and β-galactosidase deficiencies (galactosialidosis) with mild clinical courses. In: Sialidases and Sialidoses (eds. G.Tettamanti, P. Durand & S. Di Donato) Edi. Ermes, Milan, 379-395.

Bach, G., Friedman, R., Weissmann, B. & Neufeld, E.F. (1972) The defect in the Hurler and Scheie syndromes: Deficiency of α-L-iduronidase. Proc. Natl. Acad. Sci. 69, 2048-2051.

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

Barret, A.J. (1972) Lysosomal enzymes. In: Lysosomes (ed. J.T. Dingle). Elsevier/North-Holland Amsterdam, 40-109.

Barrett, A.J. & Dean, R.T. (1976) Enzymes of lysosomes. Fed. Am. Soc. Exp. Biol. 317-324.

Barret, A.J. & Heath, M.F. (1977) Lysosomal enzymes. In: Lysosomes, a laboratory handbook (ed. J.T. Dingle). Elsevier/North-Holland, Amsterdam, 2nd ed., 19-146.

Beutler, E. & Kuhl, W. (1975) Subunit structure of human hexosaminidase verified: interconvertibility of hexosaminidase isoenzymes. Nature 258, 263-264.

Beutler, E., Kuhl, W. & Comings, D. (1975) Hexosaminidase isoenzyme in type O G_{M2}-gangliosidosis. Am. J. Hum. Genet. 27, 628-638.

Blobel, G. & Dobberstein, B. (1975) Transfer of protein across membranes. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. 67, 835-851.

Blobel, G., Walter, P., Chang, C., Goldman, B., Erickson, A. & Lingappa, V.R. (1979) Symp. Soc. Exp. Biol. 33, 9-36.

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

Bootsma, D. & Galjaard, H. (1979) Heterogeneity in genetic diseases studied in cultured cells. In: Models for the Study of Inborn Errors of Metabolism (ed. F.A. Hommes). Elsevier, Amsterdam, 241-256.

Braidman, I., Carrol, M., Dance, N. & Robinson, D. (1974) Separation and properties of human brain hexosaminidase C. Biochem. J. 143, 295-301.

Brot, F.E., Glaser, J.H., Roozen, K.J., Sly, W.S. & Stahl, P.D. (1974) In vitro correction of deficient human fibroblasts by β-glucuronidase from different human sources. Biochem. Biophys. Res. Commun. 57, 1-8.

Bunn, C.L., Wallace, D.C. & Eisenstadt, J.M. (1974) Cytoplasmic inheritance of chloramphenicol resistance in mouse tissue culture cells. Proc. Natl. Acad. Sci. USA 71, 1681-1685.

Campbell, P.N. & Blobel, G. (1976) The role of organelles in the chemical modification of the primary translation products of secretory proteins. FEBS Lett. 72, 215-226.

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

Champion, M.J. & Shows, T.B. (1977) Electrophoretic abnormalities of lysosomal enzymes in Mucolipidosis fibroblast lines. Am. J. Hum. Genet. 29, 149-163.

Conzelman, E. & Sandhoff, K. (1978) A B variant of infantile G_{M2}-gangliosidosis: deficiency of a factor necessary for stimulation of hexosaminidase A - catalyzed degradation of G_{M2} and glycolipid G_{A2}. Proc. Natl. Acad. Sci. USA 75, 3979-3983.

Czichi, U. & Lennarz, W.J. (1977) Localization of the enzyme system for glycosylation of proteins via the lipid-linked pathway in rough endoplasmic reticulum. J. Biol. Chem. 252, 7901-7904.

Das, R.C. & Heath, E.C. (1980) Dolicholdiphosphoryloligosaccharide-protein oligosaccharyltransferase: solubilization, purification and properties. Proc. Natl. Acad. Sci. USA 77, 3811-3815.

De Duve, C., Pressman, B.C., Giannetto, R., Wattiaux, R. & Appelmans, F. (1955). Tissue fractionation studies: intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J. 60, 604-617.

De Duve, C. (1964) From cytases to lysosomes. Fed. Proc. 23, 1045.

De Duve, C. & Wattiaux, R. (1966) Functions of lysosomes. Ann. Rev. Physiol. 28, 435-492.

De Duve, C., De Barsy, T., Poole, B., Trouet, A., Tulkens, P. & Van Hoof, F. (1974) Lysosomotropic agents. Biochem. Pharmacol. 23, 2495-2531.

Den Tandt, W.R., Lassila, E. & Phillippart, M. (1974) I-cell disease: Markedly increased activity of plasma acid hydrolases. J. Lab. Clin. Med. 83, 403-408.

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

De Wit-Verbeek, H.A., Hoogeveen, A. & Galjaard, H. (1978) Complementation studies with enucleated fibroblasts from different variants of β-galactosidase deficiency. Exp. Cell Res. 113, 215-218.

Distler, J., Hieber, V., Sahagian, G., Schmickel, R. & Jourdian, G.W. (1979) Identification of mannose-6-phosphate in glycoproteins that inhibit assimilation of β-galactosidase by fibroblasts. Proc. Natl. Acad. Sci. USA 76, 4235-4239.

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

Elbein, A.D. (1979) The role of lipid linked saccharides in the biosynthesis of complex carbohydrates. Ann. Rev. Plant. Physiol. 30, 239-272.

Elting, J.J., Chen, W.W. & Lennarz, W.J. (1980) Characterization of a glucosidase involved in an initial step in the processing of oligosaccharide chains. J. Biol. Chem. 255, 2325-2331.

Engel, A.G., Gomen, M.R., Marjorie, E., Seybold, M.D. & Lambert, E.H. (1973) The spectrum and diagnosis of acid maltase deficiency. Neurology 23, 95-106.

Erickson, A.H. & Blobel, G. (1979) Early events in the biosynthesis of the lysosomal enzyme cathepsin D. J. Biol. Chem. 254, 11771-11774.

Erickson, A.H., Conner, G.E. & Blobel, G. (1981) Biosynthesis of a lysosomal enzyme. J. Biol. Chem. 256, 11224-11231.

Fiddler, M.B., Ben-Yoseph, Y. & Nadler, H.L. (1979) Binding of human liver hydrolases by immobilized lectins. Biochem. J. 177, 175-180.

Fischer, G. & Jatzkewitz, H. (1975) The activator of cerebroside sulfatase. Purification from human liver and identification as protein. Hoppe-Seyler's Z. Physiol. Chem. 356, 605-613.

Fischer, H.D., Gonzalez-Noriega, A. & Sly, W.S. (1980a) β-Glucuronidase binding to human fibroblast membrane receptors. J. Biol. Chem. 255, 5069-5074.

Fischer, H.D., Gonzalez-Noriega, A., Sly, W.S. & Morré, P.J. (1980b) Phosphomannosyl-enzyme receptors in rat liver. J. Biol. Chem. 255, 9608-9615.

Frisch, A. & Neufeld, E.F. (1981) Limited proteolysis of the β-hexosaminidase precursor in a cell-free system. J. Biol. Chem. 256, 8242-8246.

Fukunaga, H., Hirose, K., Beppu, H., Uono, M. & Suzuki, Y. (1976) Two siblings with mucolipidosis. Clin. Neurol. (Tokyo) 16, 566-573.

Galjaard, H., van Hoogstraten, J.J., de Josselin de Jong, J.E. & Mulder, M.P. (1974) Methodology of the quantitative cytochemical analyses of single or small numbers of cultured cells. Histochem. J. 6, 409-429.

Galjaard, H., Hoogeveen, A., de Wit-Verbeek, H.A., Reuser, A.J.J., Ho, M.W. & Robinson, D. (1975) Genetic heterogeneity in G_{M1}-gangliosidosis. Nature 257, 60-62.

Galjaard, H. & Reuser, A.J.J. (1977) Clinical, biochemical and genetic heterogeneity in gangliosidoses. In: The Cultured Cell and Inherited Metabolic Disease (eds. R.A. Harkness & F. Cockburn). MTP Press, Lancaster, 139-160.

Galjaard, H. (1980) Genetic Metabolic Diseases, early diagnosis and prenatal analysis. Elsevier/North-Holland, Amsterdam.

Galjaard, H., Hoogeveen, A., Verheijen, F., van Diggelen, O.P., Konings, A., D'Azzo, A., Reuser, A.J.J. (1981) Relationship between clinical, biochemical and genetic heterogeneity in sialidase deficiency. In: Sialidases and Sialidoses (eds. G. Tettamanti, P. Durand & S. Di Donato). Edi. Ermes, Milan, 317-333.

Goldberg, M.F., Cotlier, E., Fichenscher, L.G., Kenyon, K., Enst, R. & Borowsky, S.A. (1971) Macular cherry-red spot, corneal clouding and β-galactosidase deficiency. Arch. Inter. Med. 128, 387-398.

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

Grinna, L.S. & Robbins, P.W. (1979) Glycoprotein biosynthesis. J. Biol. Chem. 254, 8814-8818.

Grinna, L.S. & Robbins, P.W. (1980) Substrate specificities of rat liver microsomal glucosidases which process glycoproteins. J. Biol. Chem. 255, 2255-2258.

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

Halley, D.J.J. (1980) Release and uptake of lysosomal enzymes studied in cultured cells. Thesis, Erasmus University Rotterdam.

Harpaz, N. & Schachter, H. (1980) Control of glycoprotein synthesis. J. Biol. Chem. 255, 4894-4902.

Hart, G.W., Brew, K., Grant, G.A., Bradshaw, R.A. & Lennarz, W.J. (1979) Primary structural requirements for the enzymatic formation of the N-glycosidic bond in glycoproteins. Studies with natural and synthetic peptides. J. Biol. Chem. 254, 9747-9753.

Hasilik, A. (1980) Biosynthesis of lysosomal enzymes. Trends Biochem. Sci. 5, 237-240.

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

Hasilik, A. & Neufeld, E.F. (1980a) Biosynthesis of lysosomal enzymes in fibroblasts synthesis as precursors of higher molecular weight. J. Biol. Chem. 255, 4937-4945.

Hasilik, A. & Neufeld, E.F. (1980b) Biosynthesis of lysosomal enzymes in fibroblasts. Phosphorylation of mannose residues. J. Biol. Chem. 255, 4946-4950.

Hasilik, A. & von Figura, K. (1981) Oligosaccharides in lysosomal enzymes. Eur. J. Biochem. 121, 125-129.

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

Hers, H.G. (1963) α-Glucosidase deficiency in generalized glycogen storage disease (Pompe's disease). Biochem. J. 86, 1-6.

Hers, H.G. & van Hoof, F. (eds.) (1973) Lysosomes and storage diseases. Academic Press, New York.

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

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

Hieber, V., Distler, J., Myerowitz, R., Schmickel, R.D. & Jourdian, G.W. (1976) The role of glycosidally bound mannose in the assimilation of β-galactosidase by generalized gangliosidosis fibroblasts. Biochem. Biophys. Res. Commun. 73, 710-717.

Hoeksema, H.L. (1979) The genetic defects in ganglioside storage diseases. Thesis, Erasmus University Rotterdam.

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

Hoeksema, H.L., de Wit, J. & Westerveld, A. (1980) The genetic defect in the various types of human β-galactosidase deficiency. Hum. Genet. 53, 241-247.

Holtzman, E. (1976) Lysosomes: a survey. Springer-Verlag, Wien.

Honey, N.K., Miller, A.L. & Shows, T.B. (1981) The mucolipidosis: Identification by abnormal electrophoretic patterns of lysosomal hydrolases. Am. J. Med. Genet. 9, 239-253.

Hoogeveen, A., Verheijen, F.W., D'Azzo, A. & Galjaard, H. (1980) Genetic heterogeneity in human neuraminidase deficiency. Nature 285, 500-502.

Hooghwinkel, G.J.M., Veltkamp, W.A., Overdijk, B. & Lisman, J.J.W. (1972) Electrophoretic separation of hexosaminidases of human and bovine brain and liver and of Tay-Sachs tissue. Hoppe-Seyler's Z. Physiol. Chem. 353, 839-841.

Hubbard, S.C. & Robbins, P.W. (1979) Synthesis and processing of protein-bound oligosaccharides in vivo. J. Biol. Chem. 254, 4568-4576.

Hubbard, S.C. & Robbins, P.W. (1980) Synthesis of the N-linked oligosaccharides of glycoproteins. J. Biol. Chem. 255, 11782-11793.

Hubbard, S.C. & Ivatt, R.J. (1981) Synthesis and processing of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 50, 555-583.

Hunt, L.A. (1979) Biosynthesis and maturation of cellular membrane glycoproteins. J. Supramol. Struct. 12, 209-226.

Hunt, L.A. (1980) Altered synthesis and processing of oligosaccharides of vesicular stomatitis virus glycoprotein in different lectin-resistant chinese hamster ovary cell lines. J. Virol. 35, 362-370.

Ikonne, J.U., Ratazzi, M.C. & Desnick, R.J. (1975) Characterization of hex S, the major residual β-hexosaminidase activity in type O G_{M2}-gangliosidosis. Am. J. Hum. Genet. 27, 639-650.

Jackson, R. & Blobel, G. (1977) Post-translational cleavage of presecretory proteins with an extract of rough microsomes from dog pancreas containing signal peptidase activity. Proc. Natl. Acad. Sci. USA 74, 5598-5602.

Jongkind, J.F., Verkerk, A., Schaap, G.H. & Galjaard, H. (1981) Flow sorting in studies on metabolic and genetic interaction between human fibroblasts. Acta Path. Microbiol. Scand. Sect. A Suppl. 274, 164-169.

Justice, R.M., Wenger, D.A., Naidu, S. & Rosenthal, I.M. (1977) Enzymatic studies in a new variant of G_{M1}-gangliosidosis in an older child. Pediatr. Res. 11, 407.

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

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

Kornfeld, S., Li, E. & Tabas, I. (1978) The sythesis of complex-type oligosaccharides. J. Biol. Chem. 253, 7771-7778.

Koster, J.F., Niermeijer, M.F., Loonen, M.C.B. & Galjaard, H. (1976) β-Galactosidase deficiency in an adult: a biochemical and somatic cell genetic study on a variant of G_{M1}-gangliosidosis. Clin. Genet. 9, 427-432.

Leader, D.P. (1979) Protein biosynthesis on membrane-bound ribosomes. Trends Biochem. Sci. 205-208.

Ledeen, R.W. (1978) Ganglioside structures and distribution: are they localized at the nerve ending? J. Supramol. Struct. 8, 437-453.

Leroy, J.G. & De Mars, R.I. (1967) Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. Science 157, 804-806.

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

Leroy, J.G., Spranger, J.W., Feingold, M., Opitz, J.M. & Crocker, A.C. (1971) I-cell disease: a clinical picture. J. Pediatr. 79, 360-365.

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

Li, E., Tabas, I., Kornfeld, S. (1978) The synthesis of complex-type oligosaccharides. J. Biol. Chem. 253, 7762-7770.

Li, S.C., Hirabayashi, Y. & Li, Y.T. (1981) A protein activator for the enzymic hydrolysis of G_{M2}-ganglioside. J. Biol. Chem. 256, 6234-6240.

Li, Y.T. & Li, S.C. (1981) Protein activators for the enzymic hydrolysis of G_{M1}- and G_{M2}-gangliosides. In: Lysosomes and Lysosomal Storage Diseases (eds. J.W. Callahan & A.J. Lowden). Raven Press, N.Y. 173-180.

Loonen, M.C.B., van der Lugt, L. & Franke, C.L. (1974) Angiokeratoma corporis diffusum and lysosomal enzyme deficiency. Lancet ii, 785.

Loonen, M.C.B. (1979) The variability of Pompe's disease. Thesis, Erasmus University Rotterdam.

Lowden, J.A. & O'Brien, J.S. (1979) Sialidosis: a review of human neuraminidase deficiency. Am. J. Hum. Genet. 31, 1-18.

Lowden, J.A., Cutz, E. & Skomorowski, M.A. (1981) Infantile type 2 sialidosis with β-galactosidase deficiency. In: Sialidases and Sialidoses (eds. G. Tettamanti, P. Durand & S. Di Donato). Edi. Ermes, Milan 261-280.

Maroteaux, P. & Lamy, M. (1966) La pseudo-polydystrophie de Hurler. Presse Méd. 74, 2889-2892.

Martin, J.J., Leroy, J.G., Farriaux, J.P., Fontaine, G., Desnick, R.J. & Cabello, A. (1975) I-cell disease (Mucolipidosis II). Acta Neuropath. 33, 285-305.

McKusick, V.A. (1972) Heritable disorders of connective tissue, 4th ed., Mosby, St. Louis.

McKusick, V.A., Neufeld, E.F. & Kelly, T.E. (1978) The mucopolysaccharide storage diseases. In: The Metabolic Basis of Inherited Disease (eds. J.S. Stanbury, J.B. Wyngaarden & D.S. Fredrickson) McGraw-Hill Book Company, New York, 1282-1308.

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

Michael, J.M. & Kornfeld, S. (1980) Partial purification and characterization of the glucosidases involved in the processing of asparagine-linked oligosaccharide. Arch. Biochem. Biophys. 199, 249-258.

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

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

Myerowitz, R. & Neufeld, E.F. (1981) Maturation of α-L-iduronidase in cultured human fibroblasts. J. Biol. Chem. 256, 3044-3048.

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

Neufeld, E.F. & Cantz, M.J. (1971) Corrective factors for inborn errors of mucopolysaccharide metabolism. Ann. N.Y. Acad. Sci. 179, 580-587.

Neufeld, E.F., Lim. T.M. & Shapiro, L.J. (1975) Inherited disorders of lysosomal metabolism. Ann. Rev. Biochem. 44, 357-376.

Neufeld, E.F., Sando, G.N., Garvin, A.J. & Rome, L.H. (1977) The transport of lysosomal enzymes. J. Supramol. Struct. 6, 95-101.

