release and uptake of lysosomal enzymes studied in cultured cells

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE ERASMUS UNIVERSITEIT TE 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 11 JUNI 1980 DES NAMIDDAGS TE 3.00 UUR

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DIRKJE JORIJNTJE JOHANNA HALLEY

GEBOREN TE ROTTERDAM

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De elektronenmikroskopische opnamen op de kaft (W.J. Visser, Afdeling Celbiologie & Genetica, Erasmus Universiteit, Rotterdam) symboliseren een co-cultivatie van "I-cell" fibroblasten en normale fibroblasten van de mens.

I'm looking at the river
But I'm thinking of the sea

Randy Newman

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Chapter one

GENERAL INTRODUCTION

1.1 LYSOSOMES

"Lysosomes stand out in a unique fashion against all other subcellular constituents by their polymorphism and by the variety of processes, both physiological and pathological, in which they are implicated".

De Duve and Wattiaux 1966.

Lysosomes were first recognized by De Duve et al. (1955) after their isolation from rat liver by means of density gradient centrifugation. The early, biochemical and cytochemical, definitions of lysosomes refer to structures containing a variety of hydrolytic enzymes with an acidic pH optimum, which are delimited by a single unit membrane. These organelles have as their main function the digestion of intracellular material and of material that is taken up by the cell from the environment. Ultrastructural studies have contributed a great deal of knowledge on the dynamics of the lysosomal apparatus (for reviews see De Duve and Wattiaux 1966; Novikoff 1973, 1976; Holtzman 1976; Schellens et al. 1977). Different types of lysosomes were distinguished and they were found to have structural and functional associations with other organelles such as the endoplasmic reticulum, the Golgi apparatus,

and the plasma membrane. Vacuoles containing hydrolases that have not yet been engaged in digestion are called "primary" lysosomes. "Secondary" lysosomes are those in which both enzyme and substrate are present before, during, or after digestion. Lysosomes containing apparently undigestible material are named "residual bodies".

The material to be degraded may enter the cell by means of endocytosis or it may be derived from the sequestration of parts of the cell's own interior (autophagy) or from secretion products (crinophagy). All major classes of macromolecules present in the cell may be subject to lysosomal degradation. Proteins and glycoproteins, polysaccharides, nucleic acids, and glyco-, phospho-. and neutral lipids are the natural substrates for the lysosomal enzymes (reviewed by Vaes 1973, and Barrett and Heath 1977). Substrates and enzymes can be brought together by fusion of enzyme-containing vacuoles with endocytic or autophagic vacuoles or with secretion granules. In addition to fusion between different vacuoles direct binding of macromolecules at the cytoplasmic side of the lysosomal membrane and subsequent internalization has also been observed (Dean 1978).

In cells of multicellular organisms the functions of the lysosomal apparatus are manifold. De Duve and Wattiaux (1966) consider all functions of lysosomes as evolutionary derivatives of the original function of a cellular digestive system.

Digestion involves such diverse objects as macromolecules, cell organelles, whole cells and bacteria, during events of nutrition, differentiation , and cellular defence (reviewed by De Duve and Wattiaux 1966; Holtzman 1976). The role of the lysosomes in the turnover of proteins has recently been reviewed by Segal and Doyle (1978). Lysosomes have a regulatory function in the crinophagic destruction of excess secretion products (Farquhar 1969; Daems et. al. 1973; Neufeld and Cantz 1973; Ginsel 1979). Other regulatory functions involve partial degradation rather than complete digestion such as in the proteolytic activation of hormones (Novikoff 1976), the processing of intracellular liver proteins (Mortimore et al. 1978) and in the regulation of cholesterol synthesis by separating cholesterylesters from their carrier proteins (Goldstein et al. 1978,1979). It has also been proposed that the lysosomal enzymes themselves are subject to lysosomal processing (Goldstone and Koenig 1973; Vladutiu 1978; Neufeld et al. 1979; Hasilik and Neufeld 1980 a,b).

At present 60 or more enzymes have been attributed to the lysosomal system, which have their physiological substrates in all major classes of cellular macromolecules. Many of the lysosomal enzymes themselves are known or thought to be glycoproteins, as was first suggested by Barrett (1969). Most of the presently recognized lysosomal enzymes have an acidic pH optimum. With the exception of a few oxidoreductases all

lysosomal enzymes are hydrolases. The hydrolases can be classified according to the type of linkage they cleave in naturally occurring or artificial test substrate. Various carboxylic, phosphoric and sulphuric esters can be cleaved off by different hydrolytic enzymes. Peptide bonds can be cleaved near the end of polypeptides (by exopeptidases) or at a position away from the end of polypeptides (by endopeptidases). A major group of around 25 lysosomal enzymes act on glycosidic linkages (glycosidases) and their natural substrates are found among the cellular mucopolysaccharides, glycolipids and glycoproteins. The degrading capacities of lysosomal enzymes have been reviewed by Vaes (1973), Holtzman (1976) and Barrett and Heath (1977). About 30 human genetic disorders have now been associated with the deficiency of a lysosomal enzyme. Such a deficiency leads to lysosomal storage of the enzyme's undegraded substrate(s), to cellular pathology, and to clinical disease, which underlines the importance of unimpaired functioning of lysosomal enzymes.

1.2 LYSOSOMAL STORAGE DISEASES

Hers (1965) was the first to develop the concept of inborn lysosomal disease and to predict a variety of biochemical and pathological manifestations due to genetic lysosomal enzyme deficiencies. He did so at a time when only one such disease was known, i.e. glycogenosis type II, associated with the storage of glycogen as a result of a deficient activity of the lysosomal enzyme acid α-1,4-glucosidase (Pompe 1932; Cori 1957; Hers 1963). Since Hers' concept of inborn lysosomal diseases more than 30 genetic pathological conditions resulting from a deficiency of one (or several) of the lysosomal enzymes have been identified in man (Hers and Van Hoof 1973; Stanbury et al. 1978; Galjaard 1980).

Degrading enzymes usually have a relatively broad specificity (Hers 1973), which implies that the malfunction of one of them often leads to the accumulation of more than one product. Autophagy, intracellular "pinocytosis" at the lysosomal membrane, and endocytosis of macromolecules from the cell's exterior may all contribute to the accumulation. The rate of accumulation will also depend on the type of cell concerned. In secretory cells, for instance, the production rate of certain products is high and crinophagy plays an important regulatory role. If, as a result of a genetic lysosomal enzyme deficiency, crinophagy is impaired, overloading of the lysosomal apparatus is relatively fast (Daems et al. 1973;

Neufeld and Cantz 1973).

The pathogenesis of lysosomal storage diseases is related to the nature of the stored product and the rate of its accumulation. Excessive storage in a particular cell type will eventually lead to cellular death and to malfunction of organs. Gangliosides are normal constituents of nerve cell membranes. When the breakdown of gangliosides is impaired - as is the case in G_{M1}- and G_{M2}-gangliosidosis - the occurrence of the most striking pathological alterations in the central nervous system is accompanied by progressive psychomotor retardation. In glycogenosis type II (Pompe's disease) the accumulation of glycogen as a result of the deficiency of acid α-1,4-glucosidase is considered to cause the severe malfunctions of muscles. Although a number of clinical symptoms may be explained by pathological and biochemical findings, there is often not a uniform clinical picture among different patients with the same enzyme deficiency. In patients suffering from the infantile form of Pompe's disease, for instance, both skeletal and cardiac muscle are pathologically and clinically involved. In other patients, with the adult form of the disease, however, cardiac symptoms are virtually absent. The differences in clinical symptoms have been correlated with different levels of residual α-glucosidase activity in these patients (Mehler and Di Mauro 1977; Reuser 1977; Loonen 1979). Complementation studies using somatic cell hybridization techniques did not provide evidence for

different gene mutations in the infantile and adult forms (Reuser 1977). A similar situation exists in G_{M1}-gangliosidosis, where adult variant patients have been described with milder clinical symptoms than the classical form (Suzuki et al. 1977). Also in these cases a higher residual enzyme (β-galactosidase) activity was found, but complementation analysis did not reveal a different gene mutation (Suzuki et al. 1980). These examples illustrate the complex relationship between an enzyme defect and the clinical manifestations in the patients.

Genetic, biochemical or clinical heterogeneity (for recent reviews, see Galjaard and Reuser 1977; Reuser 1977; Stanbury et al. 1978; Hoeksema 1979 and Galjaard 1980) has now been observed in most lysosomal storage diseases. The study of organs and cultured fibroblasts from patients with different variants of "the same" disease may contribute significantly to our insight in the pathogenesis. A close collaboration between various disciplines and the application of different methods of investigation is required before the question can be answered why the deficiency of a lysosomal enzyme sometimes leads to a rapidly progressive fatal disease and in other instances to a late onset disease with much milder symptoms. The study of fibroblasts from patients with such different clinical courses and the analysis of the kinetic and molecular properties of residual enzyme activity in some variants may also be valuable as a background for enzyme re-

placement therapy. A major question is the minimally required intracellular activity of each lysosomal enzyme to prevent accumulation of particular substrates. In the experimental work to be described in this thesis we have used cultured fibroblasts from patients with different lysosomal storage diseases, i.e. G_{M1}- and G_{M2}-gangliosidoses, glycogenosis II, mannosidosis, mucopolysaccharidosis VII and "I-cell" disease. In this general introduction a brief description of the main clinical features and biochemical abnormalities and a few genetic data of each of these diseases will be given (see also Table 1).

G_{M2}-gangliosidosis

Patients with the classical form of this disease (Tay-Sachs disease) usually show symptoms during the first six months of life. The child is apathetic, hypotonic and a sharp sound initiates a rapid extension of the arms. During the second half of the first year motor weakness, mental deterioration and a cherry-red spot in the retina become apparent. Blindness, deafness, megalocephaly and finally decerebrate rigidity occur by two years and most patients die of bronchopneumonia before the age of three.

The biochemical abnormalities consist of accumulation of G_{M2}-gangliosides mainly in the central and peripheral nervous system, as a result of a genetic deficiency of N-acetyl-β-D-

TABLE I

CLINICAL FEATURES AND BIOCHEMICAL ABNORMALITIES IN SOME LYSOSOMAL STORAGE DISEASES.

	Main clinical symptoms	Stored material	Deficient enzyme(s)	Recent reviews
Glycogenosis type II (Pompe's disease, generalized glycogenosis)	Varying from progressive muscular weakness in the adult form to cardiac enlargement, profound muscular hypotonia and (not in all cases) neurological defects in the infantile form, where cardiorespiratory failure usually leads to death in the first year of life.	Glycogen in muscle, heart, central nervous system, liver, heterosaccharides in muscle, heart and brain.	α-1,4-glucosidase EC. 3.2.1.20	Howell, 1978
GM_1-gangliosidosis (Landing's disease, pseudo-Hurler disease, generalized gangliosidosis)	Varying degrees of psychomotor retardation, bone deformities and hepatosplenomegaly with blindness and deafness in infantile, juvenile, and adult types. Patients with the infantile form die mostly in the second year of life.	GM_1-ganglioside and asialo GM_1-ganglioside in brain, spleen and liver. Galactosecontaining mucopolysaccharides and glycoproteins in visceral tissues.	GM_1-β-galactosidase EC. 3.2.1.23	Adachi et al., 1978; O'Brien, 1978; Sandhoff and Christomanou, 1979
GM_2-gangliosidosis type 2 (type 0) (Sandhoff's disease, Sandhoff-Jatzkewitz disease)	Progressive psychomotor deterioration with blindness and developing spasticity. Death due to bronchopneumonia has usually occurred by 3 years of age.	GM_2-ganglioside and asialo GM_2-ganglioside and globoside, glycoproteins and mucopolysaccharides in brain and visceral tissues.	N-acetyl-β-D-glucosaminidases A and B EC 3.2.1.30	
Mannosidosis	Psychomotor retardation, stiff joints, skeletal deformities, hearing loss and hypotonia.	Mannose-rich oligosaccharides in liver, brain, spleen and excreted in the urine.	α-D-mannosidase EC. 3.2.1.24	Vidgoff et al., 1977
Mucopolysaccharidosis VII (Sly's disease, β-glucuronidase deficiency disease)	Six cases have now been described with different phenotypes. Different degrees of bone deformities; mental retardation in some cases, but not in all; repeated episodes of pneumonia in the first year of life in several cases.	Varying types and levels of mucopolysaccharides are stored and excreted.	β-glucuronidase EC. 3.2.1.31	McKusick et al., 1978
Mucolipidosis II (I-cell disease)	Early-onset skeletal changes and progressive psychomotor deterioration. Most of the patients die by the age of 5 or 6 years.	Mucopolyssaccharides and glycolipids. No excessive urinary mucopolysaccharides.	Multiple intracellular deficiency and extracellular accumulation of: α-L-iduronidase, iduronate sulfatase, β-glucuronidase, N-acetyl-β-D-glucosaminidase, arylsulfatase, β-galactosidase, α-mannosidase, α-L-fucosidase.	

hexosaminidase A (Okada and O'Brien 1969). The elucidation of this defect became possible after the demonstration by Robinson and Stirling (1968) that the lysosomal hexosaminidases consist of at least two isoenzymes: a thermolabile and more acidic A-form and a stable B-form which has a slower electrophoretic mobility. Later, in addition to those acidic isoenzymes a neutral hexosaminidase C was found (Hooghwinkel et al. 1972; Braidman et al. 1974; Swallow et al. 1976) and an acidic S-form which has about the same fast electrophoretic mobility but is immunologically not related to the neutral form (Ikonne and Desnick 1974; Beutler et al. 1975; Ikonne et al. 1975; Reuser and Galjaard 1976).

Gene localisation studies using rodent-human cell hybrids (for review see Hoeksema 1979) and molecular analysis (Beutler and Kuhl 1975) have subsequently shown that hexosaminidase S is a homopolymer of an α-subunit, hexosaminidase B a homopolymer of β-subunits, and hexosaminidase A is a heteropolymer of α- and β-subunits (for reviews, see Galjaard and Reuser 1977; O'Brien 1978; Galjaard 1980).

Another variant of G_{M2}-gangliosidosis is Sandhoff's disease, where the patients have similar clinical features as in Tay-Sachs disease but the enzyme defect involves both hexosaminidase A and B. In addition to G_{M2}-ganglioside accumulation there is storage of asialo-G_{M2}-ganglioside and of globosides and visceral organs are also involved.

Several patients with Tay-Sachs disease and Sandhoff's disease have been reported to have later onset and milder clinical forms and some of them have a higher residual hexosaminidase activity than patients with the classical type of the disease. Interesting observations have also been made in a number of adults who show no clinical features but nevertheless have a clearly reduced hexosaminidase activity (Dreyfus et al. 1975; O'Brien et al. 1977). These individuals most likely are compound heterozygotes where the enzyme has normal activity towards the natural substrate(s) in vivo and decreased activity towards artificial substrate in the in vitro assay.

In a few patients with massive ganglioside accumulation and decreased hexosaminidase activity the primary defect is not affecting the hexosaminidase enzyme polypeptides but an activator that is required for the in vivo interaction between the enzyme and its ganglioside substrate (Conzelmann and Sandhoff 1978).

Somatic cell hybridization studies and molecular analyses have now provided definite evidence that Tay-Sachs disease is due to a mutation affecting the α-subunit, coded for by a gene on chromosome nr.5, whereas Sandhoff disease is caused by a mutation of a structural gene on chromosome nr.15 coding for the β-subunit (for reviews, see O'Brien 1978; Hoeksema 1979; Galjaard 1980).

G_{M1}-gangliosidosis

Patients with the progressive infantile form and those with the somewhat milder juvenile form have been detected first (for review, see O'Brien 1972). In the most severe type the newborn has feeding difficulties, is inactive and has coarse facial features such as bulging forehead, depressed nasal bridge, low set ears, hypertrophied gums, macroglossia and short neck. Many, but not all patients have a macular cherry-red spot. During the first half year of life hepatosplenomegaly is noted as well as skeletal dysostosis multiplex. Later on, mental and motor development are retarded and subsequently rapidly progressing deterioration leads to death within two years. In patients with the juvenile type of G_{M1}-gangliosidosis psychomotor deterioration becomes apparent at 1 year of age or later and visceromegaly and skeletal deformities are absent. The progression of cerebral degeneration is slower and death usually occurs between 4 and 10 years of age (see for review O'Brien 1978; Galjaard 1980).

Several adults with G_{M1}-gangliosidosis have been reported (Suzuki et al. 1977; O'Brien 1978; Galjaard 1980). Most of them have visual impairment, ataxia, myoclonus, macular cherry-red spots and subnormal intelligence.

The nervous system and visceral organs of all patients show intralysosomal storage of G_{M1}-gangliosides and some cell types show accumulation of keratansulfate like mucopolysaccharides

as well. The responsible enzymic defect is a β-galactosidase deficiency (Okada and O'Brien 1968). A few patients have been reported whose fibroblasts show a combined deficiency of β-galactosidase and neuraminidase (Wenger et al. 1978; Lowden and O'Brien 1979; Hoogeveen et al. 1980). Gel filtration studies (Hoeksema 1979) and somatic cell hybridization studies (Galjaard et al. 1975; De Wit-Verbeek et al. 1978) suggested that this combined deficiency is based on a post-translational defect in the processing of β-galactosidase molecules whereas the different variants of G_{M1}-gangliosidosis result from structural mutations affecting the β-galactosidase polypeptide (for review, see Hoeksema 1979).

Glycogenosis II

Patients with the classical form of this glycogen storage disease do not grow well, show profound hypotonia and lie in flaccid position. Cardiac enlargement becomes apparent and often hepatosplenomegaly. Neurological symptoms may develop and finally most patients die in their first years of life because of cardiorespiratory failure. These clinical features are associated with massive glycogen storage, and they are caused by a deficiency of the lysosomal enzyme acid α-1.4 glucosidase (for reviews, see Hers and de Barsy 1973).

Several adults have been discovered who also

have an acid glucosidase deficiency but who only show manifestations of muscular weakness (Engel et al. 1973; Reuser 1977; Loonen 1979). The molecular basis for this heterogeneity has not yet been resolved, but fibroblasts from patients with the progressive infantile form of glycogenosis II do not produce detectable numbers of acid α-glucosidase molecules, whereas in the adult form the number of enzyme molecules is reduced (Reuser et al. 1978).

Mannosidosis

The clinical features of the patients with mannosidosis, so far described, have been quite similar. During the first year of life recurrent infections, coarsening of facial features, hepatosplenomegaly and psychomotor retardation become apparent. In some patients skeletal abnormalities are present whereas deafness and speech impairment are general features. Most patients have died before school age (for review, see Öckerman 1973) but a few others have grown up onto puberty and adult age (Booth et al. 1976; Kistler et al. 1977).

Cells from the nervous system or from visceral organs show intralysosomal storage of mannose containing oligosaccharides which are also secreted in excessive amounts in the urine. The molecular basis of this storage is a deficiency of acidic α-mannosidase (Öckerman 1967a;

Carroll et al. 1972; Hultberg et al. 1975). The residual enzyme activity which is often found is mostly due to activity of a neutral mannosidase, which enzyme has no immunological relationship with the lysosomal forms (Phillips et al. 1976). Kinetic studies pointed to a mutation in the structural gene (Beaudet and Nichols 1976; Mersman and Buddecke 1977) and it has also been suggested that the abnormal α-mannosidase is not efficiently recognized and taken up by cultured fibroblasts. On the basis of a low intracellular α-mannosidase activity in mutant cells and a relatively high activity in the culture medium above these cells Hultberg and Masson (1977) postulated a localized recognition defect comparable with that assumed for many hydrolases in "I-cell" disease.

Further studies on the exact molecular background of an α-mannosidase deficiency and on the effect of enzyme therapy are facilitated by the availability of experimental animals (Angus cattle) with mannosidosis (Jolly 1975).

Mucopolysaccharidosis type VII

Patients with the recently recognized lysosomal storage disease mucopolysaccharidosis VII (Sly et al. 1973) have coarse facial features, skeletal abnormalities, hepatosplenomegaly, retarded growth, gibbing deformity and psychomotor retardation. No neurological abnormalities have been

noted and the mental retardation has been variable among different patients. Some of the patients have attended school before deteriorating (Beaudet et al. 1975).