Neufeld, E.F. & Ashwell, G. (1980) Carbohydrate recognition systems for receptor-mediated pinocytosis. In: The Biochemistry of Glycoproteins and proteoglycans (ed. W. Lennarz). Plenum Press, N. York, 241-266.

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

Neurath, H. & Walsh, K.A. (1976) Role of proteolytic enzymes in biological regulation (a review). Proc. Natl. Acad. Sci. USA 73, 3825-3832.

Nicol, D.M., Lagunoff, D. & Pritzl, P. (1974) Differential uptake of human β-glucuronidase isoenzymes from spleen by deficient fibroblasts. Biochem. Biophys. Res. Commun. 59, 941-946.

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

Novikoff, A.B., Essner, E. & Quintana, N. (1964) Golgi apparatus and lysosomes. Fed. Proc. 23, 1010-1022.

Novikoff, A.B. (1973) Lysosomes: a personal account in lysosomes and storage diseases (eds. H.G. Hers & F. van Hoof). Academic Press, New York, 1-41.

Novikoff, A.B. (1976) The endoplasmic reticulum: a cytochemist's view (a review). Proc. Natl. Acad. Sci. 73, 2781-2787.

O'Brien, J.S. (1972) Ganglioside storage diseases. In: Advances in Human Genetics (eds. H. Harris & K. Hirschhorn), Vol. 3. Plenum Press, New York, 39-98.

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

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

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

O'Brien, J.S. (1978) The gangliosides. In: The Metabolic Basis of Inherited Disease (eds. J.B. Stanbury, J.B. Wyngaarden & D.S. Fredrickson). McGraw-Hill, New York, 841-865.

O'Brien, J.S. (1978) An explanation for complementation in G_{M1}-gangliosidosis. Biochem. Genet. 36A.

O'Brien, J.S. (1981) Molecular genetics of sialidosis. In: Sialidases and Sialidoses (eds. G. Tettamanti, P. Durand & S. Di Donato). Edi. Ermes, Milan, 207-218.

Okada, S. & O'Brien, J.S. (1969) Tay-Sachs disease, generalized absence of a β-D-N-acetylhexosaminidase component. Science 165, 698-700.

Okhuma, S. & Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA 75, 3327-3331.

Opheim, D.J. & Touster, O. (1978) Lysosomal α-D-mannosidase of rat liver: purification and comparison with Golgi and cytosolic α-D-mannosidase. J. Biol. Chem. 253, 1017-1023.

Orii, T., Minami, R., Sukegawa, K., Sato, S., Tsugawa, S., Horino, K., Muira, R. & Nakoa, T. (1972) A new type of mucolipidosis with β-galactosidase deficiency and glycopeptiduria. Tokuko J. Exp. Med. 107, 303-315.

Palade, G. (1975) Intracellular aspects of the process of protein synthesis. Science 189, 347-358.

Paigen, K. (1979) Acid hydrolases as models of genetic control. Ann. Rev. Genet. 13, 417-466.

Paigen, K. (1981) Genetic regulation of lysosomal enzymes. In: Lysosomes and Lysosomal Storage Diseases (eds. J.W. Callahan & A.J. Lowden). Raven Press, New York, 1-15.

Parodi, A.J. & Leloir, L.F. (1979) The role of lipid intermediates in the glycosylation of proteins in the eukaryotic cell. Biochim. Biophys. Acta 559, 1-37.

Pinsky, L., Miller, J., Stanfield, B., Watters, G. & Wolfe, L. (1974) G_{M1}-gangliosidosis in skin fibroblasts culture: enzymatic differences between type I and II and observations on a third variant. Am. J. Hum. Genet. 26, 563-577.

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

Rapin, I., Goldfischer, S., Katzmann, R., Engel, J. & O'Brien, J.S. (1978) The cherry-red spot myoclonus syndrome. Ann. Neurol. 3, 234-242.

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

Reitman, M.L. & Kornfeld, S. (1981b) Lysosomal enzyme targeting. J. Biol. Chem. 256, 11977-11980.

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

Reuser, A.J.J. & Galjaard, H. (1976) Characterization of β-D-N-acetyl hexosaminidases C and S in fibroblasts from control individuals and patients with Tay-Sachs disease. FEBS Lett. 71, 1-5.

Reuser, A.J.J.(1977) Clinical, biochemical and genetic heterogeneity in lysosomal storage diseases. Thesis, Erasmus University Rotterdam.

Ringertz, N.R. & Savage, R.E. (eds.) (1976) Cell hybrids. Acad. Press, New York.

Robbins, A.R. & Myerowitz, R. (1981) The mannose-6-phosphate receptor of chinese hamster ovary cells. J. Biol. Chem. 256, 10623-10627.

Robinson, D. & Stirling, J.L. (1968) N-acetyl-β-glucosaminidases in human spleen. Biochem. J. 107, 321-327.

Rosenberg, A. & Schengrund, C.L. (1976) Sialidases. In: Biological Roles of Sialic Acid (eds. A.E. Rosenberg & C.L. Schengrund). Plenum Press, New York, 295-359.

Rothman, J.E. & Lodish, H.F. (1977) Synchronised transmembrane insertion and glycosylation of a nascent membrane protein. Nature 269, 775-780.

Rothman, J.E., Katz, F.N. & Lodish, H.F. (1978) Glycosylation of a membrane protein is restricted to the growing polypeptide chain but is not necessary for insertion as a transmembrane protein. Cell 15, 1447-1454.

Rousson, R., Ben-Yoseph, Y., Fiddler, M.B. & Nadler, H.L. (1979) Demonstration of altered acidic hydrolases in fibroblasts from patients with Mucolipidosis II by lectin titration. Biochem. J. 180, 501-505.

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

Sandhoff, K. (1968) Auftrennung der Säuger N-acetyl-β-D-hexosaminidase in multiple Formen durch Elektrofokussierung. Hoppe-Seyler's Z. Physiol. Chem. 349, 1095-1098.

Sandhoff, K., Andreae, U. & Jatzkewitz, H. (1968) Deficient hexosaminidase activity in an exceptional case of Tay-Sachs disease with additional storage of kidney globoside in visceral organs. Pathol. Eur. 3, 278-285.

Sandhoff, K. (1969) Variation of β-N-acetyl-hexosaminidase pattern in Tay-Sachs disease. FEBS Lett. 4, 351-354.

Sandhoff, K. & Harzer, K. (1973) Total hexosaminidase deficiency in Tay-Sachs disease (variant O). In: Lysosomes and Storage Diseases (eds. H.G. Hers & F. van Hoof). Acad. Press, New York, 345-356.

Sandhoff, K. & Christomanou, H. (1979) Biochemistry and genetics of ganglisidoses. Hum. Genet. 50, 107-143.

Sando, G.N. & Neufeld, E.F. (1977) Recognition and receptor-mediated uptake of a lysosomal enzyme α-L-iduronidase by cultured human fibroblasts. Cell 12, 619-627.

Schaap, G.H., van der Kamp, A.W.M., Öry, F. G. & Jongkind, J.F. (1979) Isolation of anucleate cells using a fluorescence-activated cell sorter (FACS II). Exp. Cell Res. 122, 422-426.

Schachter, H. & Roseman, S. (1980) Mammalian glycosyltransferases: Their role in the synthesis and function of complex carbohydrates and glycolipids. In: The Biochemistry of Glycoproteins and Proteoglycans (ed. W. Lennarz). Plenum Press, New York, 85-160.

Schachter, H. (1981) Glycoprotein biosynthesis and processing. In: Lysosomes and Lysosomal Storage Diseases (eds. J.W. Callahan & J.A. Lowden) Raven Press, New York, 73-93.

Schellens, J.P.M., Daems, W.Th. Emeis, J.J., Brederoo, P., de Bruyn, W.C. & Wisse, E. (1977) Electron microscopical identification of lysosomes. In: Lysosomes, a Laboratory Handhook (ed. J.T. Dingle). Elsevier/North-Holland Biomedical Press, Amsterdam, 2nd ed., 147-208.

Schwarz, R.T. & Datema, R. (1980) Inhibitors of protein glycosylation. Trends Biochem. Sci. 5, 65-67.

Seglen, P.O., Grinde, B. & Solheim, A.E. (1979) Inhibition of the lysosomal pathway of protein degradation in isolated rat hepatocytes by ammonia, methylamine, chloroquine and leupeptin. Eur. J. Biochem. 95, 215-225.

Shows, T.B., Scrafford-Wolff, L.R., Brown, J.A. & Meisler, M. (1979) G_{M1}-gangliosidosis: chromosome 3 assignment of the β-galactosidase A gene (β-gal_A). Som. Cell Genet. 5, 147-158.

Sinnott, M.L. & Smith, P.J. (1976) Active-site-directed irreversible inhibition of E.coli β-galactosidase by the "hot" carbonium ion precursor β-D-galactopyranosylmethyl-p-nitrophenyltriazene. J. Chem. Soc. Chem. Commun. 223-224.

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

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

Skudlarek, M.D. & Swank, R.T. (1980) Turnover of two lysosomal enzymes in mouse peritoneal macrophages. J. Biol. Chem. 87, 305.

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

Sly, W.S., Natowicz, M.R., Gonzalez-Noriega, A., Grubb, J.H. & Fischer, H.D. (1981) The role of the mannose-6-phosphate recognition marker and its receptor in the uptake and intracellular transport of lysosomal enzymes. In: Lysosomes and Lysosomal Storage Diseases (eds. J.W. Callahan & J.A. Lowden). Raven Press, New York, 131-146.

Spranger, J.W., Wiedemann, H.R. (1970) The genetic mucolipidosis: diagnosis and differential diagnosis. Hum. Genet. 9, 113-139.

Spranger, J., Gehler, J. & Cantz, M. (1977) Mucolipidosis I-A sialidosis. Am. J. Med. Genet. 1, 21-29.

Spritz, J.W., Coates, P.M. & Lief, F.S. (1979) I-cell disease: intracellular desialylation of lysosomal enzymes using an influenza virus vector. Biochim. Biophys. Acta 582, 164-171.

Stanbury, J.B., Wyngaarden, J.B. & Fredrickson, D.S. (eds.) (1978) The metabolic basis of inherited disease. McGraw-Hill Book Company, New York.

Strawser, L.D. & Touster, O. (1980) The cellular processing of lysosomal enzymes and related proteins. Rev. Physiol. Biochem. Pharmacol. 87, 169-210.

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

Struck, D.K., Lennarz, W.J. & Brew, K. (1978) Primary structural requirements for the enzymatic formation of the N-glycosidic bond in glycoproteins. Studies with α-lactalbumin. J. Biol. Chem. 253, 5786-5794.

Struck, D.K. & Lennarz, W. J. (1980) The function of saccharide-lipids in synthesis of glycoproteins. In: The Biochemistry of Glycoproteins and Proteoglycans (ed. W. Lennarz) Plenum Press, New York, 35-83.

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

Suzuki, Y., Suzuki, K. & Kamoshita, S. (1969) Chemical pathology of G_{M1}-gangliosidosis. J. Neuropathol. Exptl. Neurol. 28, 25-30.

Suzuki, Y., Nakamura, N., Fukuoka, K., Shimada, Y. & Uono, M. (1977) β-Galactosidase deficiency in juvenile and adult patients. Report of six Japanese cases and review of literature. Hum. Genet. 36, 219-229.

Svennerholm, L. (1980) Gangliosides and synaptic transmission. In: Structure and Function of Gangliosides (eds. L. Svennerholm, P. Mandel, H. Dreyfus & P.F. Urban). Plenum Press, New York, 533-544.

Swallow, D.M., Evans, L., Saha, M. & Harris, H. (1976) Characterization and tissue distribution of N-acetylhexosaminidase C: suggestive evidence for a separate hexosaminidase locus. Ann. Hum. Genet. 40, 55-66.

Tabas, I. & Kornfeld, S. (1979) Purification and characterization of a rat liver Golgi mannosidase specific for α-1,2-linked mannose residues. Fed. Proc. 38, 291.

Tabas, I. & Kornfeld, S. (1980) Biosynthetic intermediates of β-glucuronidase contain high mannose oligosaccharides with blocked phosphate residues. J. Biol. Chem. 255, 6633-6639.

Tai, T., Yamashita, K., Ito, S. & Kobata, A. (1977) Structures of the carbohydrate moiety of ovalbumin glycopeptide III and the difference in specificity of endo-β-N-acetylglucosaminidases C_{11} and H. J. Biol. Chem. 252, 6687-6694.

Tarentino, A.L. & Maley, F. (1974) Purification and properties of an endo-β-N-acetylglucosaminidase from Streptomyces griseus. J. Biol. Chem. 249, 811-817.

Tarentino, A.L., Trimble, R.B. & Maley, F. (1978) Endo-beta-N-acetylglucosaminidase from Streptomyces plicatus. Methods Enzymol. 50, 574-580.

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

Thomas, G.H., Tipton, R.E., Chien, L.T., Reynolds, L.W. & Miller, C.S. (1978) Sialidase (α-N-acetylneuraminidase) deficiency: the enzyme defect in an adult macular cherry-red spots and myoclonus without dementia. Clin. Genet. 13, 369-379.

Tondeur, M., Vamos-Hurwitz, E., Mockel-Pohl, S., Dereume, J.P., Cremer, N. & Loeb, H. (1971) Clinical, biochemical and ultrastructural studies in a case of chondrodystrophy presenting the I-cell phenotype in culture. J. Pediatr. 79, 366-378.

Touster, O. (1973) Some aspects of the cellular biochemistry of lysosomal and related glycosidases. Mol. Cell Biochem. 2, 169-177.

Touster, O. (1978) The chemistry and turnover of lysosomal enzymes. In: Protein Turnover and Lysosomal Function (eds. H.L. Segal & D.J. Doyle). Acad. Press, New York, 231-250.

Tulsiani, D.R.P., Opheim, D.J. & Touster, O. (1977) Purification and characterization of α-D-mannosidase from rat liver Golgi membranes. J. Biol. Chem. 252, 3227-3233.

Turco, S.J. & Robbins, P.W. (1979) The initial stages of processing of protein-bound of oligosaccharides in vitro. J. Biol. Chem. 254, 4560-4567.

Ullrich, K., Mersmann, G., Weber, E. & von Figura, K. (1978) Evidence for lysosomal enzyme recognition by human fibroblasts via a phosphorylated carbohydrate moiety. Biochem. J. 170, 643-650.

Vaes, G. (1973) Digestive capacity of lysosomes. In: Lysosomes and Storage Diseases (eds. H.G. Hers & F. van Hoof). Acad. Press, New York, 43-77.

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

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

Van Diggelen, O.P., Hoogeveen, A., Reuser, A.J.J. & Galjaard, H. (1982) in press.

Van Elsen, A.F., Leroy, J.G. (1979) Lysosomal enzymes in fibroblasts: lectin affinities. In: Models for the Study of Inborn Errors of Metabolism (ed. F.A. Hommes). Elsevier/North-Holland Biomedical Press, Amsterdam, 329-332.

Varki, A. & Kornfeld, S. (1980) Structural studies of phosphorylated high mannose-type oligosaccharides. J. Biol. Chem. 255, 10847-10858.

Varki, A. & Kornfeld, S. (1981) Purification and characterization of rat liver α-N-acetylglucosaminyl phosphodiesterase. J. Biol. Chem. 256, 9937-9943.

Vladutiu, G.D. & Ratazzi, M.C. (1979) Excretion-reuptake route of β-hexosaminidase in normal and I-cell disease cultured fibroblasts. J. Clin. Invest. 63, 595-601.

Von Figura, K. & Kresse, H. (1974) Quantitative aspects of pinocytosis and intracellular fate of N-acetyl-α-D-glucosaminidase in Sanfilippo B fibroblasts. J. Clin. Invest. 53, 85-90.

Von Figura, K. & Klein, U. (1979) Isolation and characterization of phosphorylated oligosaccharides from α-N-acetylglucosaminidase that are recognized by cell surface receptors. Eur. J. Biochem. 94, 347-354.

Von Figura, K., Rey, M., Prinz, R., Voss, B. & Ullrich, K. (1979) Effect of tunicamycin on transport of lysosomal enzyme in cultured skin fibroblasts. In: Glycoconjugates - Proc. 5th Int. Sym. (eds. R. Schauer, P. Boer, E. Buddecke, M.R. Kramer, J.F.G. Vliegenthart & H. Wiegandt). George Thieme Publishers, Stuttgart, 322-323.

Waheed, A., Pohlmann, R., Hasilik, A. & von Figura, K. (1981) Subcellular location of two enzymes involved in the synthesis of phosphorylated recognition markers in lysosomal enzymes. J. Biol. Chem. 256, 4450-4452.

Walter, P., Jackson, R.C., Marcus, M., Lingappa, V.R. & Blobel, G. (1979) Tryptic dissection and reconstitution of translocation activity for nascent presecretory proteins across microsomal membranes. Proc. Natl. Acad. Sci. USA 76, 1795-1799.

Walter, P. & Blobel, G. (1980) Purification of a membrane-associated protein complex required for protein translation across the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 77, 7112-7116.

Warner, T.G. & O'Brien, J.S. (1979) Synthesis of 2^1-(4-methylumbelliferyl)-α-D-N-acetyl neuraminicacid and detection of skin fibroblasts neuraminidase in normal human and in sialidosis. Biochemistry 18, 2783-2787.

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

Wiesmann, U. & Neufeld, E.F. (1970) Scheie and Hurler syndromes. Apparent identity of the biochemical defect. Science 169, 72-74.

Wiesmann, U.N., Lightbody, J., Vasella, F. & Herschkowitz, N.N. (1971a) Multiple lysosomal enzyme deficiency due to enzyme leakage? New Engl. J. Med. 284, 109-110.

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

Wiesmann, U.N. & Herschkowitz, N.N. (1974) Studies on the pathogenetic mechanism of I-cell disease in cultured fibroblasts. Pediatr. Res. 8, 865-870.

Willingham, M.C. & Pastan, I. (1980) The receptosome: an intermediate organelle of receptor-mediated endocytosis in cultured fibroblasts. Cell 21, 67-77.

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

Yamashita, K., Tachibana, Y. & Kobota, A. (1978) The structures of the galactose-containing sugar chains of ovalbumin. J. Biol. Chem. 253, 3862-3869.

SUMMARY

The many events that must occur with some precision between translation of a lysosomal enzyme protein and its final correct sequestering and full activation within the lysosomes, offer numerous opportunities for a genetic lesion that could result in a faulty expression of the enzyme activity.

So far two inherited lysosomal storage diseases have been reported which are associated with a multiple lysosomal enzyme deficiency: mucolipidosis II ("I-cell" disease) and combined β-galactosidase/neuraminidase deficiency. They both result from a post-translational modification error.

The purpose of the experiments described in this thesis was to gain more insight into the molecular and genetic nature of these diseases.

Different approaches were used:

- Somatic cell hybridization and co-cultivation studies were performed, to clarify whether different gene mutations were responsible for the deficiency of a given enzyme in various related diseases.
- "Cybridization" studies were carried out after fusion of whole fibroblasts and enucleated cells (cytoplasts), to further investigate whether the presence of the nucleus of one of the cell types hybridized was mandatory for complementation or cytoplasmic factors were sufficient for this event to occur.
- Immunoprecipitation of radiolabelled lysosomal glycoproteins, followed by SDS gel electrophoresis, was applied as a method to verify the possible molecular and biochemical changes of a deficient enzyme (β-galactosidase) in comparison with its normal counterpart.

Cultured fibroblasts from "I-cell" patients are character-

ized by the intracellular loss of many lysosomal hydrolases which are recovered in excess in the extracellular space. "I-cells" are therefore a suitable model for complementation studies.

In the first series of experiments, "I-cells" were hybridized with cells with a single lysosomal enzyme deficiency (i.e. Sandhoff's disease: hexosaminidase A+B; G_{M1}-gangliosidosis: β-galactosidase; mannosidosis; acid α-mannosidase) and with combined β-galactosidase$^-$/neuraminidase$^-$ fibroblasts. Restoration of the intracellular enzyme activity was achieved in the various heterokaryon populations tested and in some instances (β-hexosaminidase, α-mannosidase) a normalization of the enzymic level was detected in the medium above fused cells. These findings implied the occurrence of different gene mutations and clearly inferred the correction of the "I-cell" post-translational defect.

Complementation also occurred when I-cells were fused with enucleated fibroblasts (cytoplasts) with an isolated hydrolase deficiency, due to a structural mutation. Since no increase was seen after fusion of "I-cell" cytoplasts with whole mutant fibroblasts, it was apparent that a long lasting "factor" present in enucleated cells of other genotypes enabled the correct processing of the normally synthesized lysosomal hydrolases of I-cells.

The molecular defect underlying this disease, has recently been discovered as being a deficiency of the phosphotransferase responsible for the proper biosynthesis of the mannose-6-phosphate recognition marker common to lysosomal enzymes. The restoration of the enzyme activities after somatic cell hybridization can now be interpreted as the result of the presence in other mutant fibroblasts of the phosphotransferase missing in I-cells and of the occurrence in the latter of the structural gene for a particular enzyme.

Our attention was subsequently focused on the combined β-galactosidase and neuraminidase deficiency.

A specific neuraminidase deficiency alone is well documented as mucolipidosis I (sialidosis) and similarly an isolated β-galactosidase deficiency due to a mutation at the structural gene, has been well described as classical G_{M1}-gangliosidosis. A third manifestation combines deficiencies of neuraminidase and β-galactosidase. In view of the fact that a significant residual β-galactosidase activity remains in these patients, it was first thought that neuraminidase was the primary defect in this disease.

There is now good evidence that a defect in a third gene product is likely to be the cause of the coexistent deficiency of these two enzymes. The occurrence of complementation after fusion of $\beta\text{-gal}^-/\text{neur}^-$ cells with "I-cells" and with fibroblasts with a single neuraminidase deficiency (sialidosis) pointed to three different gene mutations being responsible for these individual disorders.

It was observed that co-cultivation of $\beta\text{-gal}^-/\text{neur}^-$ fibroblasts with sialidosis cells resulted in a partial restoration of neuraminidase activity, selectively in the former cell type only. Thus sialidosis fibroblasts appeared to secrete an unidentified "corrective factor" for the combined deficiency. An enriched source of such a factor was obtained after NH_4Cl stimulation of different types of human fibroblasts including those with an isolated β-galactosidase or neuraminidase deficiency. The corrective factor was subsequently shown to be a glycoprotein, labile above 60^oC, whose uptake by $\beta\text{-gal}^-/\text{neur}^-$ cells was competitively inhibited by mannose-6-phosphate. Its corrective activity appeared to continue within the cells for at least 72 hrs after its removal from the medium. The absence of this factor in combined deficient

cells seemed to result in an excessively rapid degradation of an otherwise normally synthesized β-galactosidase. A pseudo-correction was accomplished by suppressing this degradation using the thiol protease inhibitor leupeptin.

Immunoprecipitation studies carried out on radio-labelled cells and media demonstrated that in normal fibroblasts an enzymically active 85,000-dalton precursor β-galactosidase is processed into a mature 64,000-dalton form. In addition two other related components are present, a non-enzymically active 32,000-dalton form and its 54,000-dalton precursor. In $\beta\text{-gal}^-/\text{neur}^-$ fibroblasts hardly any mature β-galactosidase is detectable and there is a complete lack of the 32,000-dalton component and of its precursor in the medium. When the mutant cells were grown in the presence of the "corrective factor" the β-galactosidase and neuraminidase activities were restored and after immunoprecipitation the mature 64,000-dalton β-galactosidase accumulated.

We propose that the combined deficiency is caused by a defective 32,000-dalton glycoprotein which is normally required to protect β-galactosidase against rapid intra-lysosomal degradation and to allow neuraminidase its catalytic activity.

The existence of such protective factors for various lysosomal enzymes may well be a common feature in the control of their half life within the lysosomes.

SAMENVATTING

De talrijke gebeurtenissen die een lysosomaal enzym met enige precisie moet ondergaan voordat het uiteindelijk in het lysosoom tot volledige activiteit komt, bieden vele mogelijkheden voor het optreden van een genetische mutatie, die kan uitmonden in een foutieve expressie van de enzymatische activiteit.

Tot nu toe zijn er slechts twee erfelijke lysosomale stapelingsziekten beschreven die als een meervoudige lysosomale enzymdeficiëntie gekarakteriseerd zijn, te weten: Mucolipidosis II (I-cell disease) en de gecombineerde neuraminidase/β-galactosidase deficiëntie. Beide aandoeningen zijn het gevolg van een post-translationele modificatiefout.

Het doel van de experimenten, beschreven in dit proefschrift, was het verkrijgen van een breder inzicht in de moleculaire en genetische achtergronden van deze ziekten.

De navolgende benaderingswijzen werden toegepast:

- Somatische celhybridisatie en samenkweken dienden ter verduidelijking of verschillende genmutaties verantwoordelijk waren voor de deficiëntie van een bepaald enzym in verschillende aangedane patiënten.
- Fusies van intacte fibroblasten met ontkernde cellen (cytoplasten) werden uitgevoerd om te bepalen of de aanwezigheid van één van de kernen van de bij de "cybridisatie" betrokken celtypen voor complementatie noodzakelijk was, of dat alleen cytoplasmatische factoren hiervoor al voldoende waren.
- Immuunprecipitatie van radioactief gelabelde lysosomale glycoproteinen, gevolgd door SDS-electroforese, werd toegepast als methode om de mogelijke moleculaire en biochemische veranderingen in een deficiënt enzym te vergelijken met een normaal enzym.

Gekweekte fibroblasten van "I-cel" patiënten worden gekenmerkt door afwezigheid van vele lysosomale hydrolases in de cellen en door overmatige afscheiding van deze enzymen aan het medium. I-cellen zijn daarom bij uitstek geschikt voor complementatieonderzoek.

In de eerste reeks experimenten werden I-cellen gehybridiseerd met cellen met een enkelvoudige lysosomale enzymdeficiëntie (bijv.: ziekte van Sandhoff: hexosaminidase A + B; G_{M1}-gangliosidose: β-galactosidase; mannosidose: zure α-mannosidase), en met gecombineerde β-galactosidase$^-$/neuraminidase$^-$ fibroblasten. Herstel van de intracellulaire enzymactiviteit werd aangetoond in de verschillende heterokaryonpopulaties en in enkele gevallen (β-hexosaminidase, α-mannosidase) werd een normalisatie van het enzymatische niveau in het medium van de gefuseerde cellen vastgesteld. De resultaten duiden op het bestaan van verschillende genmutaties en bevestigen duidelijk de correctie van het post-translationale defect in I-cellen.

Complementatie trad ook op na fusie van I-cellen met ontkernde fibroblasten (cytoplasten) met een enkelvoudige hydrolase-deficiëntie, veroorzaakt door een structurele mutatie. Daar geen toename waargenomen werd na fusie van I-cel cytoplasten met intacte mutant-fibroblasten, was het duidelijk dat een stabiele "factor", aanwezig in ontkernde cellen van een ander genotype, de correcte processing van normaal gesynthetiseerde lysosomale hydrolases in I-cellen kon bewerkstelligen.

Het moleculaire defect dat ten grondslag ligt aan deze aandoening, is kort geleden opgehelderd als zijnde een deficiënte van het fosfotransferase, dat zorg draagt voor de biosynthese van de mannose-6-fosfaat-herkenningsmarker, welke alle lysosomale enzymen bezitten. Het herstel van enzymactiviteit na somatische celhybridisatie kan nu gezien worden als het resultaat van de aanwezigheid van het fosfotransferase, dat ontbreekt in I-cellen, in de

andere mutante fibroblasten, en als de expressie in de laatstgenoemde van het structureel gen afkomstig van I-cellen.

Vervolgens richtte onze aandacht zich op de gecombineerde β-galactosidase/neuraminidase deficiëntie.

Een enkelvoudige neuraminidase-deficiëntie staat bekend als mucolipidose I (sialidose) en de enkelvoudige β-galactosidase-deficiëntie, veroorzaakt door een mutatie in het structurele gen, is uitvoerig beschreven als de klassieke G_{M1}-gangliosidose. In een derde aandoening worden deficiënties van zowel neuraminidase als β-galactosidase gecombineerd. Gezien het feit dat patiënten met deze aandoening een relatief hoge restactiviteit van β-galactosidase bezaten, was men in eerste instantie van mening dat neuraminidase het primaire defect bij deze ziekte was.

Nu zijn er echter sterke aanwijzingen dat een defect in een derde genproduct waarschijnlijk de oorzaak is van een gelijktijdig optredende deficiëntie van deze twee enzymen. Het optreden van complementatie na fusie van β-galactosidase$^-$/neuraminidase$^-$ cellen met I-cellen en met fibroblasten met een enkelvoudige neuraminidase-deficiëntie (sialidose), wees op drie verschillende genmutaties welke verantwoordelijk zijn voor deze afzonderlijke aandoeningen.

Co-cultivatie van β-gal$^-$/neur$^-$ fibroblasten met sialidose-cellen leidde tot een gedeeltelijk herstel van de neuraminidase-activiteit in de eerstgenoemde cellen. Sialidose-cellen leken dus een onbekende corrigerende factor voor de gecombineerde deficiëntie uit te scheiden. Een verrijking van een dergelijke factor werd verkregen na NH_4Cl-stimulatie in het medium van verschillende typen menselijke fibroblasten, inclusief die met een enkelvoudige β-galactosidase- of neuraminidase-deficiëntie. De corrigerende factor bleek vervolgens een hittelabiel glycoproteïne te zijn, waarvan de opname door β-gal$^-$/neur$^-$

cellen competitief geremd werd door mannose-6-fosfaat. De corrigerende werking binnen in de cel bleek tenminste nog 72 uur na verwijdering van de factor uit het medium, voort te duren. De afwezigheid van deze factor in de tweevoudige deficiënte cellen leek te leiden tot een uitermate snelle afbraak van het overigens op normale wijze gesynthetiseerde β-galactosidase. Een schijnbare correctie werd verkregen door deze afbraak te onderdrukken met de thiolprotease-remmer leupeptine.

Immuunprecipitatie, uitgevoerd met radioactief gelabelde cellen en media, toonde aan dat in normale fibroblasten een enzymatisch actieve β-galactosidase-precursor van 85.000 dalton wordt omgezet in een mature vorm van 64.000 dalton. Bovendien zijn er twee andere componenten bij betrokken, een niet-enzymatische component van 32.000 dalton en zijn precursor van 54.000 dalton. In $\beta\text{-gal}^-/\text{neur}^-$ fibroblasten is nauwelijks enig matuur β-galactosidase aantoonbaar, en de 32.000-dalton component en zijn precursor in het medium zijn volstrekt afwezig. Indien de mutante cellen werden gekweekt in aanwezigheid van de corrigerende factor, werd de activiteit van β-galactosidase en neuraminidase hersteld en werd het mature β-galactosidase van 64.000 dalton na immuunprecipitatie opgehoopt.

Wij stellen dat de gecombineerde deficiëntie veroorzaakt wordt door een defect glycoproteïne van 32.000 dalton, dat onder normale omstandigheden noodzakelijk is om β-galactosidase tegen snelle afbraak te beschermen en om neuraminidase zijn katalytische werking te laten uitoefenen. Het bestaan van dergelijke factoren voor meerdere lysosomale enzymen is wellicht een algemeen verschijnsel bij de regulering van de biologische halfwaardetijd van deze enzymen in de lysosomen.

GRAZIE

Una vita piacevolmente ricca si può ottenere in diversi modi. Sono dell'idea che un ruolo fondamentale sia giocato in questo senso dalla cerchia di persone care che ti circondano. Ho fatto per questo dell'Olanda la mia seconda casa.
I want to say grazie to Professor Galjaard for everything he did for me. Without his guidance and the right opportunities he offered me, I would not have reached this point. "Prof" Robinson grazie for the pleasant discussions, the essential help and your friendship. Grazie Professor Koster for your quick and critical remarks. Professor Fraccaro desidero ringraziarLa per avermi sempre seguita e per essere di nuovo presente in un'occasione per me importante.
Arnold and Mariette, grazie for being always present and close in any occasions. Ad, grazie per la fiducia che mi hai sempre dimostrato e per le tue forti prese di posizione. André, grazie for all the moments, very gay, enthusiastic and a little less. Dear Wilma my grazie to you goes without saying. Dicky, grazie for the hours spent together working and hoping. Grazie Elly and Jan for the long talks, the laughing and your lovely hospitality. Frans, I always enjoy your variable moods; Ruud, your laughing is contagious; Herman, you joined our lysosomal group in the most pleasant way; Otto and Henk, too far away; Han, you always look to the bright site of

life; Pim, I appreciate your slim presence and "buongiorno contessa"; Marian, your sweetness is a good "intermezzo" among these boys. Grazie to all of you.
Professor Jongkind, grazie for the artistic discussions;
Ton, grazie for your helpfull "cell sorting".
Standing next to you, Tar, I always look "upwards" to perfection. Grazie to you and Mr. Fengler.
Mariette, grazie for the quick and extremely accurate type work, done always with a yesterday deadline.
Mevr. Godijn and Jopie, grazie for doing the very necessary things, I do notice when you are not there.

Papa' e mamma miei, sorella e fratelli adorati, grazie per tutto il bene che mi volete.

I still have a dream...

Sandra

CURRICULUM VITAE

Date of birth: October 1, 1948
Place of birth: Pavia, Italy

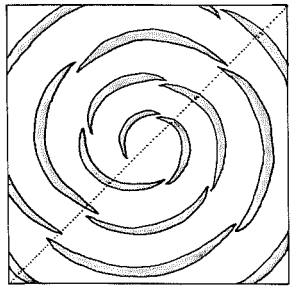
A. Education

University of Palermo (Faculty of Biology)	1966-1968
University of Milano (Faculty of Biology)	1968-1973
University of Milano (Faculty of Biology)	Ph.D.1973
University of Pavia (Medical Faculty) Specialized school in human cytogenetics	1978-1979

B. Professional positions

Research associate, University of Milano, Faculty of Biology	1974
Research associate, University of Pavia, Medical Faculty	1975-1977
Fellowship, University of Rotterdam, Medical Faculty	Feb.1977-July 1977
Research associate, University of Rotterdam, Medical Faculty	1978-present
Research associate, instructor of cell biology and histology, University of Rotterdam, Medical Faculty	1979-present

Publications 1,2,3,4,5



Publication 1

Correction of I-Cell Defect by Hybridization with Lysosomal Enzyme Deficient Human Fibroblasts

A. D'AZZO,[1] D. J. J. HALLEY, A. HOOGEVEEN, AND H. GALJAARD

SUMMARY

I-cell fibroblasts with a multiple intracellular lysosomal enzyme deficiency were hybridized with cells from patients with different types of single lysosomal enzyme defects. Fusion with G_{M2} gangliosidosis, type 2, (Sandhoff disease) fibroblasts resulted in a restoration of the hexosaminidase activity, in a normalization of the electrophoretic mobility of the isoenzymes, and in a decreased activity in the medium. Fusion of I-cells with fibroblasts from G_{M1} gangliosidosis, type 1, led to enhancement of β-galactosidase (β-gal) activity. This complementation must be the result of the presence of normal polypeptide chains in I-cells, whereas the other cell types provide a factor that causes the intracellular retention of the enzymes. Restoration of β-gal was also observed in heterokaryons after fusion of I-cells with β-galactosidase/neuraminidase-deficient (β-gal$^-$/neur$^-$) variants, indicating that the neuraminidase(s) and the posttranslational modification of β-gal are affected in a different way in I-cell disease and in β-gal$^-$/neur$^-$ variants. Fusion of I-cells with mannosidosis fibroblasts resulted in a restoration of the acidic form of α-mannosidase and in a decrease of the extracellular activity of both this enzyme and the hexosaminidase enzyme, indicating that fusion of I-cells with different types of fibroblasts with a single lysosomal enzyme deficiency not only leads to complementation for one particular enzyme but also to a correction of the basic defect in I-cells.