Storage of acid mucopolysaccharides (glycosaminoglycans) and excessive urinary excretion is a feature of all mucopolysaccharidoses and in this type VII the main types are chondroitin-4-sulfate and chondroitin-6-sulfate (Sly et al. 1973). Dermatan sulfate and heparan sulfate have, however, also been found in the urine (Gehler et al. 1974; Beaudet et al. 1975). The molecular defect is a deficiency of ß-glucuronidase which interferes with the degradation of glucuronic acid containing macromolecules (Hall et al. 1973). Fibroblasts from patients usually have a very low residual activity but immunological studies revealed the presence of normal amounts of cross-reactive material (Bell et al. 1977). Kinetic studies also point to a structural mutation interfering with the catalytic activity of the enzyme (see also reviews by Neufeld 1977 and McKusick et al. 1978).

Mucolipidosis II ("I-cell disease")

Patients with this syndrome show much resemblance with those with mucopolysaccharidosis I (Hurler's syndrome). They have severe clinical and radiological features and psychomotor retardation but there is no mucopolysaccharidiuria (Leroy and de

Mars 1967). Soon after birth the facial features are coarsening, there is hypotonia and recurrent infections. At about one year linear growth decelerates, radiological examination shows dysostosis multiplex, and the skin is thickened. Hepatomegaly occurs and psychomotor retardation progresses rapidly and patients usually die between two and eight years because of cardiorespiratory failure.

The most remarkable pathological manifestation is the extreme intralysosomal storage in cultured fibroblasts and in other cells of mesenchymal origin. Mucopolysaccharides, (glyco)lipids and oligosaccharides accumulate as a result of a multiple lysosomal enzyme deficiency (Leroy and Spranger 1970; Lightbody et al. 1971) and an increased activity of the same hydrolases is found in serum of the patients and in the medium above deficient cultured fibroblasts (Wiesmann et al. 1971a,b; Ellis et al. 1975). On the basis of studies with cultured "I-cells" and fibroblasts with a single lysosomal enzyme deficiency Hickman and Neufeld (1972) postulated a defective recognition marker which is common to many lysosomal enzymes. A large number of investigations have since been performed on the exact nature of the marker(s) that are involved in the recognition, uptake and intracellular retention of lysosomal enzymes (for review, see Sly and Stahl 1978). The results of these investigations and the most recent hypothesis that "I-cell" disease is caused by a defective processing of lysosomal enzymes

(Hasilik and Neufeld, 1980b) will be described in more detail in the last two sections of this chapter.

As is evident from the description of the various lysosomal storage diseases their clinical course is usually fatal. During the last decade several attempts have been made to substitute the deficient lysosomal enzyme by a normal enzyme. The results of these clinical trials and of model studies related to enzyme therapy will be discussed in the next section.

1.3 ENZYME THERAPY IN LYSOSOMAL STORAGE DISEASES

Substitution of the missing or defective gene product (enzyme replacement therapy) is a possible strategy for treatment of patients with inherited metabolic disorders, once the (primary) enzymatic defect has been established. Requisites for effective enzyme therapy, as summarized by Desnick et al. (1975), include a good enzyme technology (sufficient quantity and quality of the administered enzyme), appropriate enzyme administration (delivery of active enzyme to target tissues and subcellular sites), the availability of in vivo test systems and finally the demonstration of biochemical and clinical improvement after therapeutic evaluation. With respect to a correct localization of the administered enzyme in the appropriate subcellular compartment(s), lysosomal storage diseases seem particularly suitable as the normal route of proteins entering cells is by endocytosis and subsequent fusion of endocytic vacuoles with the lysosomal apparatus (De Duve 1964).

During the last decade many in vitro experiments have been performed, where purified enzyme preparations were fed to cultured fibroblasts with a deficiency of that particular enzyme (for review see Neufeld 1974; Neufeld et al. 1977; Von Figura 1977; Sly and Stahl 1978). As an alternative source of normal enzyme co-cultivations of enzyme deficient fibroblasts and normal cells or fibroblasts with another enzyme defect have

been performed. In several instances it has been shown that exogenous enzyme that is taken up by deficient cells is capable of degrading otherwise indigestible products stored in the lysosomes (Fratantoni et al. 1968, 1969; Porter et al. 1971; Hall et al. 1973; O'Brien et al. 1973; Distler et al. 1975; Cantz and Kresse 1974). Cell fractionation studies have recently confirmed that exogenous lysosomal enzymes are delivered to the lysosomes (Bach and Liebmann-Eisenberg 1979; Rome et al. 1979a).

However, clinical trials with enzyme therapy were less successful. The first attempt was by Baudhuin et al. (1964) who administered α-glucosidase from Aspergillus niger to a patient with glycogenosis type II. Although near normal levels of α-glucosidase were measured in the liver (the activity in muscle was not measured), there was no decrease of vacuolar glycogen in either liver or muscle. Many attempts at enzyme replacement therapy have since been made in different human lysosomal storage diseases and many problems have been encountered (see the reviews by Desnick et al. 1975,1976; and Gregoriadis and Dean 1979). Several strategies have been followed such as the administration of purified enzyme from various sources, infusion of whole blood or plasma, and the transplantation of cells, tissues, or whole organs.

The use of purified intravenously injected enzymes and blood or plasma has been limited by the short half-lives of the infused enzyme activ-

ities and by the predominant uptake of exogenous enzyme by cells in the liver (Schlesinger et al. 1976; Bearpark and Stirling 1977; Marinkovic and Gant 1978). Clinical improvements that have been reported in a few instances upon such treatment have been transient in a patient with Fabry's disease (Mapes et al. 1970) and in various patients with different types of mucopolysaccharidosis (DiFerrante et al. 1971; Dekaban et al. 1972; Erickson et al. 1972). Brady et al. (1974) described two patients with a relatively mild form of Gaucher's disease who showed persistent decreases of storage material after injection of human placental β-glucosidase. Recently, Håkansson (1979) has discussed the prospects of therapy in patients with a mild variant of Gaucher's disease, with special emphasis on the fact that most of the storage material is found in the spleen and in the liver.

Immunological complications may be another limitation in the use of exogenous enzymes, especially when the genetic defect is associated with the absence of crossreactive material (Boyer et al. 1973). One approach to avoid a rapid breakdown of injected enzyme has been the entrapment of enzymes in liposomes (Gregoriadis and Ryman 1972) or erythrocytes (Ihler et al. 1973). Although liposomes may provide some protection from inactivation in the circulation and may minimize immunological reactions, their present use seems limited,

since they were almost exclusively recovered from liver in experiments with animal models (Gregoriadis and Ryman 1972; Thorpe et al. 1975).

An alternative method to provide patients with a genetic defect with normal enzyme has been the transplantation of normal organs. Kidney transplantations have been applied in several patients with Fabry's disease, primarily as a treatment of renal failure. Improvement, both at the biochemical and clinical levels, was reported in some cases, but not in others (for review see Desnick et al. 1978). Renal transplantation has also been carried out in 2 patients with Gaucher's disease (Desnick et al. 1973; Håkansson 1979). Transplantation led to a slight reduction in the glucosylceramide concentration in erythrocytes of the former patient and a temporary decrease was found in the cerebroside level in the liver of the latter. Little or no effect was seen in patients with mucopolysaccharidoses after infusion of normal leukocytes (Knudsen et al. 1971; Moser et al. 1974), which may be related to the short lifespan of these cells. A more favourable situation might be the transplantation of cultured fibroblasts in patients with mucopolysaccharidosis II. Three patients have been described, who received fibroblasts from a histocompatible sib (Dean et al. 1975, 1979). Prolonged increases were noted in the activity of idurono-sulfate sulfatase and in the catabolism of heparan and dermatan sulfates in leukocytes, but no improvement of the patients' clinical condition occurred. The patients were, however,

mentally and physically severely affected at the beginning of the treatment, as has been the case in many of the other attempts at therapy. Altogether it seems too early for a conclusion about the possibillity of successful enzyme therapy; much more has to be learned from in vitro and in vivo model studies.

The tissue distribution of exogenous enzyme, either after tissue transplantation or after intravenous administration, and the long-term biochemical and pathological effects can best be studied in animal models. Several animal species with a lysosomal enzyme deficiency are now available, such as cattle with mannosidosis (Jolly et al.1976) mice with β-glucosidase- (Stephens et al. 1979) or β-glucuronidase deficiency (Thorpe et al. 1975) and cats with a β-galactosidase- (Rattazzi et al. 1979), an arylsulfatase- (Haskins et al. 1979a) or an α-L-iduronidase deficiency (Haskins et al. 1979b).

Recently the feasibility has been shown of influencing the in vivo fate of exogenous enzymes, either by enhancing the accessibility of the target organ or by modification of the enzyme. A major problem in the improvement of mental retardation as a result of brain damage in lysosomal storage diseases is the difficulty for enzymes in the circulation to cross the blood-brain barrier. A temporary modification of the blood-brain barrier was recently achieved in rats and cats and has enabled lysosomal enzymes to be taken up by brain tissue (Barranger et al. 1979; Rattazzi et al.

1979). Targeting of enzymes to specific tissues has been a subject of discussion since Ashwell and Morell discovered the specific recognition of galactosyl-terminal glycoproteins by hepatocytes (review: Ashwell and Morell 1974). As will be discussed in the next section different recognition markers have been discovered for various cell types. In principle targeting of enzymes seems possible by the attachment or uncovering of a specific carbohydrate marker (Furbish et al. 1978). A blockade of the uptake by liver may be achieved by means of a specific competitive inhibitor (Rattazzi et al. 1979). Also, enzyme carriers like liposomes and erythrocytes may be equipped with covalently coupled marker molecules to guide them to specific cell types in the body (cf. De Duve et al. 1978; Dale et al. 1979).

1.4 GLYCOPROTEINS AND RECOGNITION

Lysosomal enzymes are glycoproteins, which has been substantiated during the last few years by the analysis of the carbohydrate composition of a number of highly purified lysosomal hydrolases. α-L-Fucosidase (Alhadeff and Freeze 1977), acid phosphatase (Saini et al. 1978) and α-glucosidase (Belen'ky et al. 1979), which were isolated from liver, and β-glucuronidase (Brot et al. 1978), α-N-acetylglucosaminidase (Rührborn and Von Figura 1978) and hexosaminidase A and B (Freeze et al. 1979) purified from placenta were all found to contain mannose and N-acetylglucosamine residues, whereas the presence of fucose, galactose, glucose and sialic acid was demonstrated in some of these enzymes. The investigation of recognition and cellular uptake of lysosomal enzymes has benefitted a great deal from the studies on glycoprotein recognition in general. Recognition and uptake of glycoproteins have been studied in cells from various tissues and several receptor-ligand combinations have been identified with apparently diverse physiological significance. Ashwell and coworkers (for review see Ashwell and Morell 1974) discovered the first instance of receptor-mediated uptake of a glycoprotein by a mammalian cell. These authors demonstrated that desialylated plasma glycoproteins are rapidly cleared from the circulation of rats due to specific recognition by hepatocytes. The exposure of galactosyl residues upon removal of sialic acid from the glyco-

proteins was found to be crucial to this type of uptake, since the rapid clearance of asialoglycoproteins could be abolished by removal of the galactose groups from the molecules. Treatment of the cell surface with neuraminidase also diminished the rapid uptake of the asialoglycoproteins, which showed that sialic acid is an essential part of the hepatic cell membrane receptor. A glycoprotein receptor was subsequently isolated from rabbit liver and characterized (Hudgin et al. 1974). It has been suggested (Segal and Doyle 1978) that removal of sialic acid from serum glycoproteins uncovers galactosyl-termini to function as a "destruction marker", since the endocytosed molecules are rapidly degraded. Recently, it was shown that the majority of receptors are not located at the cell surface, but on membranes inside the cells (Tanabe et al. 1979). This may point to a more complicated function of this glycoprotein recognition system than merely the destruction of endocytosed plasma glycoproteins.

A second route for the transfer of certain glycoproteins from the circulation into the liver was first described by Stockert et al. (1976). This second uptake system was found to have specificity for glycoproteins terminating in either mannose or N-acetylglucosamine residues and it is located on reticuloendothelial cells. It is this system that accounts for the rapid clearance of intravenously administered lysosomal enzymes (Schlesinger et al. 1976; Stahl et al. 1976). The enzymes have, consequently, been shown to localize

in nonparenchymal liver cells, presumably Kupffer cells. The same receptor-mediated uptake system has recently been demonstrated in vitro on rat alveolar macrophages (Stahl et al. 1978). One possible role for the mannose/N-acetylglucosamine specific recognition would be to keep the plasma levels of lysosomal enzymes low (Sly and Stahl 1978). Other functions regarding intra- and intercellular transfer of lysosomal enzymes have not been excluded. Little is as yet known about the occurrence of receptors on intracellular membranes and the intracellular fate of lysosomal enzymes endocytosed by reticuloendothelial cells. It seems likely that the presence of pancreatic ribonuclease in hepatic Kupffer cells (Bartholeyns et al. 1975) is mediated by uptake via the mannose/N-acetylglucosamine receptor, which would illustrate the possibility that an enzyme is not immediately degraded after uptake by reticuloendothelial cells.

Neufeld and co-workers were first to demonstrate that lysosomal enzymes released from cultured skin fibroblasts, can subsequently be taken up by adjacent cells while remaining enzymically active (Fratantoni et al. 1968, 1969; Neufeld 1974; Neufeld et al. 1977). Specific recognition of extracellular enzymes by fibroblast surface receptors was deduced from experiments with fibroblasts from patients with mucolipidosis II ("I-cell disease") (Hickman and Neufeld 1972, Hickman et al. 1974). Lysosomal enzymes from the secretions of normal human fibroblasts could be taken up and retained by "I-cell" fibroblasts, but enzymes se-

creted by the latter cells failed to enter human fibroblasts. It was then postulated that the underlying defect of the multiple lysosomal enzyme deficiency in "I-cells" would be an abnormality of a recognition site that is common to those enzymes that are deficient in these cells. At the time of these experiments Neufeld et al. believed that the normal fate of lysosomal enzymes involves secretion and subsequent recognition and uptake before the hydrolases reach the lysosomes.

The recognition at the fibroblast surface was shown to involve carbohydrate moieties on the enzymes (Hickman et al. 1974). Later studies implicated phosphorylated mannose residues as the critical constituent, as the uptake of lysosomal hydrolases could be abolished by treatment of the enzyme with alkaline phosphatase, and mannose 6-phosphate and phosphorylated mannans appeared to be strong competitive inhibitors of enzyme assimilation (Kaplan et al. 1977; Sando and Neufeld 1977; Ullrich et al. 1978a). Chemical and enzymic analysis provided direct evidence for the presence of mannose 6-phosphate in high mannose type oligosaccharides from human urine α-N-acetylglucosaminidase (Von Figura and Klein 1979), bovine testicular β-galactosidase (Distler et al. 1979; Sahagian et al. 1979) and human splenic β-glucuronidase (Natowicz et al. 1979).

The uptake rates of hydrolases show great variation between different hydrolases, between different sources of one hydrolase, and even between different preparations of one hydrolase

purified from one source (Lagunoff et al. 1973; Hickman et al. 1974; Nicol et al. 1974; Brot et al. 1974; Glaser et al. 1975; Shapiro et al. 1976; Röhrborn and Von Figura 1978). The so called "high uptake" forms of the enzymes, which are taken up rapidly, were shown to be more acidic than "low uptake" forms, which are taken up at much lower rates (Glaser et al. 1975; Röhrborn and Von Figura 1978). Recently a direct correlation was found between the mannose 6-phosphate content and the susceptibility to pinocytosis of different fractions of β-glucuronidase from spleen (Natowicz et al. 1979). Furthermore, it was suggested that "high uptake" hydrolases may be polyvalent ligands whose pinocytosis depends on interaction of more than one phosphomannosyl recognition marker with pinocytosis receptors on fibroblasts (Fischer et al. 1980).

No report has yet been published on the purification and characterization of the phosphomannosyl receptor. The number of binding sites per fibroblast was calculated to be 14,000 (Rome et al. 1979b). The occurrence of these receptors on other cell types than fibroblasts was indicated since lysosomal enzyme uptake that can be inhibited by mannose 6-phosphate has also been found in rat hepatocytes (Ullrich et al. 1978b), an established epithelial rat liver cell line (Ullrich et al. 1978 b) and in Chinese hamster ovary cells (Robbins 1979).

The results so far obtained in studies on uptake of lysosomal hydrolases by cultured fibro-

blasts support Hickman and Neufeld's (1972) suggestion of a recognition marker that is common to a number of enzymes. Recent experiments by Neufeld's group provided evidence that lysosomal enzymes are synthesized in fibroblasts in mannose 6-phosphate containing precursor forms of higher molecular weight than the final products (Hasilik et al. 1979; Hasilik and Neufeld 1980 a,b). In "I-cell" fibroblasts, which have a multiple intracellular lysosomal enzyme deficiency and show extracellular accumulation of the same hydrolases, phosphorylation was absent and chain shortening was impaired. This establishes the involvement of the phosphomannosyl marker in the processing and packaging of lysosomal enzymes, which will be discussed in more detail in the next section.

1.5 PROCESSING AND TRANSPORT OF LYSOSOMAL HYDROLASES

After the suggestion by Hickman and Neufeld (1972) that the lysosomal enzymes need to be equipped with a recognition marker in order to become correctly sequestered into the lysosomal apparatus, Neufeld's group provided another contribution to an understanding of the packaging of lysosomal hydrolases by their recent studies on precursor forms of the enzymes (Hasilik and Neufeld 1980a, b). By means of pulse-labelling and immunoprecipitation these authors showed the presence of precursor chains as well as partially and fully processed chains of β-hexosaminidase, α-glucosidase and cathepsin D in normal fibroblasts, and of precursors but not of processed forms in the culture medium above them (Hasilik and Neufeld 1980a). Precursors and intermediate forms had a higher molecular weight than the mature chains, which is similar to the findings of Skudlarek and Swank (1979), who detected a high molecular weight precursor of β-galactosidase in mouse peritoneal macrophages. As the processing involved both large and small changes in molecular weight, proteolytic cleavage as well as modification of carbohydrate side chains were presumed to occur (Hasilik and Neufeld 1980a).

The precursor chains and the processed forms in fibroblasts were all found to be phosphorylated (Hasilik and Neufeld 1980b). The position of the phosphate was found to be on mannose residues. The

normal mannose contents and the absent phosphorylation of enzyme forms in "I-cell fibroblasts (Hasilik and Neufeld 1980b) were accompanied by excess of precursors in the medium, whereas the low amount of intracellular forms was poorly processed (Hasilik and Neufeld 1980a). This establishes the role of mannose 6-phosphate in the lysosomal location of hydrolases.

Tager et al. (1980) recently presented a model for the synthesis and packaging of lysosomal enzymes, which combined the above mentioned results on the biosynthesis of lysosomal enzymes with data on the synthesis of other glycoproteins. Analogous to export proteins (Campbell and Blobel 1976), precursor polypeptides of lysosomal enzymes may possess a signal peptide, which enables them to traverse the membrane of the endoplasmic reticulum and reach the cisternae where glycosylation begins. A complex type of oligosaccharide is first transferred to asparagine residues of the polypeptides by a lipid carrier (Robbins 1979; Parodi and Leloir 1979; Li et al. 1978). Mannosidases, glucosidases, glycosyltransferases and (in the case of lysosomal enzymes) a 6-phosphomannosyltransferase process this oligosaccharide until a specific type of glycopeptide results (Robbins 1979; Parodi and Leloir 1979), which bears mannose 6-phosphate. In this model the mannose 6-phosphate containing precursors bind to receptors in the Golgi apparatus, which is followed by sequestration into the future primary lysosomes and proteolytic processing. Further processing of oligosaccharides is to occur in the lysosomes.

The biogenesis of primary lysosomes proposed here is in agreement with ultrastructural studies of granulocytes and monocytes, the only cell types in which primary lysosomes have been clearly observed, and where they are thought to bud off from the Golgi apparatus (Cohn and Fedorko 1969; Nichols et al. 1971). Another site of primary lysosome formation may exist in some other cell types and this is a specialized region of the endoplasmic reticulum, which is designated as GERL (Golgi-associated Endoplasmic Reticulum from which Lysosomes form)(Novikoff 1967, 1973, 1976).