INTRODUCTION

I-cell disease, or mucolipidosis II, is an autosomal recessive disease characterized by psychomotor retardation, severe growth failure, frequent upper respiratory infections, and death, usually before age 5 [1] (see for review [2]). The pathological manifestations seem to be restricted to mesenchymal tissues where the cells show numerous enlarged lysosomes filled with storage material, as is the case in cultured skin fibroblasts [3]. Biochemical assays of these cells show a marked decrease of the intracellular activity of more than 10 lysosomal hydrolases, whereas the activity of these same enzymes is increased two- to 10-fold in the culture medium above the mutant cells [4].

The existence of a multiple-enzyme deficiency in I-cell fibroblasts has been an intriguing and as yet unsolved problem, and the I-cell fibroblasts have been a useful model for studies on the secretion, recognition, uptake, and packaging of lysosomal hydrolases. Hickman and Neufeld [5] provided evidence that I-cells can take up lysosomal enzymes normally, but that hydrolases which are secreted by I-cells are not taken up by other fibroblasts. This led to the hypothesis that lysosomal enzymes in I-cell disease are defective in a common component which normally is involved in their proper intracellular compartmentalization. Subsequent studies suggest a role of phosphorylated carbohydrate residues in the recognition and binding to the membrane of lysosomal hydrolases [6–8]. Sly et al. [9] found that Sindbis virus obtained after passage on I-cell fibroblasts acquired certain abnormalities of their glycoprotein envelope. Lloyd [10] tried to combine the various experimental observations by hypothesizing that in I-cell disease lysosomal enzymes fail to bind to the membrane during membrane recycling, and as a result, they are being lost to the extracellular environment. It is unclear whether the neuraminidase deficiency in I-cells [11–13] and the hypersialylation of various lysosomal enzymes are related to their defective intracellular retention [14–16].

This study investigates whether the unknown metabolic defect in I-cell fibroblasts could be corrected by hybridization with fibroblasts from patients with different types of single lysosomal enzyme deficiencies. For these complementation studies, we used fibroblasts from patients with G_{M2} gangliosidosis, type 2 (Sandhoff disease), and G_{M1} gangliosidosis, type 1, in which a structural mutation affects the properties of the polypeptide chain in β-*N*-acetylhexosaminidase and β-gal, respectively. In addition, we hybridized I-cells with fibroblasts from β-gal$^-$ variants [17, 18] in which a neuraminidase deficiency [19, 20] is probably responsible for a defective posttranslational modification of β-gal [21, 22]. Finally, we studied the results of fusion with mannosidosis fibroblasts because the relatively high α-mannosidase activity in the medium above these deficient cells has been explained as "a localized recognition defect" [23].

MATERIALS AND METHODS

Human skin fibroblast strains were derived from four normal adults, from patients with mucolipidosis II, G_{M2} gangliosidosis, type 2, different variants of G_{M1} gangliosidosis (infantile type 1, adult-type Rotterdam, and Andria variant), and mannosidosis. The cells were grown in Ham's F10 culture medium supplemented with 15% fetal calf serum (FCS). Cell hybridizations

were performed with 10^6 cells from each parental strain with inactivated Sendai virus 250 HAU/ml under such conditions that 60%–90% of the nuclei were present in multinuclear heterokaryons. The fusion index was determined in each experiment by counting the number of cells and nuclei in stained preparations. After fusion, the cells were seeded in 25 cm^2 Falcon flasks and grown overnight in Ham's F10 medium with 15% FCS; during the following 1–3 days, cultivation was carried out in medium with 7% FCS to decrease the proliferation of nonfused mononuclear fibroblasts. After trypsinization and rinsing in saline, the cells were suspended in 100 μl distilled water and disrupted by sonication (5 seconds). If electrophoretic studies were to be performed, the cells were suspended in 50–75 μl and homogenized by repeated freezing and thawing, and aliquots of the supernatant after centrifugation (1,400 g during 10 min) were applied.

In parallel experiments, 10^6 cells of the same pairs of cell strains were mixed and cocultivated under the same conditions as in the cell fusions. In all enzymatic analyses, the protein content was determined according to Lowry et al. [24].

For quantitative enzyme assays, 5 μl of cell homogenate was incubated with 10 μl of 4-methylumbelliferyl substrate during 1 hr at 37°C. Subsequently, 500 μl 0.5 M carbonate buffer, pH 10.7, was added, and the fluorescence was read at 448 nm using a Perkin-Elmer fluorometer. The assay conditions have been described previously for β-gal [17], β-*N*-acetylhexosaminidase [25], and α-mannosidase [26].

The activity of hexosaminidase and α-mannosidase was also measured in the culture medium above control, I-cell, mannosidosis, and Sandhoff fibroblasts after 4 days cultivation, and at 4 days after fusion of I-cells with each of the other mutant strains. For these experiments, 25 μl of the medium was incubated with 50 μl of the appropriate substrate for enzyme assays. The lysosomal enzyme activity in the FCS had previously been inactivated by incubating the serum at pH 10, 37°C for 3 hrs.

Electrophoresis of β-*N*-acetylhexosaminidase isoenzymes was carried out by applying 10–50 μg cellular protein to cellulose acetate gel (Cellogel from Chemetron, Milano, Italy) in 50 mM potassium-phosphate buffer, pH 6.6, for 1 hr at 4°C, and incubation, with methylumbelliferyl substrate according to Okada and O'Brien [27]. For electrophoresis of α-mannosidase forms, 25–60 μg cellular protein was applied, and separation was performed for 2 hrs at 4°C in 0.05 M disodium-β-glycerophosphate buffer, pH 6.2. The gels were incubated with 15 mM 4-methylumbelliferyl-α-D-mannopyranoside in 0.1 M citrate-phosphate buffer, pH 4.5.

RESULTS

In the first set of experiments, three different types of β-gal$^-$ cell strains were hybridized with I-cell fibroblasts. The results, summarized in table 1, show that the β-gal activity in I-cell fibroblasts and in cells from a patient with the early onset, progressive form of G_{M1} gangliosidosis, type 1, is less than 1% of control values. Cocultivation of these two cell strains during 4 days yields enzyme activities which are the mean of both parental strains. Cell hybridization, however, results in a five-to-16-fold increase of β-gal activity. The variation among the four experiments is due to a different proportion of heterokaryons.

The two other β-gal$^-$ cell types are derived from an adult patient with mental deficiency, angiokeratomata, myoclonus, and cerebellar ataxia [17] and from a two-year-old boy with coarse facial features, hepatosplenomegaly, and skeletal abnormalities [18, 28]. The β-gal activity in these fibroblasts is decreased to about 10% of control values, and the cells from these variants also have a neuraminidase deficiency. Again, cocultivation of these β-gal$^-$ cells with I-cell fibroblasts gives enzyme activities which are the mean of both parental cell strains, whereas hybridization results in a sixfold increase of β-gal activity.

TABLE 1

β-GAL ACTIVITY IN MULTINUCLEAR HETEROKARYONS AFTER FUSION OF I-CELL FIBROBLASTS WITH DIFFERENT TYPES OF β-GAL-DEFICIENT FIBROBLASTS

CELL TYPE	β-GAL ACTIVITY* (× 10^{-9} MOL/MG PROTEIN/HR)			
	EXPERIMENT			
	I	II	III†	IV
Controls (no. = 4)	$\bar{X}$ = 630‡			
I-cell	4	7	5	2
Type 1 G_{M1} Gangliosidosis	3	6	4	3
Adult type βGal⁻ Rotterdam	···	43	25	47
βGal⁻ Andria variant	···	35	22	38
Cocultivation:				
I-cell + Type 1	4	6	4	4
I-cell + Rotterdam	···	30	17	19
I-cell + Andria	···	17	15	18
Cell hybridization:				
I-cell × Type 1	52	87	27	52
I-cell × Rotterdam	···	162	138	83
I-cell × Andria	···	117	97	94

* β-Gal assays were carried out with 1 mM 4-methylumbelliferyl-β-D-galactopyranoside in 0.1 M acetate buffer, pH 4.2, containing 0.1 M NaCl. Variation of restored enzyme activity in the different experiments is mainly due to varying percentage of multinuclear heterokaryons (60%–90%).

† Experiment III was carried out 2 d after cell fusion, whereas other assays were performed after 4 d.

‡ Control value, $\bar{X}$ = 630, is the mean of assays of four control fibroblast strains.

The reduced activity of the acidic forms of β-*N*-acetylhexosaminidase in Sandhoff fibroblasts and in I-cells (table 2) is not changed by cocultivation of both cell strains. Fusion of I-cells with Sandhoff fibroblasts results in a restoration of hexosaminidase activity; in one of the experiments, the activity in the mixed population of multinuclear heterokaryons and nonfused mononuclear cells increased to half the control value (table 2).

Cellulose acetate gel electrophoresis of the various cell homogenates indicates that the genetic complementation also results in a change of the electrophoretic pattern of

TABLE 2

TOTAL HEXOSAMINIDASE ACTIVITY IN MULTINUCLEAR HETEROKARYONS AFTER FUSION OF I-CELLS WITH G_{M2} GANGLIOSIDOSIS, TYPE 2, (SANDHOFF) FIBROBLASTS

CELL TYPE	HEXOSAMINIDASE ACTIVITY* (× 10^{-9} MOL/MG PROTEIN/HR)	
	Experiment I	Experiment II
Control	4204	4255
I-cell	489	295
Sandhoff	199	100
I-cell + Sandhoff	335	365
I-cell × Sandhoff	1285	2090

* Hexosaminidase assays were carried out with 5 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in buffer: 20 mM phosphate, 10 mM citrate, pH 4.5.

the hexosaminidase isoenzymes (fig. 1). In Sandhoff fibroblasts, a strong band of activity is present at the HEX S/C position and a faint band at the HEX B position (lane *a*). I-cells have a residual activity at the HEX S/C and HEX B positions and show two additional bands anodal and cathodal of the HEX A position (lane *e*). Cocultivation of the two cell strains does not alter the electrophoretic pattern (lane *b*). Fusion of I-cell fibroblasts with Sandhoff cells results in an increased activity of bands near the HEX A and HEX B positions and a disappearance of the abnormally migrating bands anodal and cathodal of HEX A (lane *c*). Fusion of I-cell fibroblasts with themselves, which is a test of the hybridization process with Sendai virus, results in a slower migration of the most cathodal band near the HEX A position (lane *f*). Although genetic complementation in I-cell × Sandhoff fusions results in a normalization of the hexosaminidase isoenzyme pattern, the migration of HEX A and HEX B is somewhat slower than in control fibroblasts (compare lanes *c* and *d*).

Both in I-cell disease and in mannosidosis, we found an α-mannosidase activity of about 10% of control values (table 3). Cocultivation of both mutant cell strains did not alter the intracellular enzyme activity, but cell fusion resulted in a four- to sixfold increase of the α-mannosidase activity. This experiment was also performed with fibroblasts from another patient with I-cell disease ($I\text{-}cell_2$), and similar results were obtained (table 3). Cellulose acetate gel electrophoresis shows that the α-mannosidase deficiency involves the acidic form; by applying relatively much cellular protein, the residual acidic α-mannosidase activity in I-cells could be visualized, although it was difficult to demonstrate it photographically (fig. 2, lane *f*). It can also be seen that the genetic complementation results in a restoration of the activity of acidic α-mannosidase (fig. 2, lane *d*).

Both β-*N*-acetylhexosaminidase and α-mannosidase are relatively stable enzymes, and we have, therefore, measured their activity in the culture medium above fibroblasts from controls and from patients with I-cell disease, mannosidosis, and Sandhoff disease. The mean hexosaminidase activity expressed in nmol/methylumbelliferyl substrate hydrolyzed in 1 hr/mg of cultured cells was: 2,043 in medium above I-cells,

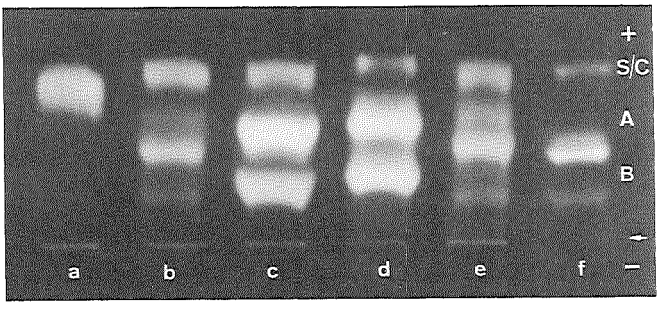


FIG. 1.—Cellulose acetate gel electrophoresis of β-*N*-acetylhexosaminidases after cell hybridization and cocultivation of human mutant fibroblasts. *lane a* = Sandhoff fibroblasts (37 μg protein), *lane b* = I-cell + Sandhoff (37 μg), *lane c* = I-cell × Sandhoff (31 μg), *lane d* = control fibroblasts (13 μg), *lane e* = I-cell fibroblasts (58 μg), and *lane f* = I-cell × I-cell (23 μg). Origin and normal positions of the different forms are indicated.

TABLE 3

α-MANNOSIDASE ACTIVITY IN MULTINUCLEAR HETEROKARYONS AFTER FUSION OF I-CELLS WITH MANNOSIDOSIS FIBROBLASTS

CELL TYPE	α-MANNOSIDASE ACTIVITY* (× 10^{-9} MOL/MG PROTEIN/HR)		
	EXPERIMENT		
	I	II	III
Controls (no. = 3)		$\bar{X}$ = 53†	
I-cell	5	···	6
I-cell$_2$	···	13	···
Mannosidosis	8	7	3
I-cell + Mannosidosis	7	···	5
I-cell$_2$ + Mannosidosis	···	11	···
I-cell × Mannosidosis	30	···	29
I-cell$_2$ × Mannosidosis	···	32	···

* α-Mannosidase assays have been carried out with 4-methylumbelliferyl-α-D-mannopyranoside, 1 mM final concentration, in 0.07 M acetate buffer, pH 4.0.

† Control value, X = 53, is the mean of assays of three control fibroblast strains.

414 above control fibroblasts, and only 13 above Sandhoff cells. The increased extracellular hexosaminidase activity of I-cells was apparently corrected by hybridization of I-cells × Sandhoff cells since the activity in the medium was 390 nmol/hr per mg cellular protein at 4 days after fusion. For α-mannosidase, similar observations were made. The mean activity in nmol/hr per mg was 63 in medium above I-cells, and 4 nmol/hr per mg at 4 days after fusion of I-cells × mannosidosis fibroblasts. Also, the extracellular activity of hexosaminidase was decreased from 2,043 above I-cells to 875 after fusion of I-cells × mannosidosis fibroblasts.

DISCUSSION

Both in G_{M1} gangliosidosis, type 1, and in I-cell disease, cultured fibroblasts show a very low (less than 1%) residual activity of β-gal (see table 1). In G_{M1} gangliosidosis, type 1, this enzyme deficiency is based on a structural mutation affecting the only polypeptide so far shown in β-gal [20, 29, 30]. In I-cells, the residual β-gal activity is lower than that of other lysosomal hydrolases [31], an observation which has also been made in a variant of mucolipidosis III [32]. An exceptional behavior of β-gal is also apparent from its low activity in some visceral organs in I-cell disease [31, 33]. Recent analysis of the properties of the residual β-gal activity in liver from a patient with I-cell disease suggests changes in the carbohydrate composition of the enzyme [34].

The intralysosomal packaging of β-gal may well proceed in a different way from that of other hydrolases. In this connection, it is interesting that some clinical variants of β-gal deficiency which were found to complement the classical types of G_{M1} gangliosidosis [17, 28] are also deficient in neuraminidase activity [19, 20]. Somatic cell hybridization studies with enucleated cells [21] and gel filtration analyses of the restored β-gal activity after complementation [22] indicate that the mutation in these β-gal$^-$/neur$^-$ variants affects a posttranslational process resulting in abnormal aggregation of the low molecular weight form. It is unclear how the neuraminidase deficiency

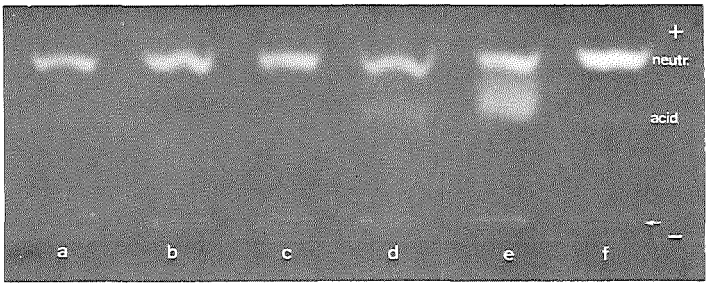


FIG. 2. —Cellulose acetate gel electrophoresis of α-mannosidases after cell hybridization and cocultivation of human mutant fibroblasts. *lane a* = I-cell × I-cell (34 μg protein), *lane b* = mannosidosis fibroblasts (31 μg), *lane c* = I-cell + mannosidosis (32 μg), *lane d* = I-cell × mannosidosis (24 μg), *lane e* = control fibroblasts (26 μg), and *lane f* = I-cell fibroblasts (58 μg). Origin and normal positions of the different forms are indicated.

is related to this process of aggregation and, may be, to the intralysosomal packaging of β-gal as well. Both in the adult type β-gal$^-$/neur$^-$ patient and in the two-year-old patient described by Andria et al. [18], the β-gal polypeptide is normal, as seems the case in I-cell disease.

The complementation observed after fusion of G_{M1} gangliosidosis, type 1, cells with I-cells (table 1) must be due to the presence of a normal β-gal polypeptide in I-cells and of a normal posttranslational factor in the G_{M1} gangliosidosis, type 1, fibroblasts. The restoration of β-gal activity in heterokaryons after fusion of I-cells with each of the β-gal$^-$/neur$^-$ variants indicates that the posttranslational modification of β-gal is affected in a different way in both types of diseases. Preliminary experiments also show a restoration of neuraminidase activity in I-cell × β-gal$^-$ variant heterokaryons [35], which suggests that neuraminidase(s) are affected in a different way in I-cell disease and β-gal$^-$/neur$^-$ variants. The same is true for mucolipidosis I where a neuraminidase deficiency is associated with a normal β-gal activity [36].