At the present time several hypotheses exist concerning the transport of lysosomal enzymes and the stage at which recognition of mannose 6-phosphate is crucial. In the model of Tager and co-authors (1980) the recognition of mannose 6-phosphate by cellular receptors occurs at the level of primary lysosome formation and packaging is an intracellular process. Hasilik and Neufeld (1980b) consider both intracellular packaging and receptor-bound transport via the cell membrane as compatible with their experimental results. Sly and Stahl (1978) regard mannose 6-phosphate as a signal to segregate secretory proteins and lysosomal enzymes, which may take place either at the level of primary lysosome formation or at the plasma membrane. Von Figura and Weber (1978) studied intracellular and extracellular activities of lysosomal enzymes in normal fibroblast cultures grown in the presence and absence of mannose 6-

phosphate. These authors proposed that the majority of lysosomal enzyme molecules cycle via the cell surface, but a small number of molecules pass through the extracellular space. The latter mechanism reflects the secretion-recapture route as originally postulated by Hickman and Neufeld (1972), where extracellular release of the enzymes occurs before the lysosomes are reached. An alternative hypothesis was given by Lloyd (1977), who assumes that lysosomal enzymes are normally present in the lysosome in a receptor-bound form. Elements from the lysosomal apparatus are constantly incorporated into the plasma membrane as a compensation for pinocytic membrane internalization. Enzymes that are less tightly bound to the membrane are consequently lost to the extracellular space but the recognition marker would enable part of the enzyme molecules to be recovered from the environment. In this model recognition plays a role in the retention of enzymes and in the (secondary) sequestration of otherwise lost molecules.

In Chapter two of this thesis the above mentioned hypotheses will be further discussed in connection with the experimental work from Appendix papers I-V. The experiments concerned the extracellular release of hydrolytic enzymes by cultured cells, the possibility of their subsequent uptake, the presence of cell surface-associated hydrolases and the possibility of restoring normal intra- and extracellular lysosomal enzyme activities in "I-cell" fibroblasts by means of somatic cell hybridization.

Chapter two

THE EXPERIMENTAL WORK: INTRODUCTION AND DISCUSSION

2.1 INTRODUCTION

In cultures of human fibroblasts the activities of lysosomal hydrolases are distributed over the intracellular fraction, an extracellular pool of enzymes released by the cells (Von Figura 1978; Willcox 1978 ; Appendix papers II-IV) and a cell surface-associated (pericellular) fraction (Appendix papers II and IV; Von Figura and Voss 1979). In the various models concerning the transport of hydrolytic enzymes different routes have been suggested to account for these different sites of enzyme activity (Fig. 2.1).

In the classical concepts by De Duve and Wattiaux (1966) and Novikoff (1973) the transport of lysosomal hydrolases was considered to be an intracellular event. Excretion before the lysosomal destination was reached, followed by lysosomal sequestration, was first postulated as a normal route for lysosomal enzymes by Hickman and Neufeld (1972). According to Lloyd (1977) excretion of hydrolases might also be the result of lysosomal involvement in plasma membrane recycling.

The presence of cell surface-associated lysosomal enzymes is not only implicated in the excretion-recapture route and in the membrane-recycling model, but also in the hypothesis that the hydrolases cycle via the plasma membrane before the

lysosome is reached (Von Figura and Weber 1978; Sly and Stahl 1978). The origin of the surface-associated molecules would, however, be different in these various models (see Fig. 2.1 and Chapter 1.5).

The purpose of the experimental work to be described here was to study some possibilities of transport of lysosomal hydrolases between the extracellular, intracellular and pericellular compartments. In these studies we used normal cultured cells and fibroblasts from patients with lysosomal storage diseases, who have a deficiency of one or more lysosomal enzymes.

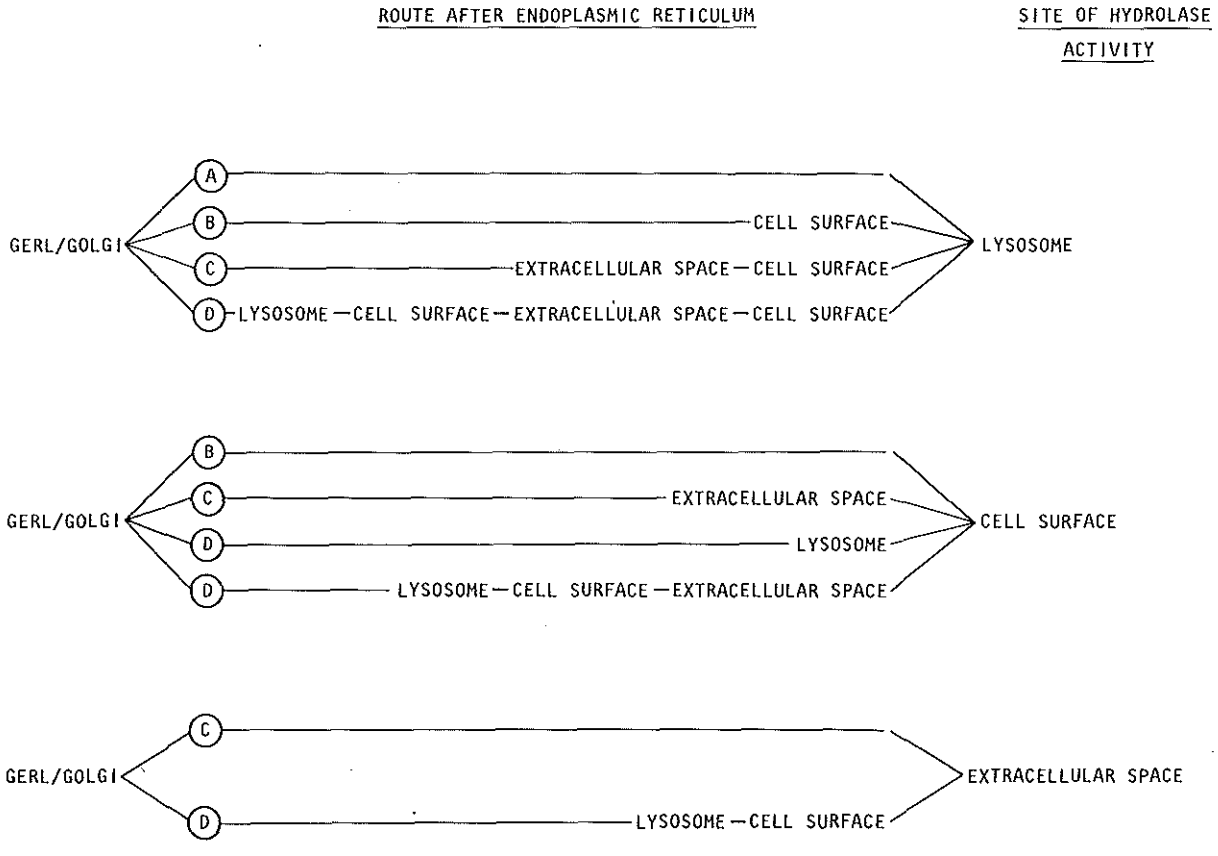
In the experiments of Appendix paper I fibroblasts from patients with a single lysosomal enzyme deficiency have been co-cultivated with normal fibroblasts. Using microchemical analyses of single cells the intercellular transfer of β-galactosidase, α-glucosidase and β-N-acetylhexosaminidase (hexosaminidase) was studied. Since transfer from normal cells to mutant fibroblasts was observed for hexosaminidase, but not for the two other hydrolases the stability of the enzymes in the culture medium, the effect of cell-to-cell contact on enzyme exchange and the distribution of the enzymes over the extracellular-, pericellular- and intracellular compartments was studied in Appendix paper II. In addition we have studied the fate of hexosaminidase after uptake by deficient fibroblasts.

The aim of the experiments described in Appendix paper III was to investigate the possible de-

Fig. 2.1

MODELS FOR THE TRANSPORT OF LYSOSOMAL HYDROLASES

The origin of hydrolase activities in different compartments



(A) De Duve and Wattiaux 1966
Novikoff 1973
Lloyd 1977
Sly and Stahl 1978
Hasilik and Neufeld 1980
Tager et al. 1980

(B) Von Figura and Weber 1978
Sly and Stahl 1978
Hasilik and Neufeld 1980

(C) Hickman and Neufeld 1972

(D) Lloyd 1977

pendence of the uptake by human deficient fibroblasts on the origin of the enzyme. Co-cultivations were carried out with fibroblasts from a variety of species and within one species -rat- with fibroblasts, hepatocytes and hepatoma cells. Recognition and uptake seem to be independent of the species whose fibroblasts are used as an enzyme source , but considerable differences in exchange of lysosomal hydrolases were observed between different cell types within one species. Rat hepatoma cells were found to release lysosomal hydrolases that are taken up very efficiently by human fibroblasts. The kinetics of the uptake of hepatoma-released lysosomal hydrolases are reported in Appendix paper III, with "I-cell" fibroblasts ("I-cells") as acceptor cells in addition to fibroblasts with a single lysosomal enzyme defect.

Appendix paper IV describes experiments on the characteristics of different forms of α-mannosidase in the intracellular-, pericellular-, and extracellular compartments of control cells and fibroblasts from patients with mannosidosis or "I-cell" disease. These studies were prompted by the observation in preliminary experiments that mannosidosis fibroblasts have a relatively high extracellular α-mannosidase activity compared with the low intracellular activity. From these experiments it was learned that a lysosomal enzyme may be exteriorized on the cell surface without being lost into the extracellular space.

The overall results described in Appendix paper I-IV suggest that lysosomal hydrolases - after the synthesis of their polypeptides, post-

translational modification and compartmentalization in the lysosomes - cycle via the plasma membrane, where enzymes may be lost to the extracellular space and partially reinternalized.

A genetic deficiency of a lysosomal enzyme will mostly be due to a structural mutation affecting its catalytic activity. There are, however, a few examples where defective posttranslational modification of a normal enzyme (precursor) polypeptide is responsible for the deficient activity. Examples are the multiple lysosomal enzyme deficiency in "I-cell" disease and probably the combined β-galactosidase and neuraminidase deficiency in a variant of sialidosis. Appendix paper V describes co-cultivation- and somatic cell hybridization studies with these mutant cells and some other fibroblast strains with a single lysosomal enzyme deficiency. It was suggested that the processing defect in "I-cells" could be corrected by cell fusion but not by co-cultivation with other cells.

In the following sections the results of the various experiments described in the Appendix papers will be discussed in relation to relevant data in the literature. This Chapter will be concluded with a model on the intracellular- and extracellular route of lysosomal hydrolases.

2.2 INTERCELLULAR TRANSFER OF LYSOSOMAL ENZYMES IN HUMAN FIBROBLASTS

When two cell populations are grown together (co-cultivated) as a mixture, the cells may communicate both through direct cellular contact and via the extracellular space, i.e. the culture medium. The experiments described in Appendix paper I involve co-cultivations of normal human fibroblasts with those from patients with a genetic, single lysosomal enzyme deficiency. The intercellular transfer of hydrolases was studied by measuring enzyme activities in single fibroblasts from both parental strains using ultramicrochemical procedures. Normal skin fibroblasts were co-cultivated during periods of 1-10 days with fibroblasts from patients with Pompe's disease (α-1,4-glucosidase deficient), G_{M1}-gangliosidosis (β-galactosidase deficient), and Sandhoff's disease (deficient in hexosaminidases A and B). Prior to co-cultivation the normal and deficient cell strains were marked with either latex or carbon particles. After co-cultivation the cells were reseeded at low density on dishes with a bottom of plastic foil, the different cell types were identified by phase-contrast microscopy and the cultures were frozen and freeze dried. Microdissection of small pieces of plastic foil containing individual fibroblasts was followed by a microchemical determination of the appropriate lysosomal enzyme activity using artificial fluorogenic 4-methylumbelliferyl substrate. (Detailed descriptions of the ultramicrochemical

analyses of cultured cells are given by Galjaard et al. (1974 b.c. 1977), Reuser et al. (1976) and Galjaard (1980)). The experiments in Appendix paper I demonstrate that the activities of β-galactosidase and α-glucosidase in the respective deficient cell strains remain unchanged during co-cultivation with normal cells. On the basis of these results it was concluded that the lysosomal enzymes β-galactosidase and α-glucosidase are not transferred from one fibroblast to another, even if the cells are in close contact.

Co-cultivation of normal and hexosaminidase deficient Sandhoff fibroblasts, however, consistently resulted in increased hexosaminidase activity of the mutant cells, associated with a decrease of the hexosaminidase activity of the normal fibroblasts. These results suggested that the deficient cells acquire active hexosaminidase molecules from the normal cells during co-cultivation in cellular contact.

In the experiments described in Appendix paper II we investigated whether cellular contact is a requirement for intercellular transfer of hexosaminidase to occur. Sandhoff fibroblasts and normal fibroblasts were co-cultivated separately on microscope slides at a constant distance of 1.5 mm in the same culture medium. After co-cultivation during 5 days the two cell populations were harvested with trypsin, homogenized, and assayed for the activities of the hexosaminidase isoenzymes A and B, and the results were expressed per milligram of cellular protein. Hexosaminidase A

and B were both found to be transferred from the normal fibroblasts to the deficient cells and in apparently equal amounts. This indicates that human fibroblasts may derive active hexosaminidase A and B molecules from an extracellular pool, which would be in accordance with an excretion-reuptake route for these isoenzymes. This type of experiment does, however, not discriminate between a lysosomal (Lloyd 1977) or a pre-lysosomal (Hickman and Neufeld 1972) origin of the excreted enzymes.

The results described in Appendix paper II permit an estimate of the contribution of the excretion-reuptake route of hexosaminidase to the maintenance of the intracellular enzyme activity. The half-life of hexosaminidase ingested by enzyme deficient Sandhoff cells was found to be 6 days (Appendix paper II). This value is very close to that of 7 days reported by Hickman et al. (1974) in experiments on uptake of high amounts of enzyme concentrated from fibroblast secretions. From the mean uptake by deficient cells co-cultivated with normal fibroblasts and the intracellular activity of the normal cells (Appendix paper III), it can be calculated that in normal cells the daily replacement of hexosaminidase is equivalent to an activity of 316 nmoles methylumbelliferyl substrate cleaved per hour per mg cellular protein, of which 35 nmoles (11%) can be accounted for by intercellular transfer. This calculation is just an estimate, since the normal hexosaminidase activity varies with time in culture and cell den-

sity (Heukels-Dully and Niermeijer 1976) and it is based on the turnover time of ingested hexosaminidase, which may not be representative for the overall turnover time of the enzyme. Similar percentages for the contribution of the excretion-reuptake route to intracellular hexosaminidase were, however, reported by Von Figura and Weber (1978) and Vladutiu and Rattazzi (1979). These authors' values ("less than 20%" and 12%, respectively) were obtained in experiments where reuptake was blocked by competitive inhibition with mannose 6-phosphate.

The lack of intercellular transfer of β-galactosidase and α-glucosidase in co-cultivations between normal fibroblasts and fibroblasts from patients with G_{M1}-gangliosidosis or glycogenosis II (Appendix paper I) might be caused by inability of the deficient cells of enzyme recognition, uptake or retention. Hieber et al. (1976), however, found that G_{M1}-gangliosidosis fibroblasts do recognize purified testicular β-galactosidase and later experiments by our own group (see Appendix paper III) showed that β-galactosidase released by rat fibroblasts or rat hepatoma cells is taken up by β-galactosidase deficient G_{M1}-gangliosidosis fibroblasts.

Another explanation for the lack of intercellular transfer of β-galactosidase and α-glucosidase in co-cultivations with normal human fibroblasts would be the very low activities of these enzymes in medium above normal fibroblasts (see Appendix paper II). The extracellular activity of hexos-

aminidase is, in contrast, relatively high and increases with the intracellular activity. Both α-glucosidase and β-galactosidase are extremely labile in the culture medium, but insufficient release by the cells cannot be excluded as the cause of the low extracellular activities. Whatever the cause, the co-cultivation studies with human fibroblasts showed that α-glucosidase and β-galactosidase do not follow an excretion-reuptake route in these cells. The far greater extracellular activity and the relative stability of β-galactosidase from rat fibroblasts and rat hepatoma cells make these cells more suitable for the study of intercellular transfer of this enzyme (Appendix paper III).

The results discussed so far permit the following conclusions about intercellular transfer of hydrolytic enzymes in human fibroblast cultures:

- the occurrence of intercellular transfer of hexosaminidases A and B from normal to enzyme deficient cells suggests an excretion-reuptake route for these isoenzymes in normal fibroblasts; this route contributes about 11% to the overall intracellular enzyme activity;
- in human fibroblasts the excretion-reuptake route is not followed by all lysosomal hydrolases. The enzymes β-galactosidase and α-glucosidase, for instance, do not show intercellular transfer, which is likely to be related to an insufficient extracellular pool of active enzyme molecules.

2.3 THE DISTRIBUTION OF HYDROLASE ACTIVITIES OVER THE INTRA-, PERI-, AND EXTRACELLULAR FRACTIONS OF HUMAN FIBROBLAST CULTURES

The presence of receptor-bound hydrolases (or their precursors) at the cell surface is implicated in a number of the current hypotheses regarding the lysosomal packaging and retention of hydrolytic enzymes (see Fig. 2.1). In Appendix paper II we describe the presence of lysosomal hydrolases in a cell-associated fraction that can be detached by treatment with trypsin. The activities of hexosaminidase, β-galactosidase and α-glucosidase in this "pericellular" fraction of normal fibroblasts was found to represent about 13% of the respective intracellular activities for all three enzymes after one week of confluent growth.

The experiments by Von Figura and Weber (1978) provided the first indication that the surface associated enzymes are receptor-bound, as they can be released by very high doses of mannose 6-phosphate. Additional evidence has been given by Von Figura and Voss (1979), who demonstrated the presence of lysosomal enzyme activities on the surface of normal cells but not on "I-cell" fibroblasts, which synthesize enzymes (or their precursors) that cannot bind to the receptors.

To investigate the interrelation between intercellular-, pericellular-, and extracellular activities of lysosomal enzymes, we have examined

whether hexosaminidase, once taken up from the extracellular pool into the cell, recycles to the pericellular and extracellular fractions (see Appendix paper II). Sandhoff cells were supplied with hexosaminidase from normal fibroblasts, trypsinized and grown in the absence of exogenous enzyme. Subsequently hexosaminidase activity was measured in the intracellular-, pericellular-, and extracellular fractions. These experiments showed that ingested enzyme reappeared both in the pericellular and in the extracellular fraction. Recently, Bach and Liebmann-Eisenberg (1979) and Rome et al. (1979a) demonstrated that exogenous lysosomal enzymes are sequestered into the secondary lysosomes of cultured fibroblasts. Consequently, our experiments in Appendix paper II indicate that hexosaminidase molecules may recycle from the secondary lysosome to the cell surface and the extracellular space. Since the relative amount of extracellular hexosaminidase after recycling in deficient cells is similar to the relative extracellular hexosaminidase activity in normal cells, it can be postulated that the majority of extracellular hydrolase molecules stem from secondary lysosomes.

Evidence for (re)cycling of constituents of secondary lysosomes to the plasma membrane was provided by Schneider et al. (1977), who showed that fibroblast plasma membrane antigens are internalized during endocytosis, become localized in lysosomes and thereafter reappear at the cell surface. Recycling of the plasma membrane receptor for lysosomal enzymes was also inferred (Rome

et al. 1979b) These authors found that the number of α-L-iduronidase molecules taken up by deficient fibroblasts exceeds the number of calculated binding sites on the cell surface and postulated that uptake is followed by regeneration of receptors by means of recycling to the plasma membrane. Lloyd (1977) regards fusion between (secondary) lysosomal membrane and plasma membrane as a continuous compensatory mechanism for endocytic plasma membrane internalization; the latter cannot be sufficiently compensated by "de novo" synthesis. Lloyd (1977) explains in this way the presence of lysosomal hydrolases at the cell surface, where part of the enzymes are lost to the extracellular space. Those enzymes that are (again) bound to the cell surface will, however, become reinternalized and in this way lysosomal enzymes may cycle between secondary lysosome, pericellular fraction and the extracellular space. Our investigations on α-mannosidase activities in cultures of human mannosidosis fibroblasts suggest that cycling does not always imply the extracellular release of a lysosomal enzyme (see section 2.5). Mannosidosis fibroblasts have an abnormal form of α-mannosidase inside the cells and exteriorized at the cell surface, but this form is not detected outside the cells.