As far as the neuraminidase deficiency in I-cell disease is concerned [11−13], no conclusive evidence has yet been presented that this is the primary defect. Electrophoretic studies showed differences between intracellular and extracellular hydrolases, and in I-cell disease, excreted lysosomal enzymes have a higher electronegative charge than in controls [14]. Although desialylation of excreted I-cell hexosaminidase by neuraminidase treatment did not alter its uptake [15], this does not exclude a possible role of neuraminidase in the intralysosomal packaging. There are, however, several indications that intracellular desialylation by exogenous neuraminidase does not correct the I-cell defect either. In our own experiments, the α2 → 3 neuraminidase of the Sendai virus used in the cell hybridization experiments does affect the electrophoretic mobility of the fast-migrating, A-like form of hexosaminidase (fig. 1, lanes *e* and *f*), but it does not result in a correction of the I-cell defect. This observation is in agreement with studies done by Spritz et al. [16] which did not find any effect of abundant intracellular α2 → 3 neuraminidase activity after infection of I-cells by influenza virus. In our own laboratory, uptake of *Clostridium perfringens* neuraminidase was not found to correct the metabolic abnormalities of I-cells.

Fusion of Sandhoff fibroblasts with I-cells results in a four- to sixfold increase of the total hexosaminidase activity (table 2). Since the hexosaminidase deficiency in Sandhoff disease is due to a structural mutation affecting the β-chain, which is present in both the HEX A and HEX B forms [20, 37], the complementation must be due to the presence of normal β-chains in I-cells, whereas the Sandhoff cells provide a factor which causes the intracellular retention of the HEX isoenzymes. The reduced activity of hexosaminidase and other hydrolases in the medium above the fused cell population also indicates that the basic defect in I-cells is corrected. Comparison of lanes *c* and *d* in figure 1 shows that both HEX A and HEX B after complementation in I-cell × Sandhoff heterokaryons have a slower electrophoretic mobility than in control fibroblasts. This might be due to extra desialylation by Sendai virus neuraminidase, in addition to the action of the cellular neuraminidase, in which activity is restored by complementation.

Immunological and kinetic studies of the 10% residual acidic α-mannosidase activity in fibroblasts from patients with mannosidosis are indicative of an altered enzyme molecule as a result of a structural mutation [38–41]. A remarkable feature is the relatively high acidic α-mannosidase activity in medium above the deficient cells, which has led to the suggestion of a ''localized recognition defect'' [23]. Our finding of restoration of acidic α-mannosidase in I-cell × mannosidosis heterokaryons (fig. 2 and table 2) shows that the molecular defects leading to a high extracellular α-mannosidase activity must be different in both diseases. The decreased extracellular activity of both α-mannosidase and of hexosaminidase after fusion of I-cells × mannosidosis is most probably due to a correction of the basic defect in the I-cells. A possible effect of Sendai virus was excluded by the unchanged activity in the medium after parental fusion of I-cells.

Little is known about the mechanism of genetic complementation. Studies on interspecies hybrids [42] suggest that the I-cell defect can be corrected by the mouse genome. The model of proliferating interspecies hybrids has, however, the disadvantage that human chromosomes are lost. Recent experiments by Hasilik et al. [43] indicate a defective phosphorylation and impairment of chain shortening of lysosomal enzymes in I-cell disease. We are now investigating the molecular properties of lysosomal enzymes after complementation, and by performing fusions with enucleated mutant fibroblasts, we hope to find whether de novo synthesis is required for correction of the I-cell defect.

ACKNOWLEDGMENTS

The mutant cells strains used in this study were kindly provided by Drs. A. Boué (Paris), G. Andria (Napoli), B. G. Winchester (London), and M. F. Niermeijer (Rotterdam). The photography was done by Mr. T. van Os.

REFERENCES

1. LEROY JG, SPRANGER JW, FEINGOLD M, OPITZ JM, CROCKER AC: I-cell disease: a clinical picture. *J Pediatr* 79:360–365, 1971
2. MCKUSICK VA, NEUFELD EF, KELLY TE: in *The Metabolic Basis of Inherited Disease*, 4th ed, edited by STANBURY JB, WYNGAARDEN JB, FREDRICKSON DS, New York, McGraw-Hill, 1978, pp 1282–1307

3. LEROY JG, DE MARS RI: Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. *Science* 157:804–805, 1967
4. WIESMANN UN, HERSCHKOWITZ NN: Studies on the pathogenetic mechanism of I-cell disease in cultured fibroblasts. *Pediatr Res* 8:865–870, 1974
5. HICKMAN S, NEUFELD EF: A hypothesis for I-cell disease: defective hydrolases that do not enter lysosomes. *Biochem Biophys Res Commun* 49:992–999, 1972
6. HICKMAN S, SHAPIRO LJ, NEUFELD EF: A recognition marker required for uptake of lysosomal enzyme by cultured fibroblasts. *Biochem Biophys Res Commun* 57:55–61, 1974
7. KAPLAN A, ACHORD DT, SLY WS: Phosphohexosyl components of lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. *Proc Natl Acad Sci USA* 74:2026–2030, 1977
8. SANDO GN, NEUFELD EF: Recognition and receptor-mediated uptake of a lysosomal enzyme, α-L-iduronidase, by cultured human fibroblasts. *Cell* 12:619–627, 1977
9. SLY WS, LAGWINSKA E, SCHLESINGER S: Enveloped virus acquires membrane defect when passaged in fibroblasts from I-cell disease patients. *Proc Natl Acad Sci USA* 73:2443–2447, 1976
10. LLOYD JB: Cellular transport of lysosomal enzymes. An alternative hypothesis. *Biochem J* 164:281–282, 1977
11. THOMAS GH, TILLER GP, REYNOLDS IW, MILLER CS, BACE JW: Increased levels of sialic acid associated with a sialidase deficiency in I-cell disease (mucolipidosis II) fibroblasts. *Biochem Biophys Res Commun* 71:188–195, 1976
12. STRECKER G, HONDI-ASSAH T, FOURNET B, ET AL.: Structure of the three major sialyl-oligosaccharides excreted in the urine of five patients with three distinct inborn diseases: "I-cell disease" and two new types of mucolipidosis. *Biochim Biophys Acta* 444:349–358, 1976
13. STRECKER G, PEERS MC, MICHALSKI JC, ET AL.: Structure of nine sialyl-oligosaccharides accumulated in urine of eleven patients with three different types of sialidosis. *Eur J Biochem* 75:391–403, 1977
14. VLADUTIU GD, RATTAZZI MC: Abnormal lysosomal hydrolases excreted by cultured fibroblasts in I-cell disease (mucolipidosis II). *Biochem Biophys Res Commun* 67:956–964, 1975
15. VLADUTIU GD, RATTAZZI MC: I-cell disease. Desialylation of β-hexosaminidase and its effect on uptake by fibroblasts. *Biochim Biophys Acta* 539:31–36, 1978
16. SPRITZ RA, COATES PM, LIEF FS: I-cell disease: intracellular desialylation of lysosomal enzymes using an influenza virus vector. *Biochim Biophys Acta* 582:164–171, 1979
17. GALJAARD H, HOOGEVEEN A, KEIJZER W, ET AL.: Genetic heterogeneity in G_{M_1}-gangliosidosis. *Nature* 257:60–62, 1975.
18. ANDRIA G, DEL GIUDICE E, REUSER AJJ: Atypical expression of β-galactosidase deficiency in a child with Hurler-like features but without neurological abnormalities. *Clin Genet* 14:16–23, 1978
19. WENGER DA, TARBY TJ, WHARTON C: Macular cherry-red spots and myoclonus with dementia: coexistent neuraminidase and β-galactosidase deficiencies. *Biochem Biophys Res Commun* 82:589–595, 1978
20. LOWDEN JA, O'BRIEN JS: Sialidosis: a review of human neuraminidase deficiency. *Am J Hum Genet* 31:1–18, 1979
21. DE WIT-VERBEEK HA, HOOGEVEEN A, GALJAARD H: Complementation studies with enucleated fibroblasts from different variants of β-galactosidase deficiency. *Exp Cell Res* 113:215–218, 1978
22. HOEKSEMA HL, VAN DIGGELEN OP, GALJAARD H: Intergenic complementation after fusion of fibroblasts from different variants of β-galactosidase deficiency. *Biochim Biophys Acta* 566:72–79, 1979
23. HULTBERG B, MASSON PK: Normal extracellular excretion of acidic α-mannosidase activity by mannosidosis fibroblast cultures. *Biochim Biophys Acta* 481:573–577, 1977
24. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with Folin

phenol reagent. *J Biol Chem* 193:265–275, 1951
25. KABACK MM: *Methods in Enzymology*, vol 28, edited by COLOWICK SP, KAPLAN NO, New York, Academic Press, 1972, p 862
26. PHILLIPS NC, ROBINSON D, WINCHESTER BG: Mannosidosis in Angus cattle. *Biochem J* 137:363–371, 1974
27. OKADA S, O'BRIEN JS: Tay-Sachs disease, generalized absence of a β-D-*N*-acetylhexosaminidase component. *Science* 165:698–700, 1969
28. REUSER AJJ, ANDRIA G, DE WIT-VERBEEK E, HOOGEVEEN A, DEL GIUDICE E, HALLEY D: A two-year-old patient with an atypical expression of G_{M1}-β-galactosidase deficiency: biochemical, immunological and cell genetic studies. *Hum Genet* 46:11–19, 1979
29. NORDEN AGW, TENNANT L, O'BRIEN JS: Ganglioside G_{M1}-β-galactosidase A: purification and studies of the enzyme from human liver. *J Biol Chem* 249:7969–7976, 1974
30. O'BRIEN JS: Molecular genetics of G_{M1}-β-galactosidase. *Clin Genet* 8:303–313, 1975
31. LEROY JG, HO MW, MAC BRINN MC, ZIELKE K, JACOB J, O'BRIEN JS: I-cell disease: biochemical studies. *Pediatr Res* 6:752–757, 1972
32. SANDMAN R, YATSIV S, ROBERTSON WVB, ERICKSON RP: A new variant mucolipidosis: biochemical investigations on two siblings. *Clin Chim Acta* 80:471–481, 1977
33. HOLMES EW, MILLER AL, FROST RG, O'BRIEN JS: Characterization of β-D-galactosidase isolated from I-cell disease liver. *Am J Hum Genet* 27:719–727, 1975
34. MILLER AL: I-cell disease: isoelectric focusing, concanavalin A-sepharose 4B binding and kinetic properties of human liver acid β-D-galactosidases. *Biochim Biophys Acta* 522:174–186, 1978
35. HOOGEVEEN AT, VERHEYEN FW, D'AZZO A, GALJAARD H: Genetic heterogeneity in human neuraminidase deficiency. Submitted for publication
36. KELLY TE, GRAETZ G: Isolated acid neuraminidase deficiency: a distinct lysosomal storage disease. *Am J Med Genet* 1:31–46, 1977
37. BEUTLER E, KUHL W, COMINGS D: Hexosaminidase isozyme in type O G_{M2} gangliosidosis (Sandhoff-Jatzkewitz disease). *Am J Hum Genet* 27:628–638, 1975
38. CARROL M, DANCE N, MASSON PK, ROBINSON D, WINCHESTER BG: Human mannosidosis. The enzymic defect. *Biochem Biophys Res Commun* 49:579–583, 1972
39. BEAUDET AL, NICHOLS LB: Residual altered α-mannosidase in human mannosidosis. *Biochem Biophys Res Commun* 68:292–298, 1976
40. MERSMANN G, BUDDECKE E: Evidence for material from mannosidosis fibroblasts crossreacting with anti-acidic α-mannosidase antibodies. *FEBS Lett* 73:123–126, 1977
41. BURDITT LJ, CHOTAI KA, WINCHESTER BG: Evidence that the mutant enzyme in fibroblasts of a patient with mannosidosis does not crossreact with antiserum raised against normal acidic α-D-mannosidase. *FEBS Lett* 91:186–189, 1978
42. CHAMPION MJ, SHOWS TB: Correction of human mucolipidosis II enzyme abnormalities in somatic cell hybrids. *Nature* 270:64–66, 1977
43. HASILIK A, ROME LH, NEUFELD EF: Processing of lysosomal enzymes in human skin fibroblasts. *Fed Proc* 38:467, 1979

Publication 2

Fusion with enucleated fibroblasts corrects "I-cell" defect

A. D'AZZO, A. KONINGS, A. VERKERK, J. F. JONGKIND and H. GALJAARD, *Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands*

Summary. Fusions have been carried out between fibroblasts from patients with "I-cell" disease and enucleated human fibroblasts with a single lysosomal enzyme deficiency derived from patients with G_{M1}-gangliosidosis, Sandhoff disease and mannosidosis. Pure cytoplasts were obtained using cytochalasin B treatment followed by fluorescence activated cell sorting. After fusion with whole "I-cells", the cybrid populations showed a restoration of deficient lysosomal enzyme activity and also the abnormal electrophoretic pattern characteristic for the residual hexosaminidase activity in "I-cells" was found to be corrected. The results described in this paper indicate that the defective post-translational modification, which is responsible for the multiple lysosomal enzyme deficiency, can be corrected by a factor that is stable for at least three days in enucleated cells. During this period the cytoplasmic factor can act without the need of de novo synthesis but the absence of correction in in vitro experiments shows that cellular integrity is required.

I-cell disease or mucolipidosis II is an autosomal recessive disease characterized by severe physical and mental handicaps and a fatal outcome at early childhood [1]. Cultured skin fibroblasts show a marked intracellular deficiency of more than ten lysosomal hydrolases but the activity of these same enzymes is increased in the culture medium above the mutant cells (for review see [2]). The responsible molecular defect in this disease has not yet been resolved but Neufeld's group recently provided evidence that a phosphorylation step common in the post-translational processing of several hydrolases is defective [3].

Correction of the "I-cell" defect was found in proliferating human×mouse hybrids [4] and in heterokaryons after fusion of "I-cells" with fibroblasts from patients with a single lysosomal enzyme deficiency [5]. The present communication deals with fusions between "I-cells" and enucleated human fibroblasts (cytoplasts) to investigate whether the presence of the cell nucleus is a prerequisite for complementation to occur.

Materials and Methods

Subcultures of normal skin fibroblasts and of cells from two patients with I-cell disease (kindly provided by Professor A. Boué, Paris) and from patients with G_{M2}-gangliosidosis type 2 (Sandhoff disease), G_{M1}-gangliosidosis type 1 and mannosidosis (all provided by Dr M. F. Niermeijer, Rotterdam) were grown in Ham's F 10 medium supplemented with 15% fetal calf serum (FCS) and antibiotics. Cells to be enucleated were labelled with green fluorescent latex spheres and enucleation was carried out using a gradient solution of 40% (v/v) (density 1.058 g/cm^3) Percoll (Pharmacia) in Ham's F 10 containing 5 μg/ml cytochalasin B (Serva). After ultracentrifugation, washing and staining with Hoechst 33342 (10 μM/ml for 30 min at 37°C) cytoplasts were isolated with a FACS II cell sorter [6, 7]. Samples containing 10^6 of pure cytoplasts, collected on agar-coated Petri dishes, were mixed with the same number of whole fibroblasts, centrifuged (5 min, 1500 rpm, 20°C) and washed with Hanks' solution. Fusion was carried out with inactivated Sendai virus (250 HAU/ml) and after subse-

quent cultivation during 1–3 days the mixed cybrid population was harvested by trypsinization.

The activity of β-*N*-acetylhexosaminidase [8], β-galactosidase [9] and α-mannosidase [10] was determined by incubating 5 μl samples of cell homogenate, prepared by 2×, 5 sec sonication, with 10 μl of the proper methylumbelliferyl substrate. The protein content was measured according to Lowry [11]. Cellulose acetate gel electrophoresis of hexosaminidase isoenzymes was performed as reported earlier [5].

Results and Discussion

All fusion experiments were repeated 2–3 times and reproducible results were obtained within the same cell strain and with the two different strains of "I-cells". The enzyme analyses were performed on a mixed cell population containing 40–60% of cytoplasmic hybrids (cybrids) (fig. 1).

The results in table 1 show that the fusion process does not affect the lysosomal enzyme activities since parental fusions between cytoplasts and whole cells give values comparable to those in the whole cells. Fusion of Sandhoff cytoplasts with whole "I-cells" leads to a 4-fold increase in hexosaminidase activity and a similar rate of complementation was observed for α-mannosidase after fusion of mannosidosis cytoplasts with "I-cells" (table 1, last column). In the latter case we also measured the α-mannosidase activity 24 h after fusion but no difference with the values after 3 days was found. Fusion of G_{M1}-gangliosidosis cytoplasts with "I-cells" leads to a 10–15-fold increase in the β-galactosidase activity. These results indicate that cytoplasts from fibroblasts with a single lysosomal enzyme deficiency are capable of correcting the defect in "I-cells".

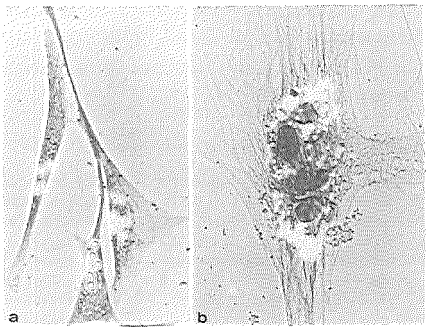


Fig. 1. Phase contrast microscopy of (*a*) cytoplasts containing green fluorescent spheres (1 day after enucleation); (*b*) cybrids obtained by fusing whole fibroblasts and cytoplasts labeled with fluorescent spheres (3 days after fusion). (*a*) ×100; (*b*) ×60.

The enzyme deficiencies in Sandhoff disease [12], G_{M1}-gangliosidosis [13] and in mannosidosis [14] are all due to a mutation in the structural gene coding for one of the enzyme polypeptides. The multiple hydrolase deficiency in mucolipidosis II fibroblasts is assumed to result from an altered post-translational modification of normally synthesized polypeptide chains [2, 3].

In our experiments we did not find complementation after fusions of I-cell cytoplasts and whole cells with a single lysosomal enzyme deficiency (table 1). This implies that mRNA for the lysosomal hydrolases tested must have a short half-life since otherwise mRNA would be translated and the enzyme proteins could be processed in a normal way.

In cybrids where complementation does occur (table 1) the presence of the I-cell nucleus will guarantee a continuous production of normal enzyme polypeptides, whereas the cytoplasm of the other fusion partner will enable their correct processing and intracellular compartmentalization. Since we observed complementation up to 3 days after fusion the post-translational processing factor, which is defective in "I-cells", must be stable in the cytoplasm for at least 3 days. Although it can act in the cybrids without the necessity of de novo synthesis, the modification apparently

Table 1. *β-N-acetylhexosaminidase, β-galactosidase and α-mannosidase activities*[a] *after fusions of cytoplasts with whole fibroblasts*

β-Hex		β-Gal		α-Man	
Controls (n=4)	$\bar{x}$=6 000	Controls (n=4)	$\bar{x}$=630	Controls (n=3)	$\bar{x}$=53
I-cells (n=2)	$\bar{x}$= 400	I-cells (n=2)	$\bar{x}$= 4.2	I-cells (n=2)	$\bar{x}$= 5.8
Sandhoff	272	G_{M1}-gangliosidosis type 1	2.0	Mannosidosis	5.2
Sandhoff cytopl.×Sandhoff	246	G_{M1} cytopl.×G_{M1}	1.7	Mann. cytopl.×Mann.	5.4
I-cells cytopl.×I-cells	257	I-cells cytopl.×I-cells	3.2	I-cells cytopl.×I-cells	6.0
Sandhoff cytopl.×I-cells	977	G_{M1} cytopl.×I-cells	38	Mann. cytopl.×I-cells	19
I-cells cytopl.×Sandhoff	270	I-cells cytopl.×G_{M1}	4.8	I-cells cytopl.×Mann.	6.9

[a] Activities were measured 3 days after fusion with 4-methylumbelliferyl substrate and expressed as 10^{-9} moles · h^{-1} · mg $proteins^{-1}$. Each value is the average of 3–10 separate experiments; when more than one cell strain is used this is indicated.

needs the "cellular machinery" because we did not find any correction after mixing cell homogenates in vitro. In heterokaryons derived from whole "I-cells" and whole fibroblasts with a single enzyme defect [5] the acid hydrolase activities are about twice as high as in the cybrids mentioned in table 1. This is probably due to a continuous production of the factors involved in the processing of the lysosomal enzymes in the former. The time needed for the post-translational modification is not exactly known but is likely to vary for different lysosomal enzymes [15]. This may explain why the complementation observed in the present studies and in those with whole cells always yields higher activities for α-mannosidase and β-hexosaminidase than for β-galactosidase, at least as a proportion of control values.

Several investigators have shown an abnormal electrophoretic pattern for a number of lysosomal enzymes in I-cell fibroblasts, which has mainly been attributed to an abnormal sialylation [16, 17]. To study whether this phenomenon could be corrected we have analysed the electrophoretic pattern of β-hexosaminidases at 3 days after fusion of "I-cells" with Sandhoff cytoplasts. The results illustrated in fig. 2 show that in the cybrids there is not only a clear increase in hexosaminidase A and B activities but also a normalization of the abnormal mobility of hexosaminidase isoenzymes. In one of the fusion experiments (lane 5) a nearly normal pattern has been attained whereas in the other fusion (lane 3) one of the bands characteristic for "I-cells" is still visible in addition to the normalized A-band.

These electrophoretic studies provide additional evidence for the correction of the basic defect in "I-cells" by fusion with enucleated fibroblasts.

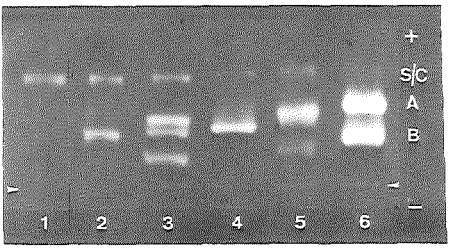


Fig. 2. Cellulose acetate gel electrophoresis of β-*N*-acetylhexosaminidase isoenzymes after fusions of cytoplasts with whole fibroblasts. *1*, Sandhoff fibroblasts; *2*, I-$cell_1$ fibroblasts; *3*, Sandhoff cytoplasts× I-$cell_1$ fibroblasts; *4*, I-$cell_2$ fibroblasts; *5*, Sandhoff cytoplasts×I-$cell_2$ fibroblasts; *6*, control fibroblasts.

References

1. Leroy, J G, Spranger, J W, Feingold, M, Opitz, J M & Crocker, A C, J pediat 79 (1971) 360.
2. McKusick, V A, Neufeld, E F & Kelly, T E, The metabolic basis of inherited disease (ed J B Stanbury, J B Wyngaarden & D S Fredrickson) 4th edn, p. 1282. McGraw-Hill, New York (1978).
3. Hasilik, A, Rome, L H & Neufeld, E F, Fed proc 38 (1979) 467.
4. Champion, M J & Shows, T B, Nature 270 (1977) 64.
5. D'Azzo, A, Halley, D J J, Hoogeveen, A & Galjaard, H, Am j hum genet 32 (1980). In press.
6. Schaap, G H, Verkerk, A, van der Kamp, A W M, Öry, F G & Jongkind, J F, Acta pathol microbiol Scand, suppl. In press.
7. Schaap, G H, van der Kamp, A W M, Öry, F G & Jongkind, J F, Exp cell res 122 (1979) 422.
8. Kaback, M M, Methods in enzymology (ed S P Colowick & N O Kaplan) vol. 28, p. 862. Academic Press, New York (1972).
9. Galjaard, H, Hoogeveen, A, Keijzer, W, De Wit-Verbeek, H A, Reuser, A J J, Ho, M W & Robinson, D, Nature 257 (1975) 60.
10. Phillips, N C, Robinson, D & Winchester, B G, Biochem j 137 (1974) 363.
11. Lowry, O H, Rosebrough, N J, Farr, A L & Randall, R J, J biol chem 193 (1951) 265.
12. Beutler, E, Kuhl, W & Comings, D, Am j human genet 27 (1975) 628.
13. O'Brien, J S, Clin genet 8 (1972) 579.
14. Carrol, M, Dance, N, Masson, P K, Robinson, D & Winchester, B G, Biochem biophys res commun 49 (1972) 579.
15. Neufeld, E F, Hasilik, A & Rome, L H, Glycoconjugates, proc IVth int symp (ed R Schauer, P Boer, E Buddecke, M F Kramer, J F G Vliegenthart & H Wiegandt) p. 320. Kiel, September 1979. G Thieme, Stuttgart (1979).
16. Vladutiu, G D & Rattazzi, M C, Biochem biophys res commun 67 (1975) 956.
17. Champion, M J & Shows, T B, Am j human genet 29 (1977) 149.

Publication 3

Genetic heterogeneity in human neuraminidase deficiency

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

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

---

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

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

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	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.

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

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

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

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

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

Publication 4

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

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

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

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

SUMMARY

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

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

Correction

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

Correction was also studied by addition of a Concanavalin A preparations of human placenta and liver. Concanavalin A - sepharose 4B (Pharmacia, Uppsala) was used according to a procedure described earlier (11). After elution at 20°C with 0.75 M α-methylglucoside and dialysis against 10 mM Na-phosphate buffer pH.7 the preparation was concentrated on an Amicon PM 10 filter till a final protein concentration of 15 mg/ml. An aliquot of 50 µl of this preparation was added to 1 ml medium.

Inhibition of β-galactosidase activity

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

RESULTS

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

INTRODUCTION

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

When fibroblasts with a combined deficiency of β-galactosidase and neuraminidase ($\beta\text{-gal}^-/\text{neur}^-$) were co-cultivated with other human fibroblasts, including those with an isolated neuraminidase deficiency (neur^-), a slight increase of neuraminidase activity in the $\beta\text{-gal}^-/\text{neur}^-$ cells was observed (4). Preliminary experiments suggested that this partial correction was due to the uptake of a glycoprotein that is secreted by other cells into the culture medium.

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

MATERIALS AND METHODS

Cell cultivation and analysis

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

Table I

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

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

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

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

To achieve a more efficient uptake of the correction factor another source of glycoproteins was found in media of various types of human fibroblasts that were treated with NH_4Cl to stimulate the secretion of precursor forms of glycoproteins (14). The medium containing these "high-uptake" forms of glycoproteins was then concentrated and dialyzed on an Amicon pM 10 filter, to remove most of the molecules with a molecular weight lower than 20 x 10^3. The concentrated medium obtained in this way was added to the culture medium above various types of human mutant fibroblasts and left for 2-3 days. Table II summarizes the effect of concentrate on the β-galactosidase and neuraminidase activity in the different cell types. There was no effect on fibroblasts with an isolated β-galactosidase deficiency (β-gal$^-$) nor on neur$^-$ cells. In two different strains of β-gal$^-$/neur$^-$ fibroblasts, however, the activity of both β-galactosidase and neuraminidase increases markedly and approaches low control values. In the

Table II

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

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

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

experiment illustrated in Table II concentrated medium containing "corrective factor" was prepared from NH_4Cl stimulated medium of G_{M1}-gangliosidosis fibroblasts (β-gal$^-$), but similar results were obtained with concentrates from normal and mucolipidosis I media.

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

The reappearance of newly synthesized β-galactosidase molecules after correction of β-gal$^-$/neur$^-$ cells could be studied with the use of an irreversible inhibitor of β-galactosidase (10,

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

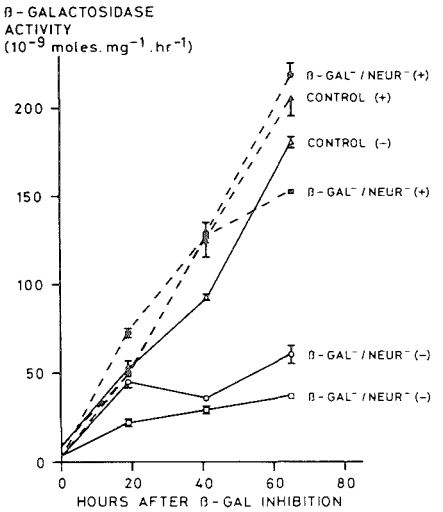


Fig. 1 CORRECTION FOR β-GALACTOSIDASE ACTIVITY IN β-GAL⁻/NEUR⁻ FIBROBLASTS.

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

□ = β-gal⁻/neur⁻ fibroblasts (8)

○ = β-gal⁻/neur⁻ fibroblasts (3)

△ = normal fibroblasts.

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

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

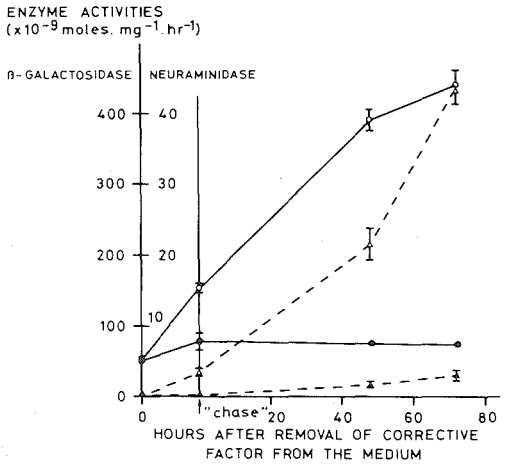


Fig. 2 ACTIVITIES OF β-GALACTOSIDASE AND NEURAMINIDASE IN β-GAL⁻/NEUR⁻ FIBROBLASTS AFTER ADDITION AND "CHASE" OF CORRECTIVE FACTOR.

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

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

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

DISCUSSION

The complementation for β-galactosidase after fusion of different types of human β-galactosidase deficient cell strains (5) was surprising in view of the fact that normal human β-galactosidase consists of one polypeptide of about 70,000 m.w. which also occurs in an aggregate of molecular weight 700,000 (16). Later, Wenger et al. (1) found a coexistent deficiency of neuraminidase in one of their atypical patients with β-galactosidase deficiency. Fibroblasts from several other patients were subsequently found to have this combined β-galactosidase/neuraminidase deficiency (2, 4). Analysis of interspecies and intraspecies hybrids, Hoeksema et al. (17, 18) showed that the isolated β-galactosidase deficiency in classical forms of G_{M1}-gangliosidosis is due to a structural mutation in the gene on chromosome 3 coding for the β-galactosidase polypeptide chain. In variants, later identified as β-gal$^-$/neur$^-$, the residual β-galactosidase activity consists of the monomeric form of β-galactosidase and aggregation to higher molecular weight forms seems to be impaired.

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

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

corrective factor (Fig. 1) is similar to that in control fibroblasts.

The corrective factor or its precursor form is secreted by various types of human fibroblasts including those from G_{M1}-gangliosidosis (β-gal$^-$) and mucolipidosis I (neur$^-$). No correction is obtained with concentrate prepared from medium after NH_4Cl stimulation of β-gal$^-$/neur$^-$ fibroblasts. This points to the specific nature of the corrective factor. The experimental results presented indicate that the corrective factor is a macromolecular glycoprotein, excluded by an Amicon PM 10 filter, which is stable at 50°C but loses its activity at 60°C. I-cell secreted glycoproteins which are not phosphorylated, are not taken up by other fibroblasts (19). Our observation that I-cell medium cannot correct β-gal$^-$/neur$^-$ fibroblasts (4) is in agreement with the phosphoglycoprotein nature of the "corrective factor" described in the present paper. This is also supported by the fact that mannose-6-phosphate inhibits the uptake of the "corrective factor" (14, 20, 21, 22).

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

Acknowledgements

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

REFERENCES

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

Publication 5

MOLECULAR DEFECT IN COMBINED β-GALACTOSIDASE AND NEURAMINIDASE DEFICIENCY IN MAN

(precursor β-galactosidase/leupeptin/corrective factor)

D'Azzo, A., Hoogeveen, A., Robinson, D. & Galjaard, H.

SUMMARY

The nature of the combined neuraminidase/β-galactosidase deficiency in human fibroblasts was investigated. Addition of the thiol protease inhibitor leupeptin resulted in a restoration of β-galactosidase activity in the mutant cells. Administration of NH_4Cl to $\beta\text{-gal}^-/\text{neur}^-$ fibroblasts showed similar amounts of precursor β-galactosidase in the medium as in control cells. The kinetic properties of the precursor and its uptake and retention by G_{M1}-gangliosidosis fibroblasts were identical in

mutant and control cells. The excessive degradation of β-galactosidase in β-gal$^-$/neur$^-$ cells is therefore not due to abnormal properties of the precursor. Immunoprecipitation studies using antiserum raised against purified placental β-galactosidase on ^{3}H-leucine labelled cells showed that in normal fibroblasts an enzymically active 85,000-dalton precursor form is processed via a number of intermediates into mature 64,000-dalton β-galactosidase. In addition there is a non-enzymically active 32,000-dalton component and its 54,000-dalton precursor. In β-gal$^-$/neur$^-$ fibroblasts hardly any mature β-galactosidase was present and there was a complete lack of the 32,000-dalton component and of its precursor in the medium. Leupeptin caused accumulation of 85,000 precursor and of a partially processed 66,000-dalton form. When mutant cells were grown in the presence of a "corrective factor" purified from NH_4Cl stimulated cultures β-galactosidase and neuraminidase activities were restored to low control levels and after immunoprecipitation mature 64,000-dalton β-galactosidase accumulated. We propose that the combined β-galactosidase/neuraminidase deficiency is caused by a defective 32,000-dalton glycoprotein which is normally required to protect β-galactosidase and neuraminidase against excessive intralysosomal degradation and to give these enzymes their full hydrolytic activity.

INTRODUCTION

Studies on acid β-galactosidase in human liver (1) and cultured fibroblasts (2) showed it to consist of a 70,000-dalton monomeric form and a high molecular weight aggregate of the same polypeptide (600,000 to 800,000 dalton). Since the observation by Okada and O'Brien (3) that the autosomal recessive disease G_{M1}-gangliosidosis is due to a β-galactosidase deficiency, a variety of

patients with infantile, juvenile and adult forms of this disease have been reported (4-7). Analyses of interspecies hybrids indicated that the β-galactosidase deficiency in the various forms of G_{M1}-gangliosidosis is based on a mutation of a structural gene on chromosome 3 coding for the enzyme polypeptide (8).

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

A lack of neuraminidase activity can, however, also occur without any abnormality of β-galactosidase as in various types of patients with Sialidosis (formerly classified as Mucolipidosis I) (12-15). Complementation studies after somatic cell hybridization (10) indicated that three different gene mutations are responsible for the enzyme deficiencies in G_{M1}-gangliosidosis ($\beta\text{-gal}^-$), Sialidosis ($neur^-$) and combined β-galactosidase and neuraminidase deficiency ($\beta\text{-gal}^-/neur^-$).

The turnover time of β-galactosidase has been measured in different cell strains (16, 17). In normal fibroblasts and in G_{M1}-gangliosidosis cells the enzyme has a half-life of approximately 10 days, whereas in $\beta\text{-gal}^-/ neur^-$ fibroblasts it is less than 24 hours. Subsequent studies revealed that this reduction is due to enhanced degradation of β-galactosidase that is synthesised at a normal rate (18). It was also shown that both the β-galactosidase and neuraminidase activities in $\beta\text{-gal}^-/neur^-$ fibroblasts could be restored by a "corrective factor" of a glycoprotein nature that is produced by normal fibroblasts and other mutant cells, including

G_{M1}-gangliosidosis fibroblasts (19). Since the latter do not contain any measurable amount of β-galactosidase, a convenient source of this "factor" is the medium obtained when these mutant cells are cultured in the presence of ammonium chloride. It is well documented (20-22) that NH_4Cl diverts newly synthesized precursor forms of glyco proteins into the medium thus enhancing their secretion.