Our experimental data on the fate of hexosaminidase ingested by Sandhoff fibroblasts:

- are in good agreement with the membrane recycling model proposed by Lloyd (1977);
- on the other hand, our data make it unlikely that excretion of lysosomal enzymes is an

important part of the route before the lysosomal destination, as was postulated by Hickman and Neufeld (1972).

2.4 INTERCELLULAR EXCHANGE OF LYSOSOMAL ENZYMES BETWEEN HUMAN FIBROBLASTS AND CELLS FROM OTHER SPECIES

Experiments have been carried out to investigate the tissue and species specificity of the intercellular transfer of lysosomal enzymes. No striking differences were observed in the uptake by human deficient fibroblasts of hexosaminidase and β-glucuronidase released by cultured fibroblasts from different species. Mammalian fibroblasts, such as those from Chinese hamster, rat and mole, as well as chicken fibroblasts all released lysosomal enzymes that were taken up by human cells, as could be demonstrated by quantitative enzyme assays and by electrophoretic identification of the ingested enzyme. Electrophoretic studies also revealed that the transfer of hydrolases in such interspecies co-cultivations is reciprocal, since bands with the mobility of human enzymes could be observed in the electrophoretic patterns of the animal fibroblasts (unpublished results).

In later experiments, which are described in Appendix paper III, we have investigated intercellular transfer of lysosomal enzymes in relation to different cell types within one species. Different types of rat cells were compared in their ability to supply human deficient fibroblasts with lysosomal enzymes. The use of rat cells was advantageous, because they were found to release relatively large quantities of active hydrolases including β-galactosidase and β-glucuronidase. These

high extracellular activities make these cells more suitable for the study of intercellular transfer of these enzymes than normal human fibroblasts.

In addition to fibroblasts, hepatocytes and hepatoma cells from the rat have been investigated both as donor cells and as recipient cells. As is described in Appendix paper III human fibroblasts with a single deficiency of either hexosaminidase, β-glucuronidase or β-galactosidase were used as recipient cells as well as "I-cells", which have a multiple lysosomal enzyme deficiency.

Fibroblasts and hepatocytes bear different recognition systems for glycoproteins (see also Chapter one, section 4). That of fibroblasts recognizes mannose 6-phosphate and hepatocytes have a specificity for terminal galactose residues (see Ashwell and Morell 1974), although also the mannose/N-acetylglucosamine and mannose 6-phosphate specific recognition systems were recently shown to be present in minor amounts in rat hepatocytes (Ullrich et al. 1979). In co-cultivations of rat hepatocytes and human fibroblasts (see Appendix paper III) we found no transfer of hexosaminidase or β-galactosidase in either direction. This suggests that there might be a relation between the uptake properties of the lysosomal enzymes released by a certain cell type and the predominant glycoprotein receptor system expressed on the surface of those cells.

Rat hepatoma cells, on the other hand, were found to be capable of taking up human fibroblast lysosomal enzyme and they release lysosomal enzymes

that are ingested by deficient human fibroblasts with a high efficiency. Inhibition studies with mannose 6-phosphate established that β-galactosidase and β-glucuronidase from the medium of rat hepatoma cells have uptake properties comparable to those of purified "high uptake" forms of β-glucuronidase (Kaplan et al. 1977) and α-L-iduronidase (Sando and Neufeld 1977).

Rat hepatoma cells were shown to ingest not only normal fibroblast hexosaminidase, but also that released by "I-cells". This is somewhat surprising since enzymes from these cells, which are now known to be defectively processed (Hasilik and Neufeld 1980 a,b) are poorly taken up by human fibroblasts (Hickman and Neufeld 1972) and by non-parenchymal rat liver cells (Ullrich and Von Figura 1979). An interesting observation was made by Ullrich et al. (1978 b), who studied an established epithelial rat liver cell line, in which the mannose 6-phosphate specific recognition system that is characteristic for fibroblasts, but not for hepatocytes, mediates the binding of lysosomal enzymes. Hickman and Ashwell (1974) showed that rat hepatomas have diminished binding activities for galactose-terminal glycoproteins compared with hepatocytes.

It seems as if transformation of hepatocytes is associated with alterations of the receptor system(s) at the cell surface, as well as with changes in the carbohydrate parts of released lysosomal enzymes. Transformation involves a change from a non-proliferating system to a rapidly

dividing cell population. Furthermore, the amounts of several membrane-associated proteins, the glycosylation of glycoproteins, and the types of secreted proteins are altered upon transformation (for review see Tumanova 1978; Yamada and Pouyssegur 1978). Unpublished experiments showed that mouse neuroblastoma cells and human HeLa cells also released appreciable amounts of lysosomal enzymes, but their uptake was not better than that of human fibroblast enzymes. The release of "high uptake" forms of lysosomal hydrolases is therefore not a consistent feature of transformed cells.

In the experiments described in Appendix paper III it was indicated that β-galactosidase from rat hepatoma cell medium is taken up better by deficient human fibroblasts than β-glucuronidase. This suggests heterogeneity in the uptake properties of different lysosomal enzymes from one cellular source, although a definite conclusion awaits turnover studies of the ingested enzymes.

The properties of the membrane, the release of lysosomal hydrolases with "high uptake" properties for cultured fibroblasts and the possibility to study heterogeneity in the uptake properties of different lysosomal enzymes from one cellular source, make rat hepatoma cells an interesting model for the study of recognition and uptake of lysosomal enzymes.

2.5 INTRACELLULAR AND EXTRACELLULAR α-MANNOSIDASES IN FIBROBLAST CULTURES FROM PATIENTS WITH MANNOSIDOSIS OR MUCOLIPIDOSIS II

In our studies on recognition, secretion and uptake of lysosomal enzymes, investigation of cultured fibroblasts from patients with mannosidosis seemed of particular interest since we found a relatively high activity of α-mannosidase in the culture medium, despite the fact that the intracellular activity of this enzyme was very low in the mutant fibroblasts. This observation was also described by Hultberg and Masson (1977), who suggested that mannosidosis might represent a specific "recognition defect", comparable to the general recognition defect that is known to exist in mucolipidosis II ("I-cell" disease) (see Chapter one, sections 2 and 5).

In addition to lysosomal α-mannosidase which has an acidic pH optimum (Carroll et al. 1972; Chester et al. 1975), two other types of α-mannosidase occur in mammalian tissues; one type is located in the cytosol and has a neutral pH optimum (Marsh and Gourlay 1971), and another type has been purified from Golgi membranes (Tulsiani et al. 1977) and has its optimum intermediate between acidic and neutral pH (Snaith 1973; Phillips et al. 1974). The three types of activity can also be distinguished by their characteristic response to Co^{2+} and Zn^{2+} ions (Hirani et al. 1977).

A specific deficiency of acidic α-mannosidase is associated with the human storage disease

mannosidosis (Öckerman 1967a,b) and a mutation in the structural gene has been proposed as the primary defect in the disease (Beaudet and Nichols 1976). Such a mutation might also affect the structure of the enzyme in such a way that it is not properly recognized and compartmentalized in the cell (Hultberg and Masson 1977).

In Appendix paper IV we describe experiments comparing some characteristics of the intra- and extracellular α-mannosidase activities in fibroblast cultures from patients with mannosidosis or mucolipidosis II with the corresponding activity in normal controls. In addition, we have attempted to ascertain whether the activity that is associated with the external surface of the fibroblasts originates by readsorption of exocytosed enzyme from the medium or by exteriorisation of intracellular activity.

In the medium above mannosidosis fibroblasts the activity of acidic α-mannosidase was found to be lower than above control cells, but in proportion to the low intracellular activity in the mutant cells the extracellular activity was high. A similar but more pronounced situation existed in cultures of "I-cells", where this abnormal distribution reflects defective recognition. However, both the extracellular and the total (intra- + extracellular) acidic α-mannosidase activities are lower than normal in mannosidosis cell cultures, whereas "I-cells" seem to have a quantitatively normal total production of α-mannosidase molecules.

The combined data of kinetic studies, the application of metal ions, and co-cultivation experiments (see Appendix paper IV) suggest that:

- two types of acidic α-mannosidase are generated by mannosidosis fibroblasts. One of these types is the lysosomal form that is affected by the gene mutation and as a result of this has a decreased catalytic activity but an unchanged affinity for the enzyme receptor. This enzyme is found inside the cells and in the pericellular fraction. The other type - that which is secreted - has normal enzymic properties but its affinity for the receptors is low.

The precise nature of the extracellular activity is not clear. In normal cells a varying proportion of both intracellular and extracellular acidic α-mannosidase consists of such "low uptake" forms (Mersmann et al. 1978; Winchester et al. unpublished) The exact fate of "low uptake" forms in general is still unclear. They may result from microheterogeneity in the carbohydrate structure of the molecule, and not be capable of joining in receptor-bound cellular transport to the lysosome. Alternatively, low or non-uptake forms may arise from removal of the recognition marker after lysosomal sequestration (Glaser et al. 1975; Sly and Stahl 1978) and get lost from the cells during lysosomal involvement in plasma membrane recycling (Lloyd 1977). Although the precise role of the "low uptake" form in the medium of mannosidosis fibroblasts is not yet understood, our experimental data provide support for membrane cycling in the localization of lysosomal enzymes and for recognition between cell receptors and markers on lysosomal enzymes.

2.6 CORRECTION OF "I-CELL" DEFECT BY HYBRIDIZATION WITH HUMAN MUTANT FIBROBLASTS

The recognition of many lysosomal enzymes is disturbed in "I-cell" disease as has become evident from the multiple intracellular lysosomal enzyme deficiency, the high extracellular activities of the same enzymes and by a variety of experiments described in Chapter one, sections 4 and 5. The experiments described in Appendix paper V were performed to investigate whether it is possible to correct this "recognition defect" by somatic cell hybridization or co-cultivation with other fibroblasts.

Complementation, i.e. the restoration of a genetic metabolic defect after hybridization has first been observed in micro-organism (for review see Fincham 1966). In these organisms complementation may occur by the combination of different genes (intergenic complementation) or by the combination of different mutations affecting the same gene (intragenic or interallelic complementation). Somatic cell hybridization in the study of clinical heterogeneity in inborn errors of metabolism (for review see Bootsma and Galjaard 1979) was first successfully applied by Bootsma's group with fibroblasts from patients with different types of the autosomal recessive disorder xeroderma pigmentosum, which is associated with defective DNA repair (De Weerd-Kastelein et al. 1972; Bootsma 1978; Keijzer et al. 1979). Hybridization studies have also demonstrated the existence of different gene muta-

tions within lysosomal storage disorders: G_{M2}-gangliosidosis (Galjaard et al. 1974a; Thomas et al. 1974) and Niemann-Pick disease (Besley et al. 1980), and among different patients with a deficiency of β-galactosidase (Galjaard et al. 1975) or neuraminidase (Hoogeveen et al. 1980).

Recent experiments by Hasilik and Neufeld (1980 a,b) have provided evidence that the metabolic abnormalities in "I-cell" disease are due to a defective posttranslational modification of the enzymes. These authors' results show that precursor polypeptide chains are normally synthesized but that absent phosphorylation and impaired processing of the molecules is accompanied by loss of the precursors to the extracellular space (see Chapter one, section 5).

In a number of lysosomal storage diseases associated with a single lysosomal enzyme deficiency, experimental evidence has been obtained that they are due to a structural mutation affecting the catalytic activity of the hydrolase's polypeptide chain. For instance, in G_{M2}-gangliosidosis type 0 (Sandhoff's disease), immunological investigations and reassociation studies (Ropers and Schwantes 1973; Beutler et al. 1975) as well as gene localization studies (for review see Hoeksema 1979) have indicated that the simultaneous deficiency of hexosaminidase A and B is due to a mutation in a structural gene, localized on chromosome 5, that is coding for the common β-subunit of these isoenzymes. The β-galactosidase deficiency in the classical forms of G_{M1}-gangliosidosis seems also

to be due to a structural mutation affecting the catalytic activity of the 70,000 MW monomeric β-galactosidase molecule (Norden et al. 1974; Galjaard and Reuser 1977; O'Brien 1978). The α-mannosidase deficiency in mannosidosis has likewise been attributed to a structural alteration of the enzyme's polypeptide (Beaudet and Nichols 1976).

In the experiments described in Appendix paper V fibroblasts from patients with these single lysosomal enzyme deficiencies have been fused with fibroblasts from patients with "I-cell" disease. A clear restoration of hexosaminidase, β-galactosidase or α-mannosidase activity was found 1-3 days after fusion of the deficient parental cell strains. On the other hand, co-cultivation or mixing of cell homogenates of the mutant cell strains did not change the enzyme activity. This indicates that:

- correction of the metabolic defect can only be achieved within the integrity of the heterokaryon. Since the polypeptides of hexosaminidase, β-galactosidase, and α-mannosidase are structurally altered in Sandhoff's disease, classical G_{M1}-gangliosidosis, and mannosidosis respectively, the complementation must be due to a restoration of the processing defect in "I-cells" by the mutant cells with a single lysosomal enzyme deficiency.

The correction of the processing and recognition defect in "I-cells" after cell hybridization was also studied by investigating the extracellular activity and electrophoretic mobility of some of the lysosomal hydrolases. For hexosaminidase and α-mannosidase a partial normalization of

the increased extracellular activity in the culture medium was found after fusion. This indicates that the correction of the defective processing leads to a normal distribution of the enzymes between the intra- and extracellular compartments. The nearly normal electrophoretic pattern of hexosaminidases after fusion of "I-cells" with Sandhoff fibroblasts is supportive evidence for normalized processing of "I-cell" polypeptides in the heterokaryons.

We have also performed fusions of "I-cells" with fibroblasts from a patient with a combined deficiency of neuraminidase and β-galactosidase. Previous experiments in our laboratory (De Wit-Verbeek et al. 1978; Hoeksema et al. 1979; D'Azzo et al., to be published) suggested that the β-galactosidase deficiency in this patient with sialidosis is either due to an incorrect processing of this enzyme related to the simultaneously occurring neuraminidase deficiency, or to a defect in a processing step that is common to both these hydrolases (Hoogeveen et al. 1980). The fact that fusion of these sialidosis cells with "I-cells" was found to result in a restoration of β-galactosidase acitivity (see Appendix paper V) indicates that:

- the processing defects in "I-cell" disease and the combined β-galactosidase/neuraminidase deficiency are at different levels and arise by mutation of different genes.

Similar fusions also resulted in complementation for neuraminidase (Hoogeveen et al. 1980), but whether restoration of neuraminidase activity

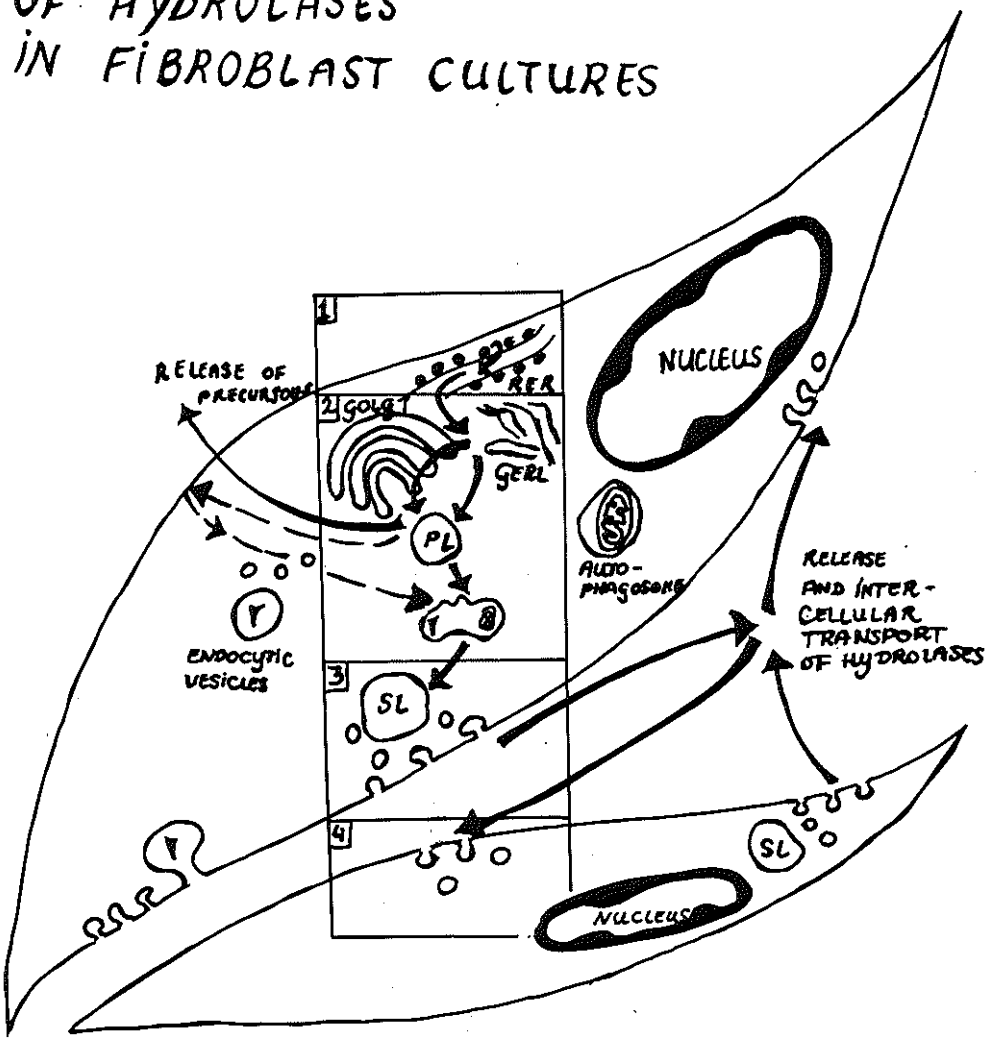
precedes that of β-galactosidase or whether the expression of both enzymes is corrected simultaneously is beyond the scope of such complementation studies. In this respect, the observations of Hoogeveen et al. (1980) are of interest, who showed that the neuraminidase deficiency in fibroblasts of the β-galactosidase/neuraminidase deficient genotype can be corrected by a "factor" (presumably a glycoprotein) taken up from the medium of normal fibroblasts. Since this "factor" was found to be absent from "I-cells", it appears that the phosphorylation step, which is defective in "I-cell" disease (Hasilik and Neufeld 1980b), also acts on the generation of this "factor", which seems to be genetically affected in the combined β-galactosidase/neuraminidase deficiency.

The various single lysosomal enzyme deficiencies, which are based on mutations in structural genes, the combined β-galactosidase/neuraminidase deficiency and "I-cell" disease represent genetic defects affecting the "realization" of lysosomal hydrolases. However, enzyme realization is not limited to the determination of enzyme primary structure and the processing of precursor forms, but it seems to be far more complex. In man, the need for activator proteins required for the in vivo interaction between some enzymes and their natural substrates is an example of this (e.g. Conzelmann and Sandhoff 1978). Studies on inbred mice provided evidence for the existence of systemic as well as temporal genes regulating the rate of synthesis of lysosomal enzymes (see the

review by Paigen 1979). At yet another level of lysosomal enzyme realization the production of receptor molecules may be genetically affected. Since a correct localization of lysosomal enzymes seems to depend on receptor-mediated transport, mutations affecting the receptor molecules would result in a disturbance of enzyme transport comparable with that in "I-cell" disease.

Although the full complexity exemplified above is not reflected in human models, the scope of investigation of lysosomal enzyme realization in man has been extended by the availability of mutant fibroblasts with a posttranslational defect, such as "I-cells" and cells with a combined β-galactosidase/neuraminidase deficiency. The precise nature of the molecular events involved in the processing of lysosomal hydrolases and their intra-, peri- and extracellular sites is the subject of research of several groups including our own. A better understanding of the normal synthesis, processing, compartmentalization and degradation of lysosomal enzymes seems necessary as a solid basis for clinical attempts at enzyme replacement therapy.