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

MATERIALS AND METHODS

Cell culture

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

Preparation and assay of NH_4Cl-induced secretions

Normal culture medium above confluent cultures was replaced by serum-free medium supplemented with NH_4Cl at a final concentration of 10 mM. Two days later the medium was collected, centrifuged (90 g, 5 min) and enzymic activities measured in aliquots of 20 µl. The cells were harvested by trypsinization, rinsed in saline and disrupted in distilled water. Enzymic analyses were carried out using the appropriate 4-methylumbelliferyl substrates (Koch-Light) as described by Galjaard (25).

Enzyme purification

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

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

Uptake studies

Cells were seeded at near confluency in 24 well plates (Costar) and cultured for 3-4 days. The above purified β-galactosidase (2.5×10^{-5} units) was added to the medium (one unit hydrolyzes 1 µmol of 4-methylumbelliferyl-β-D-

galactopyranoside per min). After 16 hours incubation in the presence of exogenous enzyme, the cells were cultured in fresh medium for two days prior to analysis. Where appropriate mannose-6-phosphate was used at a final concentration of 1 mM.

Immunoprecipitation of labelled β-galactosidase

Immunoprecipitation of labelled β-galactosidase from normal and mutant fibroblasts was carried out using purified specific immunoglobulins immobilized to sepharose-4B beads. The rabbit antiserum used for their production was raised against a highly purified human placental β-galactosidase prepared by affinity chromatography. It was virtually homogenous and showed one band of 64,000 dalton on polyacrylamide gel electrophoresis corresponding to the known size of mature human β-galactosidase with traces of a smaller molecular weight band of 30,000 dalton. The amount of coupled IgG that precipitated 95% of the β-galactosidase in the cells was judged from a previously determined enzyme activity/IgG ratio.

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

Polyacrylamide gel electrophoresis - Fluorography

Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed on 12% slab gels according to Laemmli (26), except that the ratio of acrylamide to methylene-bisacrylamide was changed to 30: 0.312. The gels were prepared for fluorography as previously described (27). (^{14}C)Methylated protein molecular weight standards were: phosphorylase B, 92,000 dalton; bovine serum albumin, 69,000 dalton; ovalbumin, 46,000 dalton; carbonic anhydrase, 30,000 dalton (Amersham Radiochemical Center).

Protease inhibition

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

Correction studies

The "corrective factor" from G_{M1}-gangliosidosis fibroblasts was prepared as previously described (19). β-Gal$^-$/neur$^-$ fibroblasts were incubated with concentrated "corrective factor" for two days in the presence of ^{3}H-leucine and the radiolabelled products were then examined by SDS electrophoresis.

RESULTS

Since the β-galactosidase deficiency in β-gal$^-$/neur$^-$ cells is known to be due to enhanced degradation the effect of leupeptin, a thiol protease inhibitor, was studied. Table 1 shows that it is able to prevent an otherwise excessive rate of decomposition of β-galactosidase in cells of the β-gal$^-$/neur$^-$ strains. When these were grown for 5 days in the presence of leupeptin the intracellular β-galactosidase activity rose to low control

Table 1. EFFECT OF LEUPEPTIN ON THE ACTIVITY OF β-GALACTOSIDASE AND NEURAMINIDASE IN HUMAN MUTANT FIBROBLASTS

Type of cell strain	Enzyme activity* (nmoles/hr/mg protein)			
	β-GALACTOSIDASE		NEURAMINIDASE	
	endogenous	+leupeptin	endogenous	+leupeptin
CONTROL	565 (530-600)	595 (550-640)	90 (80-100)	110 (90-130)
COMBINED $\beta\text{-gal}^-/\text{neur}^-$ (1)	40 (25-54)	305 (210-400)	1.7 (0.4-3)	8.4 (4.8-12)
COMBINED $\beta\text{-gal}^-/\text{neur}^-$ (2)	47 (26-68)	315 (260-370)	0.9 (0.4-1.5)	1.2 (0.8-1.7)
ADULT G_{M1}-GANGLIOSIDOSIS	49 (45-53)	53 (50-57)	118 (100-137)	142 (120-165)
SIALIDOSIS	1037 (854-1340)	1234 (998-1470)	0.9 (0.6-1.2)	1.3 (0.6-2.0)

* Activity expressed as the mean of 5 separate experiments, range in parentheses.

The effect of leupeptin (0,02 mM) for 5 days on control fibroblasts was measured by comparisons in the same cell strain.

(1) and (2) refer to two different cell strains (21, 22).

values. No such effect could be demonstrated with G_{M1}-gangliosidosis fibroblasts. The neuraminidase activity was only slightly increased in one of the β-gal$^-$/neur$^-$ strains tested.

Addition of NH_4Cl to the medium above normal fibroblasts and β-gal$^-$/neur$^-$ cells resulted in a significant increase in the extracellular β-galactosidase which was otherwise barely measurable (Table 2). Neuraminidase activity could not be detected in medium above any of the cells tested. The amount of β-galactosidase activity produced was similar to that found in the corresponding secretions from control cells, or sialidosis fibroblasts. The ability of β-gal$^-$/neur$^-$ cells to synthesise β-galactosidase precursor is therefore not impaired.

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

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

Both preparations were taken up when administered to medium above G_{M1}-gangliosidosis fibroblasts, and this uptake could be strongly inhibited by the presence of 1 mM mannose-6-phosphate in the medium (Table 3). A similar amount of β-galactosidase was ingested after a 16 hr incubation period when either preparation was used, and the levels in the recipient cells remained unchanged when cultured for a further 48 hours in fresh medium. When, however, purified β-galactosidase precursor from control fibroblasts was administered to β-gal$^-$/neur$^-$ fibroblasts no increase in enzyme activity could be detected after a

Table 2. INTRACELLULAR AND EXTRACELLULAR ACTIVITIES OF β-GALACTOSIDASE MEASURED BEFORE AND AFTER NH_4Cl TREATMENT

Type of cell strain	Extracellular activity* (nmoles/hr/ml medium)		Intracellular activity (nmoles/hr/mg protein)	
	no addition	+ NH_4Cl	no addition	+ NH_4Cl
CONTROL	0.4	11.6	762	639
COMBINED β-gal$^-$/neur$^-$ (1)	0.3	10.0	50	53
COMBINED β-gal$^-$/neur$^-$ (2)	0.3	14.7	46	62
INFANTILE G_{M1}-GANGLIOSIDOSIS	N.D.	0.2	2	1
ADULT G_{M1}-GANGLIOSIDOSIS	N.D.	1.1	36	20
SIALIDOSIS	0.4	11.9	706	520

* The values are the average of 5 separate experiments. The activity measured in 1 ml medium is derived from approximately 10^6 fibroblasts corresponding to about 0.3 mg of cellular protein.

N.D. = not detectable.

Table 3. UPTAKE OF β-GALACTOSIDASE PRECURSOR PURIFIED* FROM CONTROL MEDIUM AND β-GAL$^-$/NEUR$^-$ MEDIUM BY HUMAN MUTANT FIBROBLASTS

Type of cell strain	Incubation period	β-galactosidase activity (nmoles/hr/mg protein)		
		no addition	addition of β-gal. from control medium	addition of β-gal. from β-gal$^-$/neur$^-$ medium
G_{M1}-GANGLIOSIDOSIS (β-gal$^-$)	16 hrs	3.7	13.0	15.1
	16 hrs followed by 48 hrs "chase"	3.3	12.6	14.5
	16 hrs in the presence of 1 mM M-6-P	3.0	3.9	3.8
COMBINED β-gal$^-$/neur$^-$	16 hrs	40.5	42.0	39.5

* The purification of β-galactosidase secreted by control fibroblasts and β-gal$^-$/neur$^-$ cells after NH_4Cl treatment is described under "Materials and Methods".

Each value is the average of 2-3 separate experiments.

16-hour incubation period (Table 3). The excessive degradation of β-galactosidase in β-gal$^-$/neur$^-$ cells can therefore not be due to obvious mutant properties of the β-galactosidase precursor.

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

Control fibroblasts

After incubation for 24 hours in the presence of ^3H-leucine, control fibroblasts showed four radiolabelled immunoprecipitable components (Fig. 1, lane C). Two of these corresponded to the components of the placental preparations that showed a prominent 64,000-dalton form and traces of a 32,000-dalton form in Coomassie blue stained gels (Fig. 1, lane A). The 85,000-dalton precursor form that was also seen and the 64,000-dalton mature β-galactosidase are enzymically active. The 51,000 and 32,000 dalton components are inactive, as can also be deduced from their presence in G_{M1}-gangliosidosis fibroblasts which have less than 1% enzyme activity (to be published elsewhere). After NH_4Cl stimulation the immunoprecipitable forms in the medium had apparent molecular weights of 88,000 and 54,000 (Fig. 1, lanes D and E), while the cells under this treatment lacked the 64,000 and 32,000-dalton components. This suggests that the medium forms represent precursors of the mature 64,000 dalton β-galactosidase and of the non enzymically active 32,000-dalton component.

The sequence of events and relationship between these different constituents was clarified in pulse-chase

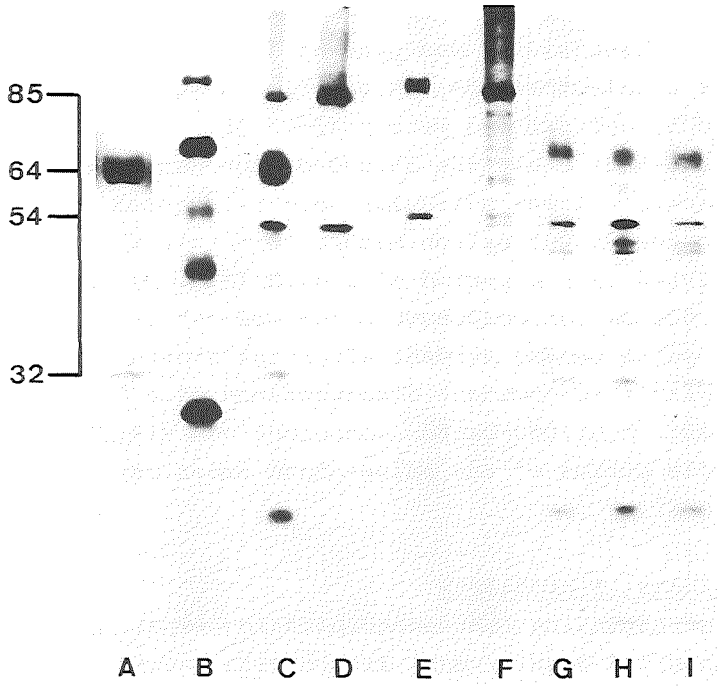


Fig. 1. Polyacrylamide gel electrophoresis in SDS of β-galactosidase from normal fibroblasts and culture medium immunoprecipitated with antiserum after ^{3}H leucine labelling.
Lane A, placental β-galactosidase (40 µg) stained with Coomassie blue; lane B, radiolabelled marker proteins; lane C, control fibroblasts labelled for 24 hrs; lane D, control fibroblasts labelled for 24 hrs in the presence of NH_4Cl; lane E, medium from control cells labelled for 24 hrs in the presence of NH_4Cl.
Lane F - lane I, pulse-labelling of β-galactosidase from control fibroblasts: lane F, 3 hrs labelling; lane G, 3 hrs labelling followed by 5 hrs chase in the presence of cold leucine; lane H, 3 hrs labelling and 20 hrs chase; lane I, 3 hrs labelling and 70 hrs chase. The experimental conditions are described under "Materials and Methods".

experiments. After a 3-hour pulse almost all immunoprecipitable radioactivity was present in the 85,000-dalton form of β-galactosidase; in addition a series of bands down to 50,000 daltons were visible (Fig. 1, lane F). After a 5-hour chase the 85,000-dalton band disappeared and transient lower molecular weight intermediates gave rise to the 64,000-dalton mature form that was fully established over a period of 3 days (Fig. 1, lanes G-I). The 32,000-dalton component which was not seen after the 3-hour pulse became evident after the shortest period of chase with a concomitant disappearance of a 54,000-dalton component. A 51,000-dalton component was visible during the whole period of the pulse-chase experiment (Fig. 1, lanes F-I).

β-Gal$^-$/neur$^-$ fibroblasts

Corresponding immunoprecipitation experiments with radiolabelled β-gal$^-$/neur$^-$ fibroblasts showed clear differences from control cells. Intracellularly the mature 64,000-dalton β-galactosidase was barely visible and the 32,000-dalton component was absent (Fig. 2, lane B). Like the controls 85,000 and 51,000-dalton bands were visible. After NH_4Cl stimulation the intracellular pattern remained unchanged. Extracellularly, unlike the control, the 54,000-dalton band was absent and the only detectable constituent had a M_r of 88,000 (Fig. 2, lanes C and D).

In pulse-chase experiments on β-gal$^-$/neur$^-$ cells the labelling pattern after a short pulse of 3 hours was similar to that in control cells. After a 5-hour chase, when 85,000-dalton precursor β-galactosidase was still present, a 66,000-dalton form appeared (Fig. 2, lanes E and F). During the subsequent period of chase both these bands disappeared but, unlike the controls, mature 64,000-dalton β-galactosidase did not accumulate in significant quantity (Fig. 2, lanes F-H). Another feature was the

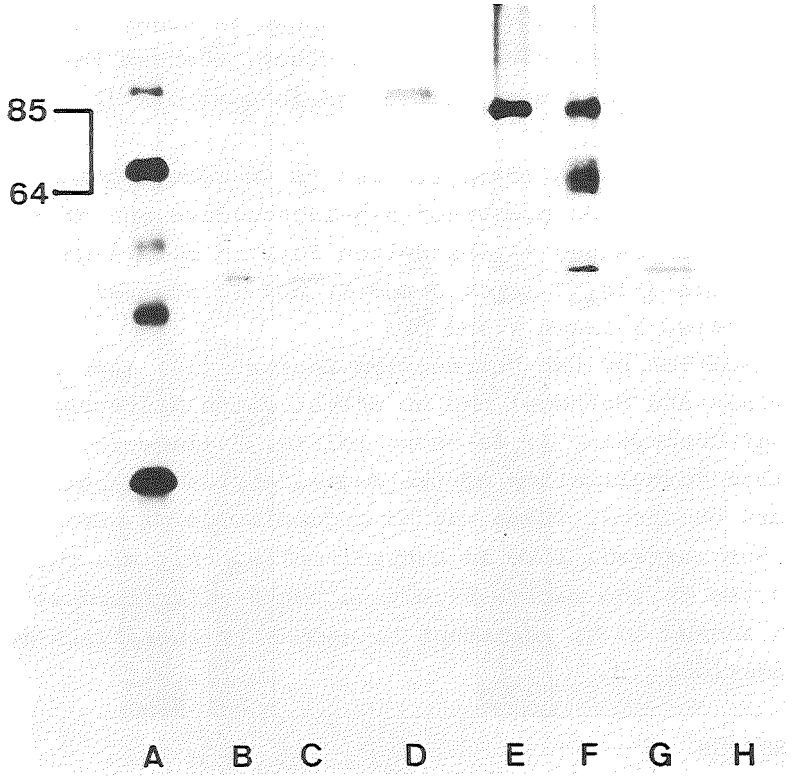


Fig. 2. Polyacrylamide gel electrophoresis in SDS of β-galactosidase from β-gal$^-$/neur$^-$ fibroblasts and culture medium immunoprecipitated with antiserum after ^{3}H leucine labelling.
Lane A, radiolabelled marker proteins; lane B, β-gal$^-$/neur$^-$ cells labelled for 24 hrs; lane C, β-gal$^-$/neur$^-$ cells labelled for 24 hrs in the presence of NH_4Cl; lane D, medium from β-gal$^-$/neur$^-$ cells labelled for 24 hrs in the presence of NH_4Cl.
Lane E - lane H, pulse-labelling of β-galactosidase from β-gal$^-$/neur$^-$ fibroblasts: lane E, 3 hrs labelling; lane F, 3 hrs labelling followed by 5 hrs chase; lane G, 3 hrs labelling and 20 hrs chase; lane H, 3 hrs labelling and 70 hrs chase.

absence of the 32,000-dalton component in β-gal$^-$/neur$^-$ cells during the whole period of chase, whereas the 51,000-dalton band was visible, as in controls (Fig. 2, lanes E-H).

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

Addition of the "corrective factor" (19) (see also Materials and Methods) had an effect quite different from that of leupeptin. As is shown in Fig. 3 (lane F), a complete normalization of the β-galactosidase labelling pattern occurred. There was no accumulation of precursor form but instead, like in control cells, this was rapidly converted to the mature 64,000-dalton β-galactosidase which seemed to be resistent to excessive intralysosomal degradation.

DISCUSSION

Previous studies (2, 7, 17) indicate that the 10-15% residual β-galactosidase activity in β-gal$^-$/neur$^-$ fibroblasts has the normal properties of mature 64,000-dalton β-galactosidase but that there is an excessive intralysosomal degradation of β-galactosidase shortening its half-life about tenfold (17, 18). The experiments described in the present paper show that in normal human fibroblasts mature 64,000-dalton β-galactosidase is produced via an 85,000-dalton precursor form. Similar observations have been made for β-galactosidase in mouse macrophages (28). We have shown that in β-gal$^-$/neur$^-$ fibroblasts the 85,000-dalton form is synthesized normally and that all properties tested are similar to those of precursor β-galactosidase in control cells. The excessive degradation of β-

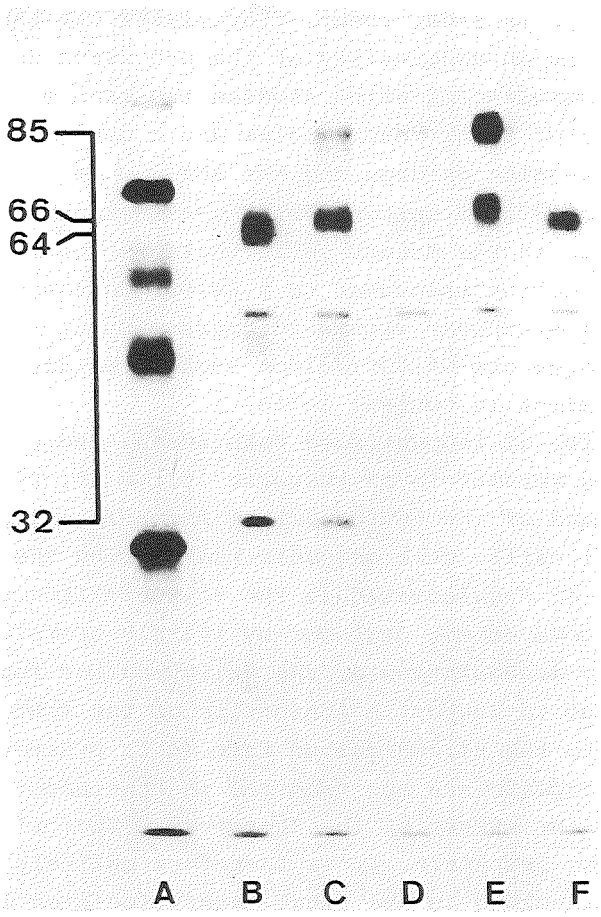


Fig. 3. Effect of leupeptin and of "corrective factor" on the SDS polyacrylamide pattern of immunoprecipitated radiolabelled β-galactosidase from control fibroblasts and $\beta\text{-gal}^-/\text{neur}^-$ cells.
Lane A, radiolabelled marker proteins; lane B, control fibroblasts; lane C, control fibroblasts plus leupeptin; lane D, $\beta\text{-gal}^-/\text{neur}^-$ cells; lane E, $\beta\text{-gal}^-/\text{neur}^-$ cells plus leupeptin; lane F, $\beta\text{-gal}^-/\text{neur}^-$ fibroblasts plus "corrective factor". The cells were grown in the presence of leupeptin or "factor" for two days.

galactosidase in β-gal$^-$/neur$^-$ fibroblasts can therefore not be due to an abnormality of the precursor form.

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

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

Our immunoprecipitation studies demonstrate a complete lack of a component with a molecular weight of 32,000 which is present in control cells (Figs. 1 and 2) and in other mutant cells (to be published). Similarly in the culture medium above β-gal$^-$/neur$^-$ cells there is an

absence of a 54,000-dalton component. Together with the results of pulse-chase experiments, this indicates that the latter larger molecular weight form is a precursor of the 32,000-dalton component. The exact relationship between intracellular 51,000 and 54,000-dalton forms is not yet understood. Neither of the components mentioned above have β-galactosidase activity but apparently they are co-purified with the precursor and mature forms of β-galactosidase.

We propose that the 32,000-dalton component is the genetically defective factor causing the combined β-galactosidase/neuraminidase deficiency and is identical to the "corrective factor" described by Hoogeveen et al. (19). This "corrective factor" could be precipitated with high concentration of the antiserum used in the present immunoprecipitation studies. Addition of the factor (Fig. 3) results in a complete normalization of the β-galactosidase labelling pattern. Unlike the effect of leupeptin, it allows complete processing of the precursor form to a 64,000-dalton mature β-galactosidase which can accumulate normally.

We are now studying the exact nature of the 32,000-dalton component, which we already know is a glycoprotein that is lacking in cells from patients with "I-cell" disease. It apparently plays a role in a final intralysosomal step leading to protection of β-galactosidase against excessive proteolytic degradation. It also stabilizes neuraminidase but, unlike β-galactosidase, which can be stabilised by protease inhibition, the presence of the 32,000-dalton glycoprotein seems to be essential to allow neuraminidase its catalytic activity.

It may well be that the 32,000-dalton glycoprotein normally is required to unite β-galactosidase monomers and neuraminidase in a complex attaching it to the lysosomal membrane. Further studies on the nature of this mutation

which affects the protection of lysosomal enzymes against the aggression of neighbouring "colleague enzymes" are likely to give more insight in the normal cell biology of lysosomal enzymes.

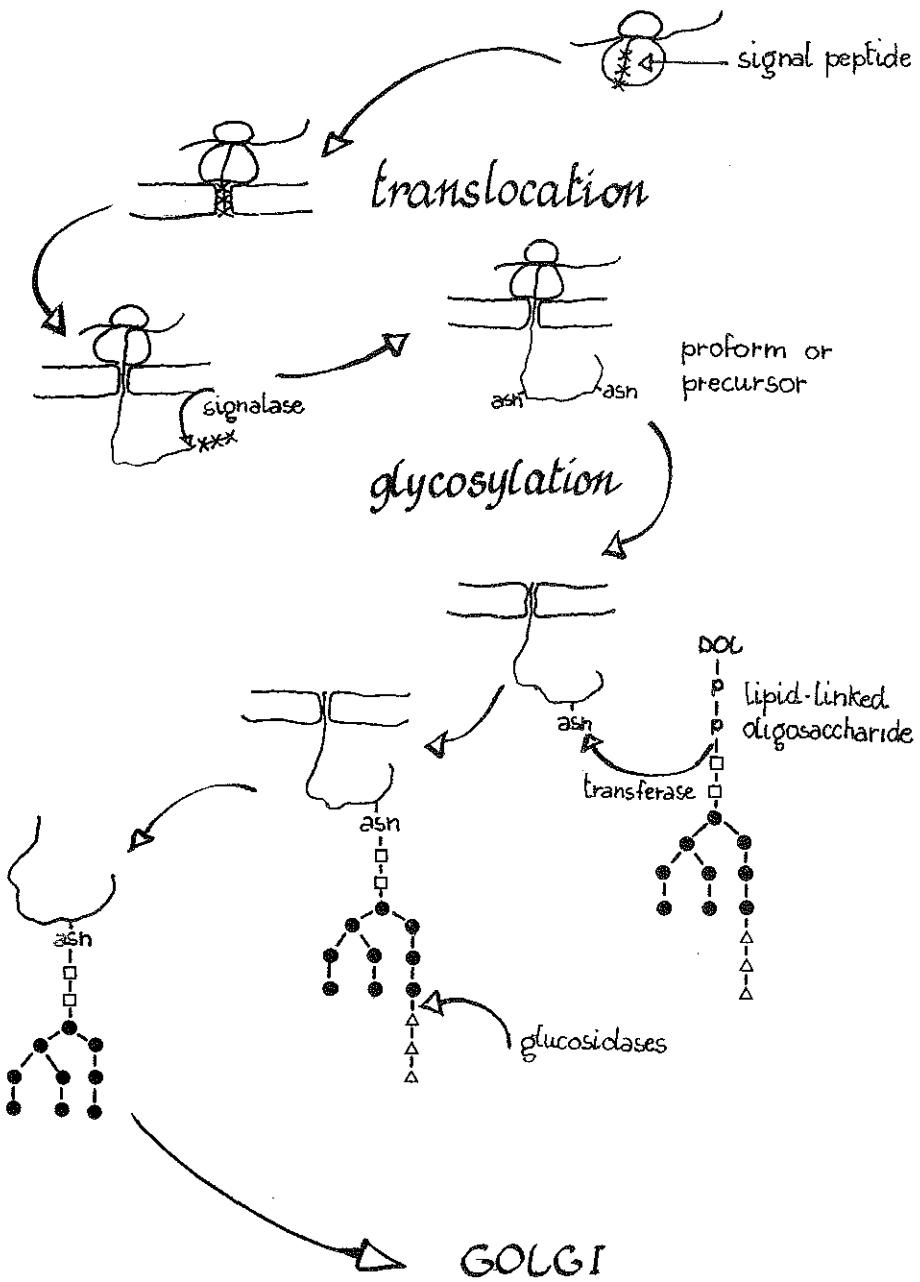
REFERENCES

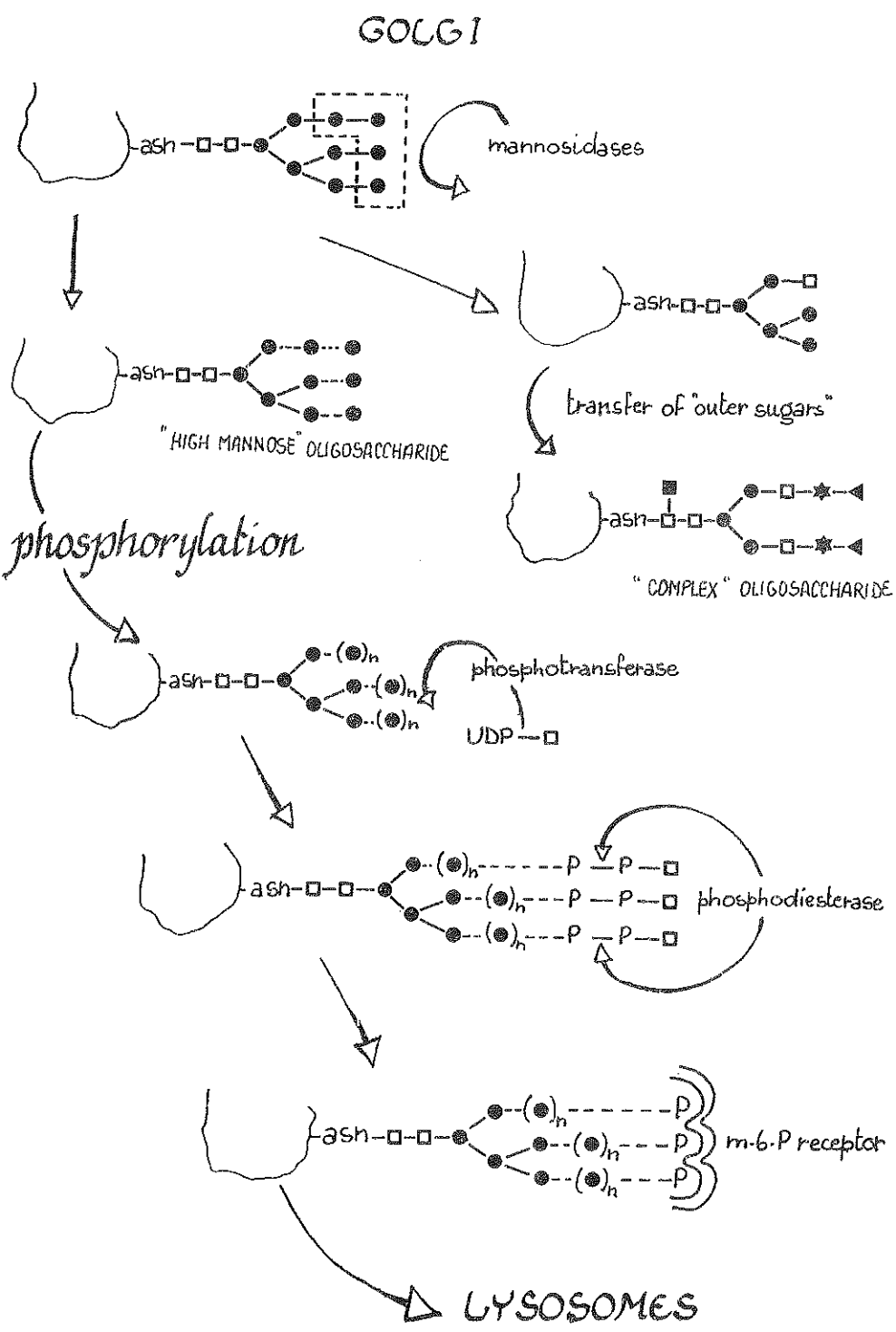
1. Norden, A.G.W., Tennant, L.L. & O'Brien, J.S. (1974) J. Biol. Chem. 249, 7969-7976.
2. Hoeksema, H.L., Van Diggelen, O.P. & Galjaard, H. (1979) Biochim. Biophys. Acta 566, 72-79.
3. Okada, S. & O'Brien, J.S. (1968) Science 160, 1002-1004.
4. Suzuki, Y., Nakamura, H., Fukuoka, K., Shimada, Y. and Uono, M. (1977) Hum. Genet. 36, 219-229.
5. O'Brien, J.S. (1978) in The Metabolic Basis of Inherited Disease, eds. Stanbury, J.B., Wyngaarden, J.B. & Frederickson, D.S. (McGraw-Hill, New York) pp. 841-865.
6. Lowden, J.A. & O'Brien, J.S. (1979) Am. J. Hum. Genet. 31, 1-18.
7. Galjaard, H., Hoogeveen, A., Verheijen, F., Van Diggelen, O.P., Konings, A., D'Azzo, A. and Reuser, A.J.J. (1981) in Perspectives in Inherited Metabolic Disease (Edi Ermes, Milan) Vol. 4, pp. 317-333.
8. Hoeksema, H.L., De Wit, J. & Westerveld, A. (1980) Hum. Genet. 53, 241-247.
9. Wenger, D.A., Tarby, T.J. & Wharton, C. (1978) Biochem. Biophys. Res. Commun. 82, 589-595.
10. Hoogeveen, A.T., Verheijen, F.W., D'Azzo, A. & Galjaard, H. (1980) Nature 285, 500-502.
11. Galjaard, H. & Reuser, A.J.J. (1977) in The Cultured Cell and Inherited Metabolic Disease, eds. Harkness R.A. & Cockburn, F. (MTP Press, Lancaster) pp. 139-160.

12. Cantz, M., Gehler, J. & Spranger, J.W. (1977) Biochem. Biophys. Res. Commun. 74, 732-738.
13. Thomas, G.H., Tiller, G.E., Reynolds, L.W., Miller, C.S. & Bace, J.W. (1976) Biochem. Biophys. Res. Commun. 71, 188-194.
14. Thomas, G.H., Tipton, R.E., Ch'ien, L.T., Reynolds, L.W. & Miller, C. (1978) Clin. Genet. 13, 369-379.
15. Durand, P., Gatti, R., Cavalieri, S., Borrone, C., Tondeur, M., Michalski, J.C. & Strecker, G. (1977) Helv. Paediat. Acta 32, 391-400.
16. Van Diggelen, O.P., Galjaard, H., Sinnott, M.L. & Smith, P.J. (1980) Biochem. J. 188, 337-343.
17. Van Diggelen, O.P., Schram, A.W., Sinnott, M.L., Smith, P.J., Robinson, D. & Galjaard, H. (1981) Biochem. J. 200, 143-151.
18. Van Diggelen, O.P., Hoogeveen, A.T., Smith, P.J., Reuser, A.J.J. & Galjaard, H. Biochim. Biophys. Acta, in press.
19. Hoogeveen, A., D'Azzo, A., Brossmer, R. & Galjaard, H. (1981) Biochem. Biophys. Res. Commun. 103, 292-300.
20. Hasilik, A. & Neufeld, E.F.(1980) J. Biol. Chem. 255, 4937-4945.
21. Gonzalez-Noriega, A., Grubb, J.H., Talkad, V. & Sly, W.S. (1980) J. Cell Biol. 85, 839-852.
22. Sly, W.S., Natowicz, M., Gonzalez-Noriega, A., Grubb, J.H. & Fischer, H.D. (1981) in Lysosomes and Lysosomal Storage Diseases, eds. Callahan, J.W. & Lowden, G.A. (Raven Press, New York) pp. 131-146.
23. Andria, G., Del Giudice, A. & Reuser, A.J.J. (1978) Clin. Genet. 14, 16-23.
24. Kleijer, W.J., Hoogeveen, A., Verheijen, F.W., Niermeijer, M.F., Galjaard, H., O'Brien, J.S. & Warner, T.G. (1979) Clin. Genet. 16, 60-61.

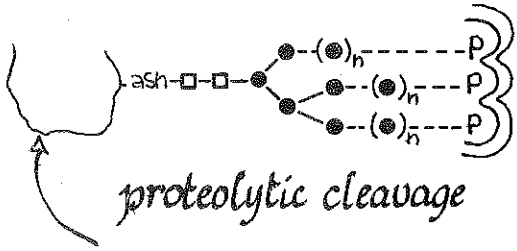
25. Galjaard, H. (1980) in Genetic Metabolic Diseases (Elsevier-North-Holland Biomed. Press) pp. 817, 825.
26. Laemmli, U.K. (1970) Nature 227, 680-685.
27. Bonner, W.M. & Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88.
28. Skudlarek, M.D. & Swank, R.T. (1979) J. Biol. Chem. 254, 9939-9942.
29. Frisch, A. & Neufeld, E.F. (1981) J. Biol. Chem. 256, 8242-8246.

Fig. 1 : processing of a lysosomal enzyme.

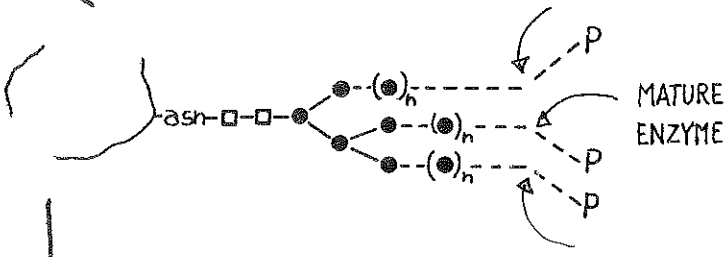



GOLGI
asn
mannosidases
"HIGH MANNOSE" OLIGOSACCHARIDE
transfer of "outer sugars"
"COMPLEX" OLIGOSACCHARIDE
phosphorylation
phosphotransferase
UDP
phosphodiesterase
m-6-P receptor
LYSOSOMES

LYSOSOMES



proteolytic cleavage



a association with "protective" proteins
b aggregation
c conformational changes
d association with cell components

proteolytic degradation

- □ N-acetylglucosamine
- ● mannose
- △ glucose
- ✱ galactose
- ◀ sialic acid
- ■ fucose