INTRA- AND INTERCELLULAR TRANSPORT OF HYDROLASES IN FIBROBLAST CULTURES



RER : ROUGH ENDOPLASMIC RETICULUM

GERL : GOLGI-ASSOCIATED ENDOPLASMIC RETICULUM FROM WHICH LYSOSOMES FORM

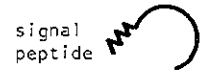
PL : PRIMARY LYSOSOME ; SL : SECONDARY LYSOSOME

RELEASE OF PRECURSOR MOLECULES - ACCORDING TO HASILIK AND NEUFELD 1980 a.

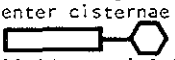
CYCLING VIA CELL SURFACE - - - - → - ACCORDING TO SLY AND STAHL 1978; VON FIGURA AND WEBER 1978

1

ENTRY OF POLYPEPTIDE INTO THE CISTERNAE OF THE ENDOPLASMIC RETICULUM, INITIAL GLYCOSYLATION AND FINAL TRANSLATION



incomplete polypeptide containing signal peptide to enter cisternae



+ final steps of translation

(Campbell and Blobel 1976; Robbins 1979; Tager et al. 1980)

2

PROCESSING OF POLYPEPTIDE AND CARBOHYDRATE CHAINS OF PRECURSORS

proteases

glycosidases
glycosyltransferases
phosphomannosyl-transferase(?)

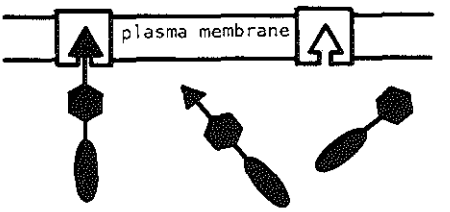
processed form of hydrolase containing mannose 6-phosphate to ensure binding to membrane-inserted receptor during primary lysosome formation

(Robbins 1979; Hasilik and Neufeld 1980 a,b; Tager et al. 1980)

3

PLASMA-MEMBRANE RECYCLING; RECAPTURE OF EXTRACELLULAR AND CELL-SURFACE BOUND HYDROLASE MOLECULES

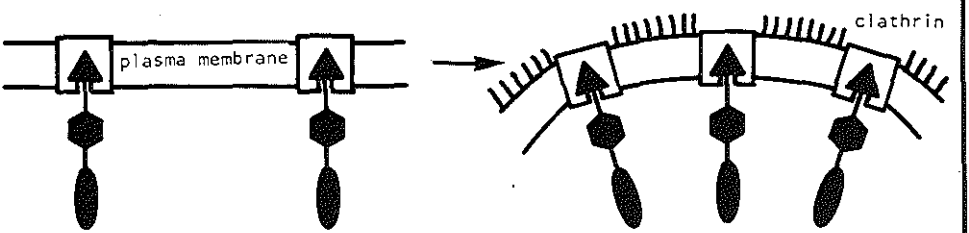
vesicles derived from secondary lysosomes containing receptor-bound and non-bound (dephosphosphorylated ?) hydrolases fuse with plasma membrane; reuptake contributes to intracellular activity



(Lloyd 1977; Sly and Stahl 1978; this thesis)

4

RECEPTOR-MEDIATED ENDOCYTOSIS BY MEANS OF COATED PITS AND COATED VESICLES



at random distributed receptors become clustered into "coated pits"; clathrin interacts with a cytoplasmic ("internalization") site of the receptor and an intracellular "coated vesicle" is formed; this mechanism has been proposed as a general phenomenon in receptor-mediated endocytosis

(Goldstein et al. 1979)

SUMMARY

Lysosomes are membrane-bound organelles, which contain hydrolytic enzymes. Their function is the continuous degradation or processing of cellular constituents and of exogenous macromolecules. These processes may have a regulatory effect on the cellular metabolism and they also function in the turnover of the cellular contents (see Chapter 1.1). In man about 30 hereditary disorders are associated with the deficiency of one (or more) lysosomal enzyme(s). Such a deficiency results in the lysosomal accumulation of molecules, which are the substrate(s) of this particular enzyme in normal cells (Chapter 1.2). Lysosomal storage leads to damage and functional disturbance of cells, tissues, and ultimately of whole organs. Depending on the genetically deficient enzyme, its residual activity, and the amount of storage product(s), different organs are involved to a varying degree and this will determine the clinical symptoms. In most patients with lysosomal storage diseases physical and mental deterioration ultimately result in death. Within each of the lysosomal storage disorders rapidly progressing infantile forms, where death of the patient occurs at very early age, as well as later onset juvenile and still milder adult variant forms have been identified. Although a great deal of knowledge has been acquired on the molecular background of the various lysosomal storage disorders our insight in the pathogenetics is still very limited.

In principle treatment of patients with these disorders seems possible by substitution of the missing or defective enzyme (see Chapter 1.3). During the last decade many attempts at enzyme replacement therapy have been carried out by intravenous injection of purified enzymes or by transplantation of cells, tissues or whole organs; the latter were hoped to be constant sources of normal enzyme. Unfortunately, most of the attempted enzyme substitutions had no or only transient beneficial effects on the patients' condition.

One of the major problems in enzyme therapy is the delivery of exogenous enzymes to those tissues and organs, in which lysosomal storage is most prominent and where sufficient enzyme activity is most urgently required. Enzymes administered by intravenous injection are rapidly cleared from the circulation and appear to be captured mainly by reticuloendothelial cells in the liver. Furthermore, the blood-brain barrier is a major obstacle for enzymes to reach the nervous tissue, which is seriously involved in most lysosomal storage disorders. Also, immunological re-

actions may complicate attempts at enzyme therapy. It is clear that a variety of problems has to be solved by basic research on model systems. Both investigations on in vitro cultured cells and on animals with a genetic deficiency of a lysosomal enzyme may contribute to our insight in the recognition and uptake of lysosomal enzymes. In the future, when sufficient knowledge has been acquired, it may be possible to target lysosomal enzymes to certain tissues by a modification of their molecular structure resulting in the recognition and uptake of the enzyme by one or more specific cell type(s).

Specific recognition of lysosomal enzymes and other glycoproteins has been a subject of research since the discovery by Ashwell and Morell that certain plasma glycoproteins are efficiently taken up by hepatocytes upon removal of terminal sialic acid residues (see Chapter 1.4). A variety of investigations have subsequently established the role of different carbohydrate residues of glycoproteins as recognition signals for their uptake by different cell types such as hepatocytes, reticuloendothelial cells or cultured skin fibroblasts.

The recognition signal mannose 6-phosphate mediates the efficient uptake of lysosomal enzymes by fibroblasts. Experimental evidence has recently been presented that mannose 6-phosphate is not only involved in the uptake of exogenous molecules, but that this recognition site may primarily function in the cellular transport of newly synthesized lysosomal enzymes to their lysosomal destination (see Chapter 1.5). Analogous to the synthesis of other glycoproteins, the production of lysosomal enzymes is presumed to involve a series of posttranslational modifications. After the initial glycosylation of polypeptides, processing of these lysosomal enzyme precursor forms is thought to include the sequential action of a number of glycosyltransferases, glycosidases, proteases, and, in the case of fibroblasts, presumably a phosphomannosyltransferase. Phosphorylated mannose on the molecules seems to ensure their final processing and their compartmentalization into lysosomes.

The lysosomal storage disease mucolipidosis II ("I-cell" disease), which is associated with serious physical and mental abnormalities resulting in an early death, is characterizes by a multiple intracellular lysosomal enzyme deficiency in cultured fibroblasts together with high activities of the same enzymes in the culture medium above the cells and in the body fluids of the patients. Cultured fibroblasts from patiens with this disorder have widely been used as a model to study recognition and uptake of lysosomal enzymes.

Recently, Hasilik and Neufeld (1980) attributed the faulty localization of lysosomal enzyme activities in "I-cell" disease to absent phosphorylation of mannose residues resulting in the absence of the correct recognition signal on several hydrolases.

Different hypotheses have been presented with regard to the normal route of lysosomal enzymes and the stage(s) at which mannose 6-phosphate acts as a traffic signal (Chapters 1.5 and 2.1):

- the transport of the enzymes to the (secondary) lysosomes is an entirely intracellular event and mannose 6-phosphate acts at the level of primary lysosome formation by the anchorage of (pro)enzymes;
- the transport of the enzymes to the lysosomes occurs via the cell surface and mannose 6-phosphate may segregate lysosomal enzymes from secretory proteins;
- the transport of the enzymes to the lysosomes includes the extracellular space and mannose 6-phosphate ensures the re-uptake of secreted molecules and their localization in lysosomes;
- lysosomes are involved in a continuous process of fusion with the plasma membrane and binding through mannose 6-phosphate minimizes the extracellular loss of enzymes that had already reached the lysosome. The mechanism of fusion between lysosomal membranes and plasma membrane is thought to compensate for the continuous internalization of plasma membrane material during endocytosis.

The purpose of the experimental work described in this thesis was to investigate some aspects of the release and uptake of lysosomal enzymes. The experiments involved the use of normal human and animal fibroblasts and some other cell types such as hepatocytes and hepatoma cells as sources of hydrolytic enzymes, and fibroblasts from patients with lysosomal storage diseases associated with a single lysosomal enzyme deficiency and with "I-cell" disease as recipient cells. In a number of studies co-cultivation of normal and mutant cells was applied as a means to study the release and uptake of lysosomal enzymes at physiological concentrations. We have also studied some characteristics of released lysosomal enzyme activities in normal and mutant cell cultures in comparison with the corresponding intracellular activities. Finally, we have used somatic cell hybridization to investigate the genetic background of the metabolic abnormalities in some of the human mutant cells. Our studies have provided information on the normal transport of lyso-

somal enzymes within the cell and between cells. Furthermore, we hope that more knowledge about recognition and uptake of lysosomal enzymes by deficient cells from patients with lysosomal storage diseases will have future implications for enzyme therapy.

The intercellular transfer of lysosomal enzymes from normal to enzyme deficient fibroblasts cultured in close contact was studied and the results are described in Appendix paper I. Single cells were assayed for enzyme activity using microchemical methods after co-cultivation of normal and deficient cells in a mixed culture. Fibroblasts from a patient with Sandhoff's disease were found to ingest hexosaminidase released by normal fibroblasts. Intercellular transfer was, however, not a consistent feature of lysosomal enzymes in cultured fibroblasts, since α-glucosidase and β-galactosidase were not transferred to deficient cells. Experiments reported in Appendix paper II showed that transfer of hexosaminidase also occurs in the absence of cellular contact between normal and deficient cells. This implies that an extracellular pool of enzyme is an intermediate in the cell-to-cell transport of lysosomal enzymes. The contribution could be estimated of uptake from the extracellular space to the maintenance of the overall intracellular activity of hexosaminidase in normal cells and this amounted to about 11% (Chapter 2.2.).

An interesting finding was that exogenous hexosaminidase that had been ingested by deficient fibroblasts reappeared outside the intracellular compartment, i.e. in the cell surface-associated and extracellular fractions (Appendix paper II). This observation probably reflects the involvement of secondary lysosomes in recycling of membrane material from the cell surface after its internalization. It seems that the extracellular activities of lysosomal hydrolases, measured in normal fibroblast cultures, are mainly derived from such a recycling mechanism. This implies that intercellular transfer of lysosomal enzymes, which seems to be dependent on this extracellular pool, represents a part of the lifecycle of lysosomal enzymes after they had already reached the lysosome. The experiments of Appendix paper II further indicated that the lack of intercellular transfer of α-glucosidase and β-galactosidase is probably related to an insufficient extracellular pool of active enzyme molecules.

In the experiments described in Appendix paper III we investigated the possibility of intercellular transfer of

lysosomal hydrolases between human fibroblasts and other cell types. Three different cell types from the rat, fibroblasts, hepatocytes, and their transformed counterparts, hepatoma cells, were compared in their ability to supply human deficient fibroblasts with lysosomal enzymes. In addition, the different electrophoretic mobilities of the human and rodent lysosomal enzymes provided a qualitative means to study the uptake of human enzyme by rat cells. Difference in species had no influence on the efficiency of uptake of hexosaminidase by deficient human fibroblasts. Differences were, however, found between the different cell types within one species, the rat, both in the uptake properties of the cells and in the characteristics of the lysosomal enzymes they release. Cultured hepatocytes did not detectably ingest human fibroblast hexosaminidase, and the enzymes released by liver cells were poorly taken up by human fibroblasts. Rat hepatoma cells, on the other hand, appeared to be capable of ingesting hexosaminidase from normal fibroblasts. Surprisingly, hepatoma cells were also found to ingest abnormal hexosaminidase from the culture medium above "I-cell" fibroblasts. Experiments by Neufeld's group had previously demonstrated that lysosomal enzymes released by "I-cells" lack the recognition signal that is required for their proper intracellular localization and for their uptake by fibroblasts from the extracellular space. The experiments reported in Appendix paper III also showed that lysosomal enzymes released by rat hepatoma cells were taken up by human deficient fibroblasts with greater efficiency than those from normal human fibroblasts. These results suggest that rat hepatoma cells have remarkable properties both in their uptake of exogenous enzymes and in the characteristics of the lysosomal enzymes they release. This makes rat hepatoma cells an interesting model for further studies on recognition and uptake of lysosomal enzymes.

A valuable model for studying the mechanism of localization of lysosomal enzymes is a mutant fibroblast strain from a patient with the lysosomal storage disease mannosidosis, which is associated with a deficiency of acidic α-mannosidase. Experiments described in Appendix paper IV indicated that these cells generate two types of acidic α-mannosidase, which are localized in different compartments. A small amount of activity with abnormal kinetic properties remains in the cell and may be exteriorized on the cell surface. The latter provides additional evidence for membrane cycling in the localization of lysosomal enzymes. The majority of enzyme activity in mannosidosis fibroblast cultures has normal kinetics but the enzyme is released into

the medium and apparently lacks the essential properties for binding to the membrane and uptake into the cells. These results suggest that the mannosidosis mutation does not simultaneously affect the catalytic and the recognition properties of one lysosomal enzyme molecule, which might also have resulted in the observed distribution of acidic α-mannosidase activity between the intra- and extracellular compartments.

Most lysosomal storage disorders are associated with a deficiency of one lysosomal enzyme as a result of a mutation in the gene that codes for the polypeptide part of that particular enzyme. The processing machinery required to modify precursor forms into mature (active and properly localized) lysosomal enzymes is normally present in cells from these patients. In "I-cell" disease, however, the genes coding for the polypeptides of the individual lysosomal enzymes are normal, but the genetic defect affects a common modification step resulting in the lack of the correct recognition site on various lysosomal enzymes. In the experiments described in Appendix paper V we investigated whether this "I-cell" defect could be corrected by fusion of "I-cell" fibroblasts from patients with a single lysosomal enzyme deficiency. After cell hybridization using inactivated Sendai virus 60-90% multinucleate cells were formed which contain the genetic information of both parental cell strains. Enzyme analyses were performed on such mixed heterokaryon populations after fusion of "I-cells" with fibroblasts with different types of β-galactosidase deficiency (from patients with G_{M1}-gangliosidosis or sialidosis) or a hexosaminidase deficiency (from a patient with G_{M2}-gangliosidosis type 2 (Sandhoff's disease)) or an α-mannosidase deficiency (from a patient with mannosidosis). A clear restoration of enzyme activity was found in all fusion combinations, but not after co-cultivation of the same parental cell strains. Since the cells with a single lysosomal defect are not capable of synthesizing catalytically active enzyme protein, we may assume that precursor forms produced by the "I-cell" fibroblasts are processed by the modifying enzymes of the fusion partner to form functioning lysosomal enzymes. The electrophoretic abnormalities of hexosaminidase and the extracellular loss of some lysosomal enzymes were also found to be (partly) corrected after hybridization of "I-cells" with other mutant cells. Further investigations are presently carried out by our group on the precise nature of the modification defect in "I-cell" fibroblasts and in cells from a patient with sialidosis where a combined β-galactosidase and neuraminidase deficiency also seems to be caused by a genetic defect in one of the modification steps for the enzymes.

SAMENVATTING

Lysosomen zijn door een membraan omgeven onderdelen van de cel waarin zich hydrolytische enzymen bevinden. Hun funktie is de voortdurende afbraak van bestanddelen van de cel zelf en van stoffen die van buiten worden opgenomen. Deze afbraakprocessen kunnen onder andere een regulerende invloed hebben op de cellulaire stofwisseling en zijn ook van belang bij het regelmatig vernieuwen van de inhoud van de cel (zie Hoofdstuk 1.1). Bij de mens is op het ogenblik een dertigtal erfelijke ziekten bekend, waar één (of meer) van de lysosomale enzymen niet aktief is (zijn). Dit leidt tot ophoping in de lysosomen van die molekulen die in normale cellen door dit enzym zouden worden afgebroken (Hoofdstuk 1.2). Cellen, weefsels en hele organen kunnen ten gevolge van een dergelijke lysosomale stapeling ernstig beschadigd worden. Afhankelijk van de aard van het enzym dat genetisch afwijkend is, de eventuele restaktiviteit en -daarmee samen hangend- soort en hoeveelheid stoffen, welke zich in de lysosomen ophopen, vertonen patiënten met "lysosomale stapelingsziekten" ziektebeelden waarbij verschillende orgaansystemen zijn betrokken en waarbij de lichamelijke en geestelijke achteruitgang snel of minder snel tot de dood leidt. Van de meeste lysosomale stapelingsziekten zijn nu zowel progressief verlopende infantiele varianten bekend, waarbij de patiënt reeds op jonge leeftijd overlijdt, als langzamer verlopende juveniele vormen en, nog mildere, volwassen typen. Hoewel veel bekend is geworden over de biochemische etiologie van de verschillende lysosomale stapelingsziekten is ons inzicht in de pathogenese nog zeer beperkt.

In principe lijkt het mogelijk om patiënten met lysosomale stapelingsziekten te behandelen door toediening van het enzym dat ze zelf niet in aktieve vorm kunnen maken (zie Hoofdstuk 1.3). De afgelopen 15 jaar zijn daartoe al vele pogingen gedaan door patiënten enzym in de bloedstroom in te spuiten of door cellen, weefsels of organen te transplanteren, in de hoop dat deze een constante bron van normaal enzym zouden zijn. Helaas hebben verreweg de meeste pogingen tot "enzymtherapie" geen of slechts een tijdelijk effekt gehad op de toestand van de patiënt. Er zijn nog zoveel uiteenlopende problemen op te lossen dat eerst meer informatie moet worden verkregen uit wetenschappelijk onderzoek met modelsystemen. Hierbij kan zowel onderzoek van in vitro gekweekte cellen als van proefdieren met erfelijke afwijkingen, die grote overeenkomst vertonen met sommige lysosomale stapelingsziekten van de mens, een belangrijke bijdrage leveren.

Eén van de grote problemen bij enzymtherapie is dat van buitenaf toegediende enzymen meestal niet in die weefsels en organen terechtkomen, waarvan de cellen het meeste gestapeld produkt bevatten en waar dus voldoende enzymaktiviteit het meest dringend gewenst is. Exogene enzymen blijken voornamelijk door cellen in de lever te worden weggevangen; daarnaast wordt het bereiken van de hersencellen verhinderd door de bloed-hersenbarrière. Het is in de toekomst misschien mogelijk om bepaalde weefsels specifiek bereikbaar te maken door de molekulaire struktuur van lysosomale enzymen zodanig te veranderen dat ze alleen door een bepaald type cellen worden herkend en opgenomen.

Specifieke herkenning van lysosomale enzymen en andere glycoproteïnen (suikerketens bevattende eiwitten) is een gebied van onderzoek dat de laatste jaren sterk in de belangstelling is gekomen, sinds de ontdekking door Ashwell en Morell dat sommige in plasma voorkomende glycoproteïnen na afsplitsing van terminale sialzuurgroepen met grote efficiëntie door levercellen (hepatocyten) worden opgenomen (zie Hoofdstuk 1.4). Uit verschillende onderzoekingen is gebleken dat bij de opname van glycoproteïnen door uiteenlopende celtypen, zoals hepatocyten, reticuloendotheliale cellen en gekweekte huidcellen (fibroblasten), bepaalde suikergroepen als herkenningstekens een belangrijke rol spelen.

Fibroblasten kunnen op efficiënte wijze lysosomale enzymen opnemen die als herkenningsteken mannose 6-phosphaatgroepen dragen. Recent onderzoek heeft uitgewezen dat dit herkenningsteken niet alleen de opname van uit de buitenwereld afkomstige molekulen bepaalt, maar waarschijnlijk vooral van belang is bij het transport van door de cel gesynthetiseerde lysosomale enzymen naar het lysosoom. Lysosomale enzymen worden in meerdere synthesestappen gemaakt (zie Hoofdstuk 1.5); na synthese van één of meer polypeptiden vindt aanhechting van suikerketens plaats. Zowel het eiwit- als het suikergedeelte van deze "precursor" molekulen zijn vermoedelijk onderhevig aan een reeks van modifikaties, waarvan het aanbrengen van mannose 6-phosphaat er één is. Deze modifikaties zijn waarschijnlijk essentiëel voor een juiste intracellulaire lokalisatie van de enzymen.

Bij de lysosomale stapelingsziekte mucolipidosis II ("I-cell" disease), die gepaard gaat met ernstige lichamelijke en geestelijke afwijkingen resulterend in een vroege dood, bestaat een multiple lysosomale enzymdeficiëntie in gekweekte huidfibroblasten, terwijl juist een hoge aktiviteit van deze enzymen in het kweekmedium boven de cellen voorkomt. Gekweekte fibroblasten van patiënten met deze ziekte zijn de afgelopen jaren veelvuldig gebruikt als model om de herkenning en opname van lysosomale enzymen te bestu-

deren. Recent kon door Hasilik en Neufeld de foutieve lokalisatie van lysosomale enzymaktiviteiten in "I-cell" disease worden geassocieerd met het ontbreken van het korrekte herkenningsteken, gephosphoryleerd mannose, op de molekulen. Over de normale route die lysosomale enzymen volgen en het stadium waarin mannose 6-phosphaat als richtingaanwijzer fungeert zijn verschillende hypothesen geformuleerd:

- transport van lysosomale enzymen naar het lysosoom speelt zich geheel binnen de cel af; d.m.v. mannose 6-phosphaat kunnen (pro)enzymen worden verankerd in pasgevormde (primaire) lysosomen;
- transport van lysosomale enzymen verloopt via het buitenoppervlak van de cel; mannose 6-phosphaat zou funktioneren bij het "sorteren" van glycoproteïnen die de cel moeten verlaten en de lysosomale enzymen die naar het lysosoom moeten worden getransporteerd;
- transport van lysosomale enzymen verloopt via de extracellulaire ruimte; mannose 6-phosphaat zorgt voor binding aan het buitenoppervlak van de cel, waarna opname gevolgd wordt door lokalisatie van de enzymen in het lysosoom;
- lysosomale enzymen die zich al in het lysosoom bevinden, kunnen naar het buitenoppervlak van de cel en naar de extracellulaire ruimte getransporteerd worden wanneer membranen van lysosomen fuseren met de membraan die de cel omgeeft; een dergelijk mechanisme zou noodzakelijk zijn i.v.m. de hoge turnover van de celmembraan; binding d.m.v. mannose 6-phosphaat beperkt het verlies van enzymmolekulen aan de extracellulaire ruimte.

Het doel van het in dit proefschrift beschreven onderzoek was enkele aspekten te bestuderen van de cellulaire afgifte, herkenning en opname van lysosomale enzymen. Normale fibroblasten van de mens en van enkele diersoorten en daarnaast enige andere celtypen zoals hepatocyten en hepatomacellen (levercellen en levertumorcellen) werden gebruikt als donorcellen. De opname van de enzymen werd bestudeerd in fibroblasten van patiënten met een lysosomale stapelingsziekte die òf één lysosomale enzymaktiviteit missen òf deficiënt zijn in meerdere lysosomale enzymen ("I-cell" disease). In een aantal studies werd het systeem van samenkweken van normale en mutante cellen gekozen om de afgifte en opname van lysosomale enzymen in fysiologische concentraties te kunnen bestuderen. Onze studies hebben een bijdrage geleverd aan het inzicht in het normale proces van transport van lysosomale enzymen binnen de cel en tussen cellen. Daarnaast hopen we dat meer kennis omtrent herkenning en opname van

enzymen door deficiënte cellen, afkomstig van patiënten met een erfelijke stofwisselingsziekte, op langere termijn van betekenis zal zijn voor klinisch toegepaste enzymtherapie.

In Appendix paper I wordt beschreven hoe normale fibroblasten van de mens werden samengekweekt met enzymdeficiënte fibroblasten. Na samenkweek in een gemengde culture werd in individuele cellen de te onderzoeken enzymaktiviteit bepaald met behulp van microchemische technieken. Op deze wijze kon worden aangetoond dat het lysosomale enzym hexosaminidase van normale naar deficiënte cellen kan worden overgedragen. Deze intercellulaire overdracht bleek geen gemeenschappelijk kenmerk van alle lysosomale enzymen te zijn, want β-galactosidase en α-glucosidase werden niet overgedragen naar deficiënte cellen. Overdracht van hexosaminidase bleek ook mogelijk als de normale cellen en de deficiënte cellen werden samengekweekt zonder cellulair kontakt (Appendix paper II). Dit houdt in dat enzymmolekulen die in het kweekmedium zijn uitgescheiden kunnen worden opgenomen. Uit deze proeven kan ook de bijdrage van opname uit de extracellulaire ruimte aan het instandhouden van de hexosaminidase aktiviteit in normale cellen worden berekend; deze bleek ongeveer 11% van het totaal te zijn. Verder vonden we aanwijzingen dat het ontbreken van aantoonbaar intercellulair transport van α-glucosidase en β-galactosidase te wijten is aan een te gering aantal aktieve enzymmolekulen uitgescheiden door de normale cellen.

Uit de experimenten beschreven in Appendix paper II bleek de mogelijkheid van transport van door deficiënte cellen opgenomen hexosaminidase - dat waarschijnlijk een lysosomale lokalisatie heeft - naar de buitenkant van de cel en naar de extracellulaire ruimte. De mate van transport van het intra- naar het extracellulaire compartiment deed vermoeden dat in normale celkweken de extracellulaire aktiviteit van lysosomale enzymen voor het grootste deel een lysosomale herkomst heeft. Dit betekent dat intercellulaire overdracht van lysosomale enzymen - een verschijnsel dat afhankelijk lijkt te zijn van die extracellulaire aktiviteiten - niet plaatsvindt vóór maar nadat de enzymen het (secundair) lysosoom bereikt hebben.

In de experimenten die in Appendix paper III zijn beschreven hebben we onderzocht of intercellulaire overdracht van lysosomale enzymen ook mogelijk is tussen fibroblasten en andere celtypen. Fibroblasten, hepatocyten en hepatoma cellen van de rat werden vergeleken als donorcellen van normaal enzym, waarbij deficiënte menselijke fibroblasten

als acceptor cellen werden gebruikt. Daarnaast was het mogelijk, door de verschillende electrophoretische mobiliteit van hexosaminidase in cellen van de mens en van de rat, om na te gaan of deze verschillende celtypen van de rat enzym kunnen opnemen van menselijke fibroblasten. Het verschil in species had geen invloed op de uitwisseling van enzymen tussen fibroblasten van de rat en van de mens. De verschillende typen cellen van de rat bleken onderling echter wel duidelijk verschillende eigenschappen te vertonen, zowel in hun capaciteit om zelf enzymen op te nemen als in de opname-efficiëntie van door deze cellen uitgescheiden enzymen. Gekweekte hepatocyten namen geen hexosaminidase op van normale menselijke fibroblasten en scheidden zelf enzymen uit die niet of nauwelijks door fibroblasten konden worden opgenomen. Hepatomacellen bleken echter wel in staat om normaal hexosaminidase van menselijke fibroblasten op te nemen. Een onverwachte bevinding was dat hepatomacellen ook in staat waren tot opname van hexosaminidase dat uitgescheiden was door "I-cell" fibroblasten. Uit experimenten van andere onderzoekers, o.a. de groep van Neufeld, was gebleken dat de mutante lysosomale enzymen van deze fibroblasten niet door andere menselijke fibroblasten herkend en opgenomen kunnen worden. Ook de lysosomale enzymen die door hepatomacellen worden uitgescheiden vertoonden opmerkelijke eigenschappen; deze werden namelijk zelfs efficiënter door deficiënte menselijke fibroblasten opgenomen dan enzym afkomstig van normale fibroblasten. De lysosomale enzymen van hepatomacellen vertoonden onderling verschillende opname-efficiënties. Deze kenmerken maken rat hepatomacellen tot een interessant modelsysteem om herkenning en opname van lysosomale enzymen te bestuderen.

Een andere cellijn, die we hebben gebruikt voor de bestudering van de intra- en extracellulaire lokalisatie van een lysosomaal enzym, is een huidfibroblastenlijn afkomstig van een patiënt met de lysosomale stapelingsziekte mannosidosis (Appendix paper IV). Door deze cellen worden waarschijnlijk twee typen zure α-mannosidase geproduceerd die in verschillende compartimenten worden aangetroffen. De ene vorm heeft als gevolg van een genmutatie verminderde katalytische aktiviteit ten opzichte van het substraat van het enzym (wat bij deze patiënten leidt tot stapeling van mannose bevattende verbindingen), maar beschikt wel over normale herkenningseigenschappen. Deze vorm komt voornamelijk in de cellen voor en wordt, waarschijnlijk ten gevolge van fusie van lysosomale- en buitenmembraan, ook op het oppervlak van de cellen gevonden. Een tweede type zure α-mannosidase vertegenwoordigt het grootste deel van de enzym-

aktiviteit in deze celkweken. Deze vorm kan wel normaal het substraat afbreken, maar mist de essentiële kenmerken voor herkenning en opname; dit type α-mannosidase kon alleen in de extracellulaire ruimte (het kweekmedium boven de cellen) worden gedetekteerd.

De meeste onderzochte lysosomale stapelingsziekten zijn geassocieerd met een deficiëntie van één lysosomaal enzym, die berust op een mutatie in het gen dat kodeert voor het eiwitgedeelte van dat bepaalde enzym. De enzymsystemen die het aanvankelijk gevormde genprodukt moeten modificeren tot het uiteindelijke enzymmolekuul zijn normaal aanwezig in de cellen van deze patiënten. Bij "I-cell" disease daarentegen zijn de genen die koderen voor de polypeptiden van de afzonderlijke lysosomale enzymen normaal, maar het genetisch defekt heeft tot gevolg dat een gezamenlijke stap in het modifikatieproces gestoord is, resulterend in het ontbreken van het juiste herkenningsteken op meerdere lysosomale enzymen. In de experimenten, beschreven in Appendix paper V, hebben we onderzocht of het "I-cell" defekt gecorrigeerd kan worden door "I-cell" fibroblasten te fuseren met fibroblasten die een deficiëntie van één lysosomaal enzym hebben. Na celhybridisatie door middel van geïnaktiveerd Sendai virus ontstonden 60-90% multinucleaire cellen met de genetische informatie van beide ouderlijnen. Enzymanalysen werden uitgevoerd na hybridisatie van "I-cell" fibroblasten met fibroblasten met verschillende typen β-galactosidase deficiëntie (afkomstig van patiënten met G_{M1}-gangliosidosis of sialidosis) of een hexosaminidase deficiëntie (van een patiënt met G_{M2}-gangliosidosis type 2 (ziekte van Sandhoff)) of een α-mannosidase deficiëntie (van een patiënt met mannosidosis). In alle gevallen werd, enkele dagen na fusie van de deficiënte ouderlijnen, gevonden dat herstel optrad van de aktiviteit van het onderzochte enzym. Omdat de cellen met een geïsoleerde lysosomale enzymdeficiëntie zelf geen aktief enzymeiwit kunnen maken, wijst dit er op dat door de "I-cell" fibroblasten gevormde eiwitketens kunnen worden verwerkt tot funktionele lysosomale enzymen met behulp van de modificerende enzymen van de fusiepartner. Bestudering van het electroforetische patroon van hexosaminidase en meting van extracellulaire lysosomale enzymaktiviteiten in het kweekmedium toonden aan dat bepaalde molekulaire afwijkingen en het cellulaire verlies van lysosomale enzymen werden gecorrigeerd na celhybridisatie van de "I-cell" fibroblasten met andere mutante cellen. Op het ogenblik wordt in ons laboratorium verder onderzoek uitgevoerd naar de precieze aard van het modifikatiedefekt in "I-cell" fibroblasten en in cellen van een patiënt met sialidosis, waar-

bij een gekombineerde β-galactosidase en neuraminidase deficiëntie het gevolg lijkt van een genetisch defekt in één van de modifikatiestappen voor deze enzymen.

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Dicky

CURRICULUM VITAE

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1 januari 1980	- indiensttreding bij de Stichting Klinische Genetica, Regio Rotterdam, waar onder leiding van Prof.Dr.H. Galjaard, Prof.Dr.D.Bootsma en Dr.A. Hagemeijer-Hausman onderzoek zal worden verricht naar chromosomale afwijkingen bij patiënten met leukemie

paper I

INTERCELLULAR EXCHANGE OF LYSOSOMAL ENZYMES:

ENZYME ASSAYS IN SINGLE HUMAN FIBROBLASTS AFTER CO-CULTIVATION

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SUMMARY

Intercellular exchange of N-acetyl-β-D-glucosaminidase (EC 3.2.1.30) β-galactosidase (EC 3.2.1.23) and acid α-glucosidase (EC 3.2.1.20) was studied after co-cultivation of normal and enzyme deficient human fibroblasts in confluent cultures. Enzyme activities were measured in single cells using microchemical procedures. After co-cultivation of normal control fibroblasts and those from a patient with Sandhoff's disease an increase of activity of N-acetyl-β-D-glucosaminidase was found in Sandhoff cells, together with a decrease of activity in normal control cells. After co-cultivation of normal fibroblasts and those from patients with glycogenosis II and GM1-gangliosidosis, no indication was found for intercellular transfer of acid α-glucosidase and β-galactosidase respectively. The significance of the results is discussed in respect of the hypothesis of Hickman and Neufeld about secretion and uptake of lysosomal enzymes.

INTRODUCTION

Cultures of human fibroblasts have proven to be a useful model system for the study of lysosomal storage diseases. Correction of the metabolic defect by administration of enzyme preparations to fibroblasts in culture was reported in several studies (1-7). It has been suggested that enzyme replacement might be a feasible therapy in lysosomal storage diseases.

A more fundamental interest in uptake and secretion of lysosomal enzymes was raised in studies with cultured cells from patients with I-cell disease. For several acid hydrolases increased levels of activity were found in the medium of cultured cells from these patients, together with decreased levels of intracellular activities (8, 9). Hickman and Neufeld suggested that lysosomal enzymes have to be secreted and subsequently are taken up via specific recognition sites in order to reach their

lysosomal destination. I-cell disease would be the result of a defective uptake. To investigate the general validity of this hypothesis we have studied the intercellular exchange of three acid hydrolases in mixed confluent cultures of normal and enzyme deficient cells. In this way administration of enzyme preparations was avoided whereas the conditions for transfer of enzyme were optimal by close contact of donor and acceptor cells. The exchange of N-acetyl-β-D-glucosaminidase (further to be referred as hexosaminidase), β-galactosidase and acid α-glucosidase was studied using fibroblasts from patients with deficiencies for these enzymes as acceptor cells. Enzyme activities were measured in single cells after co-cultivation with normal cells.

METHODS

Cell culture and isolation: Cells were cultured in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics. In addition to fibroblasts from control subjects fibroblasts were cultured from patients with Sandhoff's disease (deficient in both lysosomal forms of hexosaminidase), GM1-gangliosidosis type 1 (deficient in lysosomal β-galactosidase) and glycogenosis II (deficient in lysosomal α-glucosidase). Preceeding co-cultivation cells from different strains were labeled with either latex or carbon particles. Suspensions of latex particles were obtained from Serva (Dow latex, 0.79 μ) and were added at a concentration of 0.02 ml to 2×10^6 cells in 10 ml culture medium. Suspensions of carbon particles were obtained from George T Gurr (Indian Ink, cat no 51400) and were added at a concentration of 0.1 ml. The medium was removed after one day culturing, the cells were washed three times with 0.9% NaCl and trypsinized. Equal numbers of normal and enzyme deficient cells were mixed and confluent cultures were initiated. Growth in confluency was allowed for one to ten days. The cultures were trypsinized after this period of co-cultivation and reseeded in low density in petri dishes with a thin plastic foil (mylar) bottom, to enable single cell analysis. After cultivation for 20 hrs. differently labeled cells were identified with phase contrast microscopy and localized using a micro grid. Cultures were then freeze dried, whereupon preselected cells could be isolated by microdissection as described by Galjaard et al. (11).

Enzyme assays: 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-galactopyranoside and 4-methylumbelliferyl-α-D-glucopyranoside were used as substrates for the determination of hexosaminidase, β-galactosidase and α-glucosidase activity respectively. Detailed conditions for the assay of β-galactosidase are described by Galjaard et al (12). A method for the single cell analysis of α-glucosidase is described by Reuser et al (13). To assay hexosaminidase activity single fibroblasts were incubated in 0.1 μl phosphate (20 mM)-citrate (10 mM) buffer pH 4.5 containing 5 mM substrate and 0.02%, heat inactivated, bovine serum albumin. The reaction was allowed to proceed for one hour at 37°C and stopped by the addition of 1 μl, 0.5 M carbonate buffer

pH 10.7. Fluorescence was measured in glass capilleries as described for β-galactosidase. Enzyme activities were calculated using a standard curve of methylumbelliferone (MU). Empty pieces of plastic foil were dissected to serve as blanks.

RESULTS

Normal control fibroblasts and enzyme deficient cells were labeled with either latex or carbon particles. Control experiments showed that marker material did not influence the enzyme activities measured. In several experiments equal numbers of fibroblasts from a control subject and from a patient with Sandhoff's disease lacking both lysosomal forms of hexosaminidase were mixed and co-cultivated in confluent cultures for various time periods. As a control both cell lines were cultured separately for the same period. In the experiment described, the cells were co-cultivated for ten days as a confluent culture and then reseeded in low density in petri dishes. After a subsequent cultivation for 20 hours the cells were localized, freeze dried and isolated selectively as described in Methods. Hexosaminidase activity was assayed in single cells of both types.

Fig. 1A shows the frequency distribution of hexosaminidase activity in fibroblasts from control subject and patient when cultured separately. Low residual activity was present in Sandhoff cells (mean activity 0.06×10^{-12} moles MU per hr) whereas in normal fibroblasts the mean activity was 4.6×10^{-12} moles MU per hr. After ten days of co-cultivation marked changes were noticed in the distributions as shown in Fig. 1B. The mean activity of Sandhoff cells was increased to 0.7×10^{-12} moles MU per hr whereas the mean activity of control cells was decreased to 1.7×10^{-12} moles MU per hr. The same effect was found after three days of co-cultivation although less prominent.

In similar experiments exchange of β-galactosidase was studied. Fibroblasts from a patient with GM1-gangliosidosis, lacking lysosomal β-galactosidase, were co-cultivated with normal human fibroblasts in equal numbers for eight days. The frequency distributions of enzyme activity for both types of cells when cultured separately are

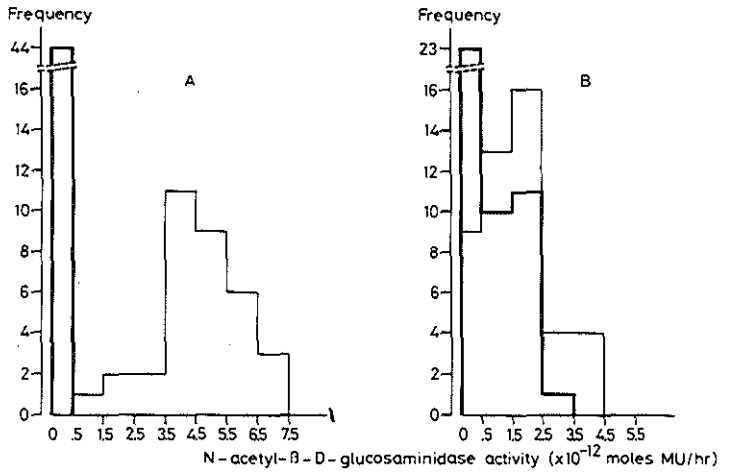

Fig. 1. Frequency distributions of hexosaminidase activity in single human fibroblasts derived from a control subject and from a patient with Sandhoff's disease.
A. Separate cultivation
B. Co-cultivation for 10 days in confluency
— patient — control

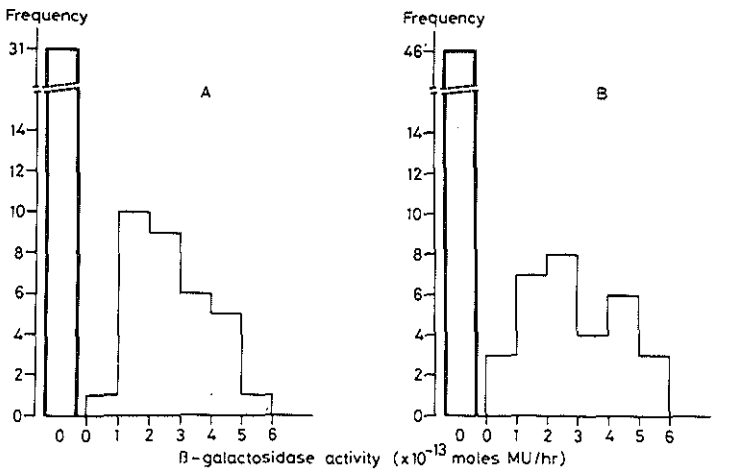

Fig. 2. Frequency distributions of β-galactosidase activity in single human fibroblasts derived from a control subject and from a patient with GM1 gangliosidosis.
A. Separate cultivation
B. Co-cultivation for 8 days in confluency
— patient — control

given in Fig. 2A. Distributions after co-cultivation are shown in Fig. 2B. In neither case β-galactosidase activity could be demonstrated in fibroblasts from the patient. The mean activity of normal cells was 1.36×10^{-13} and 1.38×10^{-13} moles MU per hr. respectively. This indicates that no intercellular exchange of β-galactosidase occurred.

Fibroblasts from a patient with glycogenosis II lacking acid α-glucosidase were used as acceptor cells to study exchange of this enzyme. The experiments were done in a similar way as described above. Cells were co-cultivated for seven days. The frequency distributions of α-glucosidase activity of normal and deficient cells in separate and mixed cultures are shown in Fig. 3A and 3B respectively.

No enzyme activity could be demonstrated in fibroblasts from the patient with glycogenosis II. The mean activity of normal cells was 4.7×10^{-14} moles MU per hr. when cultured alone and 3.9×10^{-14} after co-cultivation.

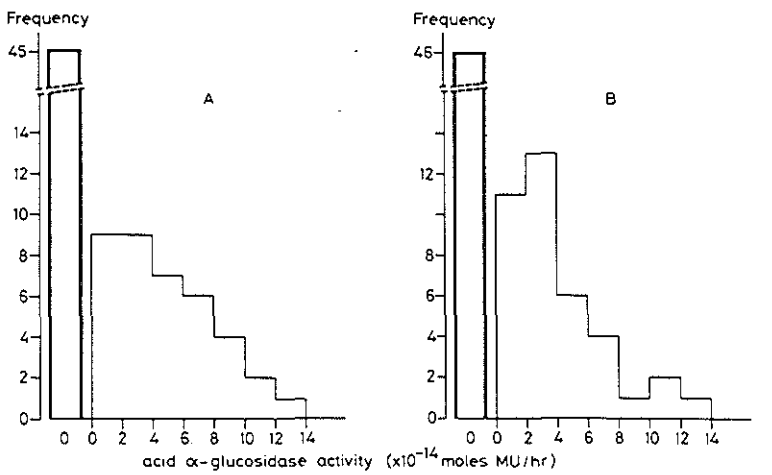

Fig. 3. Frequency distributions of acid α-glucosidase activity in single human fibroblasts, derived from a control subject and from a patient with glycogenosis II.
A. Separate cultivation
B. Co-cultivation for 7 days in confluency
patient — control

No transfer of acid α-glucosidase activity from normal to deficient cells was found.

DISCUSSION

Selective and bulk pinocytosis are distinguished for uptake of extracellular material. The former process requires specific recognition sites on the cell surface as well as on the molecule that is taken up (14). According to the hypothesis of Hickman and Neufeld (10) secretion followed by selective uptake is essential for packaging of lysosomal enzymes. This implies the possibility of transfer of enzyme from one cell to another. Mutual correction of the metabolic defect in cultured fibroblasts from clinically different patients with mucopolysaccharidosis was demonstrated to be the result of free exchange of lysosomal enzymes (1, 2, 3). The hypothesis of Hickman and Neufeld is mainly based on studies with I-cells and the authors indicate that the theory might not apply to all lysosomal enzymes. Strong evidence has been presented for the uptake of hexosaminidase via specific recognition sites (15).

We have studied exchange of hexosaminidase, β-galactosidase and α-glucosidase by co-cultivation of normal control and enzyme deficient cells in confluent cultures thereby trying to approach the in vivo situation, avoiding the administration of enzymes in quantities far exceeding the intracellular activity.

The clear transfer of hexosaminidase activity from normal cells to enzyme deficient cells fits in with the Hickman and Neufeld hypothesis. Interestingly the increase in hexosaminidase activity in Sandhoff cells, as a result of co-cultivation, is accompanied by a loss of activity in normal fibroblasts. No compensation seems to occur for the loss of intracellular activity from normal cells.

No transfer of β-galactosidase or α-glucosidase activity could be demonstrated in our experiments. This might indicate that for these enzymes the packaging into lysosomes is different compared to hexosaminidase. In this case the simultaneous lack of β-galactosidase and hexosaminidase in I-cell disease can not be explained by a mutation affecting recognition sites on the enzyme molecules.

A different explanation for our results might be that the enzyme deficiencies in GM1-gangliosidosis and glycogenosis II are caused by a defect in the recognition sites on the cell surface, preventing uptake of β-galactosidase and α-glucosidase respectively. No cross-reacting material could be demonstrated in liver cells of a patient with glycogenosis II (16) which may support this hypothesis. Detection of cross-reacting material in the liver cells of a patient with GM1-gangliosidosis (17) however, points to a structural mutation in the enzyme molecule rather than to a defect in the uptake of β-galactosidase.

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paper 4

THE DISTRIBUTION OF HYDROLYTIC ENZYME ACTIVITIES IN HUMAN FIBROBLAST CULTURES AND THEIR INTERCELLULAR TRANSFER

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SUMMARY

Both N-acetyl-β-D-glucosaminidase A and B (EC 3.2.1.30) are continuously secreted by normal cultured fibroblasts and can be taken up by deficient Sandhoff cells without cellular contact. The absence of intercellular transfer of β-galactosidase (EC 3.2.1.23) and acid α-glucosidase (EC 3.2.1.20) in co-cultivations of normal and deficient fibroblasts is accompanied by very low extracellular activities of these enzymes in cultures of normal fibroblasts. For each of the hydrolases tested an appreciable amount of activity was found in the "pericellular" fraction. N-acetyl-β-D-glucosaminidase which has been taken up by deficient Sandhoff cells has an intracellular half-life of 6 days. The ingested intracellular enzyme, which is presumably localized in the lysosomes, is partly transferred to the pericellular fraction, and to the extracellular fraction. The results are discussed in relation to the secretion-recapture model proposed by Hickman and Neufeld.

INTRODUCTION

According to Hickman and Neufeld's hypothesis (1, 2) the introduction of hydrolytic enzymes into the lysosomes of normal human fibroblasts implies intercellular exchange of these enzymes. In this hypothesis the enzymes are equipped with a recognition marker, subsequently secreted and recaptured by a receptor-mediated endocytosis, and only then packaged into lysosomes. In the recessive genetic diseases Mucolipidosis II and III the increased extracellular levels and the decreased intracellular activities of a number of lysosomal enzymes would be the result of a mutation affecting the common recognition marker of these enzymes.

Specific recognition of several lysosomal enzymes by human fibroblasts has been described (e.g. 3, 4, 5, 6). Each of these studies involved the addition of exogenous enzyme to deficient fibroblasts in amounts far exceeding the normal activities in human cells.

To avoid the administration of unphysiologically high activities, Reuser et al. (7) have studied the cell-to-cell transfer of hydrolases. The intercellular transfer of N-acetyl-β-D-glucosaminidase, β-galactosidase and acid

α-glucosidase was tested after co-cultivation of normal and enzyme deficient fibroblasts and subsequent enzyme analysis of single cells. In this way the transfer of N-acetyl-β-D-glucosaminidase (further to be referred to as hexosaminidase) was clearly demonstrated, but no transfer was found for β-galactosidase and α-glucosidase.

The purpose of the present study was to investigate the background of the different behaviour of these three hydrolases in co-cultivation experiments. The activities were measured in the intracellular, pericellular, and extracellular fractions of fibroblast cultures. Co-cultivation was performed without cellular contact between normal and enzyme deficient cells. Finally we studied the fate of hexosaminidase taken up by deficient Sandhoff fibroblasts.

METHODS

Preparation of cell material: Fibroblasts from control subjects and patients with Sandhoff's disease were cultured in Ham's F10 medium supplemented with 10% fetal calf serum and antibiotics. When harvesting of pericellular and extracellular fractions was required lysosomal enzyme activities in the fetal calf serum were destroyed by incubating the serum at pH 10, 37°C for 3 hours. Medium samples were collected from confluent cultures, centrifuged at 450g for 10 min, and concentrated up to 100x with polyethylene glycol. Pericellular fractions were prepared by incubating the cells for 10 min with 0.25% trypsin. After resuspending the cells in inactivated fetal calf serum and centrifuging at 140g for 5 min the supernatant served as pericellular fraction. Cell pellets were washed, and homogenized by freezing and thawing.

Normal and Sandhoff cells were co-cultivated as separate populations in the same culture medium. Cells were seeded onto 75x35 mm microscope slides in 90 mm plastic petri dishes. After the cells had settled, slides with deficient cells were placed upside down on top of slides with normal cells, the slides being separated by 1.5 mm stainless steel rods. Normal and deficient cells in separate dishes were treated in the same way and served as controls.

To study the fate of secreted hexosaminidase taken up by Sandhoff cells confluent cultures of Sandhoff fibroblasts were incubated with medium previously conditioned for 10 days by normal or Sandhoff cells. After 2 days incubation cells were trypsinized and aliquots were reseeded as confluent cultures and grown in fresh medium for 6 days. Subsequently the cells, the medium, and the newly formed pericellular fractions were analyzed.

To measure the half-life of ingested hexosaminidase a homogenate of normal cells was made in medium supplemented with inactivated fetal calf serum. After centrifugation (450g) the supernatant was applied to confluent cultures of Sandhoff fibroblasts. After 4 days the cells were washed and the medium was changed. Duplicate cultures were analyzed at different time intervals (0-22 days).

Enzyme assays: 4-Methylumbelliferyl substrates were used for the assays of hexosaminidase (8), α-glucosidase (9), and β-galactosidase (10) as described earlier. For the latter the incubation buffer was supplemented with 0.1M NaCl instead of albumine. The separate activities of hexosaminidase A and B were determined either by heat inactivation (8) or by incubation with DEAE cellulose as described by D'Azzo et al. (11). The protein content of the cell homogenates was assayed according to Lowry et al. (12). The intracellular, pericellular, and extracellular hydrolase activities were all expressed per milligram of cellular protein and are the averages of duplicate cultures. We also attempted to examine the presence of inactive β-galactosidase molecules in the culture medium with an antiserum raised against partially purified human β-galactosidase (13). The purity of the pericellular fraction was tested by comparing the

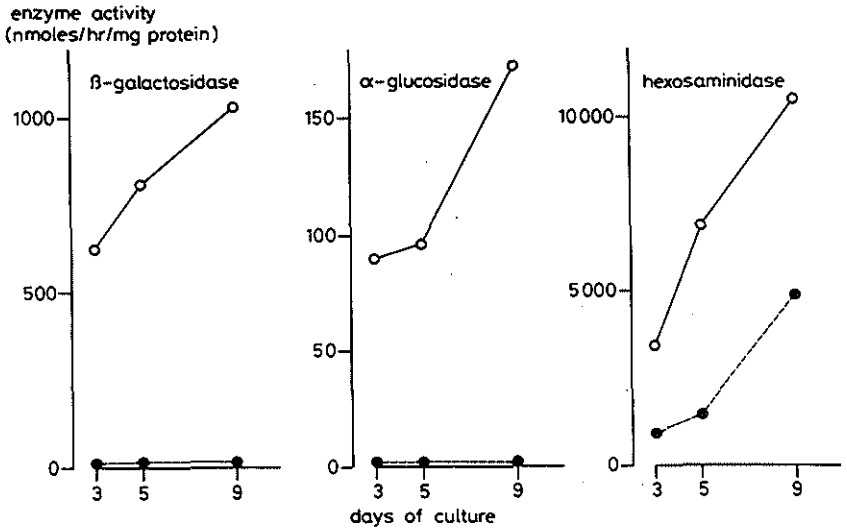

Fig.1. Hydrolase activities in confluent cultures of normal fibroblasts after different periods of cultivation: O—O = intracellular activity; ●---● = extracellular activity.

activities of the hydrolases with the cytoplasmic enzyme glucose-6-phosphate dehydrogenase assayed according to Jongkind (14).

RESULTS

HYDROLASE ACTIVITIES IN INTRACELLULAR, EXTRACELLULAR, AND PERICELLULAR FRACTIONS OF NORMAL FIBROBLAST CULTURES

The intracellular activities of hexosaminidase, β-galactosidase, and α-glucosidase show a clear increase during confluent growth (Fig. 1). The extracellular activities of β-galactosidase and α-glucosidase remain low and represent 1-3% of the intracellular activities. The hexosaminidase activity in the medium, however, is higher and amounts 27% of the intracellular activity after 3 days and increases to nearly 50% after 9 days (Fig. 1). The low extracellular levels of β-galactosidase and α-glucosidase may be due to either instability in the medium or to lack of secretion. When a normal cell homogenate is added to culture medium (pH 7, 37°C) the β-galactosidase and α-glucosidase activities decrease to 2% of the initial value after 2 and 7 hours respectively. The hexosaminidase activity is relatively stable: the A isoenzyme can be measured up to 4 days and the B form up to 12 days after addition of the homogenate. An attempt to investigate the presence of inactive (secreted) β-galactosidase molecules in the medium by means of an antiserum failed. β-Galactosidase inactivated either by heat (50°C) or by incubation in medium (pH 7, 37°C) was found to have lost its antigenic properties.

Table 1

INTRACELLULAR (I) AND PERICELLULAR (P) HYDROLASE ACTIVITIES IN CONFLUENT CULTURES OF NORMAL FIBROBLASTS

	Activity (nmoles/hr/mg protein) I	P	Ratio P/I (%)
β-Galactosidase	422	74	17
α-Glucosidase	138	9	7
Hexosaminidase	5862	964	16

Confluent normal fibroblasts were grown for 1 week. Similar results were obtained with 3 other cell strains: the ratios P/I (pericellular/intracellular) ranged from 6 - 19%.

Enzyme assays of the pericellular fraction show comparable ratios for the three hydrolases, when the activities are related to the intracellular levels (Table 1). The purity of the pericellular fractions activity was indicated by the absence of activity of the cytoplasmic enzyme glucose-6-phosphate dehydrogenase (i.e. less than 0.6% of the intracellular activity).

INTERCELLULAR TRANSFER OF HEXOSAMINIDASE AND FATE OF INGESTED ENZYME IN SANDHOFF FIBROBLAST CULTURES

Co-cultivation of separate populations of normal and Sandhoff cells in the same medium results in an increase of hexosaminidase activity in the deficient cells as is illustrated in Table 2. The residual activity in the Sandhoff cells is about 3% of control values, 83% of which is heat labile and 17% heat stable. When corrected for this endogenous activity the ingested hexosaminidase activity after co-cultivation with normal cells amounted 154 nmoles/hr/mg protein, 42% of which is heat stable. This is similar to hexosaminidase in normal fibroblasts, which indicates the transfer of both the heat labile A isoenzyme and the heat stable B isoenzyme.

The same effect could be achieved by culturing deficient cells with conditioned medium from normal fibroblasts. Increased hexosaminidase activity was found both in the intracellular and the pericellular fraction of the Sandhoff cells (Fig. 2A). Removal of the pericellular fraction and subsequent growth on fresh medium resulted in a decrease of the ingested intracellular activity, and reappearance of hexosaminidase activity in the newly formed pericellular fraction (Fig. 2B). No extracellular release of the enzyme could be detected.

The half-life of ingested intracellular hexosaminidase activity was studied after cultivation of deficient cells in medium containing normal cell homogenate, the activity of which was 2000 nmoles/hr/ml, which is 10 times

Table 2

CO-CULTIVATION OF SEPARATE POPULATIONS OF NORMAL AND SANDHOFF FIBROBLASTS IN THE SAME CULTURE MEDIUM

	Hexosaminidase activity (nmoles/hr/mg protein)		Hexosaminidase B (%)
Sandhoff + Normal	Sandhoff:	270	31
	Normal :	3380	44
Sandhoff + Sandhoff	Sandhoff:	116	17
Normal + Normal	Normal :	3507	40

Co-cultivation during 5 days. Similar results were obtained with different cell strains in 3 other experiments: the activity in the Sandhoff cells increased 2 - 3 fold during co-cultivation with normal cells.

higher than in the conditioned medium used in earlier experiments. The hexosaminidase activity taken up was 1524 nmoles/hr/mg protein and was found to have an intracellular half-life of 6 days. Transport of intracellular enzyme to the pericellular fraction was found, when the cells had been trypsinized after uptake. In this experiment extracellular release of the ingested enzyme was also noted. Two days after uptake the activity in the medium amounted 14% of the intracellular activity.

DISCUSSION

The secretion-recapture model for lysosomal enzymes described by Hickman and Neufeld (1, 2) involves both specific recognition and intercellular transfer of the enzymes. The selective uptake of exogenous enzyme by cultured fibroblasts has been described for several hydrolases, including β-galactosidase (4) and hexosaminidase (3), the specificity of which was mediated by carbohydrate moieties on the enzymes. However, co-cultivation does not result in intercellular transfer of β-galactosidase and α-glucosidase from normal to enzyme deficient fibroblasts (7). Cultures of normal fibroblasts were found to contain very low extracellular activities of these enzymes (Fig. 1), which may be due to a lack of secretion or to the apparent instability of the enzymes. In order to discriminate between these two possibilities we attempted the immunological detection of inactive β-galactosidase in the medium, but this was unsuccessful as a result of the simultaneous loss of catalytic and antigenic properties of the enzyme, as was described earlier for α-glucosidase (15).

An appreciable amount of β-galactosidase and α-glucosidase activity was found in the pericellular fraction of normal cells (Table 1). This might reflect the binding of secreted molecules to the cell surface, assuming an enhanced stability of bound enzyme compared with unbound secreted enzyme. It

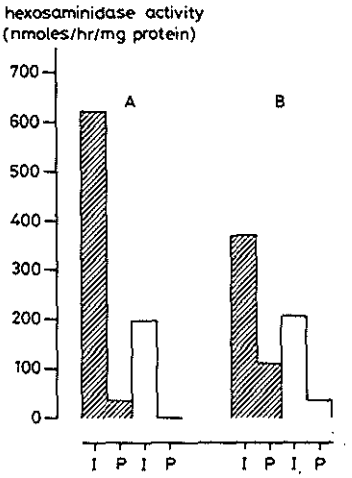

Fig.2. Distribution of ingested hexosaminidase activity during subsequent growth of Sandhoff fibroblasts. I=intracellular fraction; P=pericellular fraction.
A. Sandhoff fibroblasts incubated for 2 days with conditioned medium from normal ▨ or deficient □ cells.
B. Sandhoff fibroblasts as described in A, after removal of the pericellular fraction and 6 days of subsequent growth.

remains, however, unexplained why no intercellular transfer occurs between normal and deficient fibroblasts which have been cultured in close contact (7).

Hexosaminidase is continuously secreted by normal fibroblasts (Fig. 1) and is relatively stable in the medium. Intercellular transfer of hexosaminidase A and B occurred without cellular contact between normal and enzyme deficient cells (Table 2). This implies the possibility of transport of enzymatically active hexosaminidase through the medium, without preference for either one isoenzyme.

The intracellular half-life of ingested hexosaminidase from a normal cell homogenate was 6 days, and was similar after uptake of secreted enzyme (Fig. 2). Transport of intracelluar (presumably lysosomal) hexosaminidase was found after growth of Sandhoff cells containing ingested enzyme (Fig. 2B). Extracellular release of ingested hexosaminidase could be demonstrated after uptake of a large amount of enzyme. Both observations may reflect the mechanism of membrane reclycling as proposed by Lloyd (16). This would involve the incorporation of lysosomes into the plasma membrane as a compensation for pinocytic membrane internalization, and would thereby account for the extracellular release of lysosomal enzymes.

The secretion-recapture model implies enzyme release at a pre-lysosomal stage.

The possibility has to be considered that also in normal cell cultures extracellular hydrolase activities originate from the lysosomes.

On the basis of the present studies and those by Reuser et al. (7) an important extracellular contribution to lysosomal β-galactosidase and α-glucosidase in normal fibroblasts seems unlikely. However, for enzymes like hexosaminidase, which are both secreted and stable, such a contribution may exist.

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paper III

INTERCELLULAR EXCHANGE OF LYSOSOMAL HYDROLASES BETWEEN MUTANT HUMAN FIBROBLASTS AND OTHER CELL TYPES

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SUMMARY

Human fibroblasts with a genetic deficiency of a single lysosomal enzyme and fibroblasts from a patient with "I-cell" disease with a multiple deficiency of lysosomal hydrolases were used as recipient cells in studies on recognition and uptake of β-N-acetylhexosaminidase (hexosaminidase), β-glucuronidase and β-galactosidase. Normal human fibroblasts, and fibroblasts, hepatocytes and hepatoma cells from the rat were used as donor cells. The release of hexosaminidase was found to be similar among these different cell types, but the extracellular activities of β-glucuronidase and β-galactosidase were much higher in the rat cell cultures than in cultures of normal human fibroblasts. The enzymes released by rat fibroblasts were ingested by deficient human fibroblasts; enzyme from normal human fibroblasts was shown to be taken up by rat fibroblasts by means of electrophoresis. This indicates that reciprocal transfer of lysosomal hydrolases occurs between human and rat fibroblasts. Rat hepatocytes released hydrolases that were poorly taken up by human recipient fibroblasts and uptake of human fibroblast enzyme was not detected in the hepatocytes. Rat hepatoma cells, on the other hand, released lysosomal enzymes that were taken up by human deficient cells with a higher efficiency than those from fibroblasts. The uptake was subject to competitive inhibition by mannose 6-phosphate, the kinetics of which were comparable with those reported for "high-uptake" forms of lysosomal enzymes (1-2). Electrophoretic studies showed that rat hepatoma cells were not only capable of ingesting hexosaminidase from

normal human fibroblasts, but also defectively processed enzyme (4-5) released by "I-cells". These findings make rat hepatoma cells a useful model for the study of recognition and uptake of lysosomal enzymes.

INTRODUCTION

Recognition of lysosomal enzymes by cultured human fibroblasts is a receptor-mediated event that requires mannose 6-phosphate residues on the enzyme molecules (1-3). The important role of phosphorylated mannose in the normal process of intracellular compartmentalization of lysosomal enzymes was recently established by the demonstration that defective phosphorylation in "I-cell" disease causes a faulty distribution of lysosomal hydrolases over the intracellular and extracellular pools (4-5).

Recognition and uptake of lysosomal enzymes can be studied by the addition of purified enzymes to human fibroblasts with a genetic enzyme deficiency, as was recently reviewed by Sly et al. (6). An alternative approach is co-cultivation of normal cells and enzyme deficient fibroblasts, in which case the levels of lysosomal hydrolases, released by the normal cells, are more similar to the in vivo situation (7-9). In such experiments we showed that active hexosaminidase molecules are transported from normal human fibroblasts to mutant cells via the extracellular space (7-9). Uptake by deficient fibroblasts could not be detected for ß-galactosidase and α-glucosidase, presumably due to the low extracellular activities of these enzymes in normal fibroblast cultures (8-9).

Subsequent co-cultivation experiments (Halley et al., unpublished results) showed for several hydrolases that

human enzyme deficient cells were capable to recognize and take up enzyme released by fibroblasts from rat, Chinese hamster, mole, and chicken. In these studies it was found that the activities of some lysosomal enzymes are much higher in the culture medium above rat cells than in that above human fibroblasts. In the present study we have used these high extracellular lysosomal enzyme activities to compare fibroblasts, hepatocytes and hepatoma cells from the rat in their ability to supply human deficient fibroblasts with hexosaminidase, β-galactosidase or β-glucuronidase. As human recipient cells we used fibroblasts from patients with a single genetic deficiency of one of these enzymes, as well as fibroblasts from a patient with "I-cell" disease, which have a multiple lysosomal enzyme deficiency, including that of hexosaminidase, β-galactosidase and β-glucuronidase. The high extracellular activity of β-galactosidase and β-glucuronidase in medium above rat hepatoma cells and their efficient uptake by human deficient fibroblasts permitted a comparison of the kinetics of the uptake of these enzymes. Finally, the uptake of human enzyme by the various rat cell types was studied with electrophoretic methods.

MATERIALS AND METHODS

Cell material

All cells were cultured in Ham's F10 medium supplemented with antibiotics and 10% pH inactivated fetal calf serum (9). Human lysosomal enzyme deficient fibroblasts were derived from patients with Sandhoff's disease (deficient in β-N-acetylhexosaminidases A and B), mucopolysaccharidosis VII (MPS VII; deficient in β-glucuronidase), G_{M1}-gangliosidosis (deficient in β-galactosidase) and mucolipidosis II ("I-cell" disease; deficient in a number of lysosomal enzyme activities including β-N-acetylhexosamini-

dase, β-glucuronidase and β-galactosidase). As a source of normal enzyme, human and rat fibroblasts and rat hepatoma cells were grown. The hepatoma cells used in this study are derived from the Reuber H35 hepatoma, which is a highly differentiated hepatoma closely resembling liver in its enzymatic capacities (11). In addition rat parenchymal liver cells were isolated from Wistar rats by Dr. H.F. Bernard (Dept. of Internal Medicine III and Clinical Endocrinology, Erasmus University, Rotterdam) as described by Krenning et al. (12).

Co-cultivation

Cells were co-cultivated on microscope slides in 90 mm Petri dishes as separate populations sharing the same culture medium as described earlier (9). Twenty-five ml of medium was used per dish, with a total of about $0.5\text{-}1 \times 10^6$ cells. Recipient cells were always cultured on the top slide, which was placed upside down at a distance of 1.5 mm on a slide with donor cells. The period of co-cultivation was 3, 5 or 7 days. Medium was collected at the end of the co-cultivation and immediately centrifuged and assayed for enzyme activities. Cells were harvested by trypsinization, washed with saline and homogenized by 3 cycles of freezing and thawing using liquid nitrogen.

To test the influence of enzyme concentration in the medium on the uptake by deficient cells during co-cultivation a constant number of Sandhoff cells was co-cultivated with an increasing number of normal human fibroblasts seeded as confluent populations on several 35 mm coverslips. The numbers of normal cells in this experiment were 0.5, 1.0, 1.5, 2.5, and 4 times the number of cells normally seeded in co-cultivation studies.

Experiments using conditioned medium

Human recipient cells were also fed with culture medium that had previously been conditioned by donor cells for 3-7 days. The conditioned media from the different cell strains were diluted to yield the same enzyme activities per ml. Recipient cells in 35 mm Petri dishes were grown in 1 ml of these media over a period of 64 hrs with medium changes at 8 hr intervals. The media were stored at $4^{\circ}C$ during the course of the experiment. Control dishes were treated in the same way with fresh medium. The stability of the exogenous enzyme activities was checked after storage of the medium at $4^{\circ}C$ and after an 8 hr period

under cell culture conditions.

To test the influence of mannose 6-phosphate on enzyme uptake, recipient cells were grown in different dilutions of medium conditioned by donor cells, as described above, in the presence or absence of 0.1 mM mannose 6-phosphate (Sigma).

Enzyme assays

β-N-acetylhexosaminidase (hexosaminidase), β-glucuronidase and β-galactosidase activities were assayed with fluorogenic substrates (for review of methodology, see (12)). Ten µl of cell homogenate or culture medium was incubated at 37°C with 20 µl of the appropriate 4-methylumbelliferyl (MU) substrate solution (5 mM substrate in phosphate/citrate (20 mM/12 mM) buffer, pH 4.4, for the assay of hexosaminidase; 2 mM substrate in 0.1 M acetate buffer, pH 4.3, for β-glucuronidase; 1 mM substrate in 0.1 M acetate buffer, 0.1 M NaCl, pH 4.3, for β-galactosidase). The protein content of the homogenates was determined according to Lowry et al. (13).

Electrophoresis

Electrophoresis was carried out by applying 10-20 µl aliquots from the 3000 rpm supernatants of cell homogenates to cellulose acetate gel (Cellogel, Chemetron, Milano, Italy). Staining for hexosaminidase activity was performed with methylumbelliferyl substrate (12).

RESULTS

Normal human fibroblasts release hexosaminidase, β-glucuronidase and β-galactosidase with markedly different activities. The proportion of these activities in the medium after 3 days of culturing is 100 : 1 : 0.5 (hexosaminidase : β-glucuronidase : β-galactosidase). As a comparison the intracellular activities for these three enzymes are proportionate as 100 : 1 : 10. When the hexosaminidase activity in the medium of the normal cells is expressed per mg of cellular protein, a mean extracellular

TABLE 1 CO-CULTIVATION OF HEXOSAMINIDASE DEFICIENT HUMAN FIBROBLASTS AND NORMAL HUMAN AND RAT CELLS

Hexosaminidase activities in the medium released by normal cells and in deficient cells after enzyme uptake.

		HEXOSAMINIDASE ACTIVITY		
		MEDIUM (nmoles/hr per ml)	RECIPIENT CELLS (nmoles/hr per dish)	RATIO (act. cells : medium)
SOURCE OF MEDIUM ENZYME	RECIPIENT CELLS			
NORMAL HUMAN FIBROBLASTS	SANDHOFF			
	3 days n = 6	10.36 ± 0.96	3.13 ± 0.75	0.30 ± 0.07
	5 days n = 5	8.76 ± 1.48	5.79 ± 0.69	0.66 ± 0.04
	I-CELL			
	3 days n = 2	9.92 ± 1.24	9.20 ± 1.30	0.93 ± 0.12
	7 days	24.40	26.80	1.10
RAT FIBROBLASTS	SANDHOFF			
	5 days n = 2	24.48 ± 1.88	7.80 ± 0.80	0.32 ± 0.03
RAT HEPATOCYTES	SANDHOFF			
	3 days n = 4	9.56 ± 2.26	0	0
	I-CELL			
	3 days n = 2	5.04 ± 1.01	0	0
RAT HEPATOMA CELLS	SANDHOFF			
	3 days	1.40	3.40	2.43
	I-CELL			
	3 days n = 3	7.20 ± 2.91	45.51 ± 18.89	6.32 ± 0.27

The activity in the medium is given as the activity per ml present at the end of the co-cultivation period in non-cocultivated cultures of donor cells. The uptake by the deficient cells is expressed as the total amount of enzyme activity after correction for endogenous residual enzyme activity. The endogenous activities were determined in duplicate non-cocultivated controls in each experiment. A considerable variation in these residual activities was seen between different experiments; within one experiment the duplicate values did not differ from the mean by more than 5%. The lowest enzyme uptake was more than 4 times higher than this variation.
The mean ingested enzyme activity, the standard error of the mean and the number of experiments are shown. In the ratio of ingested and extracellular enzyme activity, the latter value is the activity at the end of the co-cultivation.

activity was measured of about 2000 nmoles MU substrate hydrolyzed per hour. Co-cultivation of fibroblasts from a patient with Sandhoff's disease and normal fibroblasts during 3 days results in increased hexosaminidase activity in the mutant cells (Table 1). When the period of co-cultivation is prolonged to 5 days, in the presence of approximately the same hexosaminidase activity in the culture medium, the Sandhoff cells accumulate more hexosaminidase activity. To investigate whether the uptake by deficient cells is dependent on the activity measured in the culture medium, we compared the hexosaminidase activity in a fixed number of deficient Sandhoff cells after co-cultivation with increasing numbers of normal human fibroblasts. As is shown in Figure 1, the uptake by deficient cells increases linearly with the activity in the medium up to an activity of 31 nmoles substrate hydrolyzed

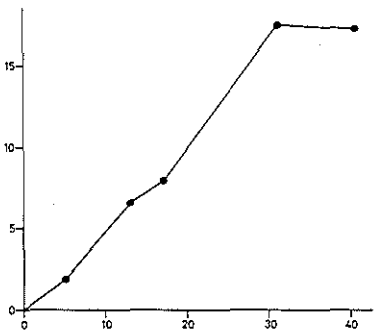

Fig. 1
Co-cultivation of Sandhoff fibroblasts with increasing numbers of normal human fibroblasts (3 days). The values are the means from duplicate cultures.
Abscissa: Hexosaminidase activity released into the medium by increasing numbers of normal human fibroblasts (nmoles/hr per ml).
Ordinate: Hexosaminidase activity in Sandhoff fibroblasts after co-cultivation (nmoles/hr per dish), corrected for endogenous activity.

per hour per ml. At still higher activities in the medium no further increase was seen after this period of co-cultivation.

We used the observed relationship between the activity in the medium and the activity taken up by deficient cells to compare co-cultivations of human hexosaminidase deficient cell strains and normal human fibroblasts or different types of rat cells (Table 1). The total activity of ingested enzyme was divided by the activity per ml of medium at the end of the co-cultivation, and this ratio served as a measure of the efficiency of uptake.

From these co-cultivation experiments (Table 1) it was learned that both rat and human fibroblasts release hexosaminidase into the medium that is taken up by human deficient Sandhoff cells. Hexosaminidase from rat hepatocytes, however, was not detectably ingested by either Sandhoff fibroblasts or I-cells (Table 1). Whereas rat hepatocytes release hexosaminidase into the medium with an activity similar to that released by human fibroblasts, the extracellular activities of β-glucuronidase and β-galactosidase are much higher in hepatocyte cultures (hexosaminidase : β-glucuronidase : β-galactosidase = 100 : 100 : 40). When we tested the uptake of these enzymes in co-cultivations with I-cells and fibroblasts from a patient with mucopolysaccharidosis VII, no uptake of β-galactosidase was detected, but β-glucuronidase was taken up with an efficiency of 0.10 ± 0.06 (n=2).

Whereas rat hepatocytes released hexosaminidase and β-galactosidase that were not taken up by human fibroblasts, the highest efficiencies of uptake of hexosaminidase in co-cultivations were found for enzyme released by rat hepatoma cells (Table 1). In this series of experiments

we noted that, whereas rat fibroblasts, rat hepatoma cells and normal human fibroblasts released hexosaminidase with a comparable activity, the amounts of β-glucuronidase and β-galactosidase released by the former are relatively large (hexosaminidase : β-glucuronidase : β-galactosidase = 100 : 50 : 30 for rat fibroblasts and 100 : 90 : 40 for hepatoma cells). In subsequent experiments we used these high extracellular activities to study the uptake of different lysosomal enzymes in a further comparison of fibroblasts and hepatoma cells as enzyme sources. In addition, we compared cell strains with either a single or a multiple lysosomal enzyme deficiency as recipient cells. These investigations were carried out with culture medium that was previously conditioned by human or rat donor cells. In this way equal amounts of exogenous enzyme activity could be applied at 8 hr intervals to I-cells and to cell strains with a single deficiency of either β-galactosidase, β-glucuronidase or hexosaminidase. Medium conditioned by normal human fibroblasts could not be used to study the uptake of β-galactosidase and β-glucuronidase because their activities were too low to allow comparison with media from rat cells. During the experiment inactivation in the media occurred for all three enzymes independently of the source of the enzyme. This resulted in a 40-50% loss of enzyme activity, measured in media collected after 64 hours, i.e. after 56 hours storage at $4^{o}C$ and 8 hours under cell culture conditions. Most of the inactivation occurred during storage at $4^{o}C$.

The results summarized in Table 2 show that the uptake of hexosaminidase from medium conditioned by rat or human fibroblasts is not clearly different when both the uptake by Sandhoff fibroblasts and by I-cells is considered.

TABLE 2 LYSOSOMAL ENZYME ACTIVITIES TAKEN UP BY DEFICIENT HUMAN FIBROBLASTS GROWN IN MEDIUM CONDITIONED BY HUMAN OR RAT DONOR CELLS

		HEXO-SAMINIDASE (nmoles/hr per dish)	RATIO (Act.cells: medium)			β-GLUCU-RONIDASE (nmoles/hr per dish)	RATIO (Act.cells: medium)			β-GALAC-TOSIDASE (nmoles/hr per dish)	RATIO (Act.cells: medium)
NORMAL HUMAN FIBROBLAST MEDIUM											
	SANDHOFF	4.49±0.06	0.22±0.01								
	I-CELL	6.92±0.38	0.35±0.01								
RAT FIBROBLAST MEDIUM											
	SANDHOFF	2.41±0.39	0.12±0.02	MPS VII		0.47±0.11	0.05±0.01	G_{M1}		0.59±0.01	0.11±0.00
	I-CELL	10.28±0.43	0.51±0.02	I-CELL		1.08±0.09	0.11±0.01	I-CELL		1.23±0.10	0.25±0.02
RAT HEPATOMA CELL MEDIUM											
	SANDHOFF	I 18.84±4.12	0.94±0.21	MPS VII	I	3.88±0.07	0.39±0.01	G_{M1}	I	3.22±0.18	0.64±0.04
		II 26.09±1.93	1.30±0.10		II	4.49±0.01	0.45±0.00		II	4.27±0.25	0.85±0.05
	I-CELL	I 21.85±1.35	1.09±0.07	I-CELL	I	4.34±0.14	0.43±0.01	I-CELL	I	5.01±0.25	1.00±0.05
		II 42.84±2.24	2.14±0.11		II	---	---		II	7.97±0.31	1.59±0.06

Human lysosomal enzyme deficient fibroblasts were fed with conditioned media from normal cells as described in Materials and Methods. The activities in the media (nmoles/hr per ml) were 20 for hexosaminidase, 10 for β-glucuronidase and 5 for β-galactosidase. The enzyme activity taken up by the deficient cells is expressed as the total amount of enzyme activity after correction for endogenous activity; the mean of duplicate cultures and the standard error of the mean are shown.
The following residual activities (nmoles/hr per dish) were measured in duplicate control cultures of deficient cells: hexosaminidase: 3.64 ± 0.03 (0.9% of the activity in normal human fibroblasts) in Sandhoff fibroblasts and 32.68 ± 0.04 (8.3% of normal) in "I-cells"; β-glucuronidase: 0.05 ± 0.00 (1.3% of normal) in fibroblasts from a patient with mucopolysaccharidosis VII (MPS VII) and 0.35 ± 0.01 (8.9% of normal) in "I-cells"; β-galactosidase: 0.32 ± 0.01 (0.8% of normal) in fibroblasts from a patient with G_{M1}-gangliosidosis (G_{M1}) and 0.45 ± 0.02 (1.2% of normal) in "I-cells". In the ratio of ingested and extracellular enzyme activity the latter value is the initial activity in the conditioned media. I and II represent two hepatoma cell lines from the same hepatoma.

Enzymes in medium conditioned by rat hepatoma cells, however, are taken up with a much higher efficiency. The activities taken up by "I-cell" fibroblasts are higher than those ingested by human cells with a single lysosomal enzyme deficiency (Table 2).

When the different doses of exogenous enzymes are

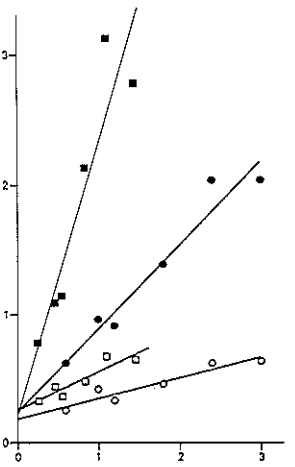

Fig. 2
Double reciprocal plots of β-galactosidase and β-glucuronidase uptake by "I-cells" from medium conditioned by rat hepatoma cells.
Abscissa: 10/activity in the medium in: 10/nmoles per hr.
Ordinate: 10/activity in the cells in: 10/nmoles per mg protein per hr.
The activity in the cells was corrected for endogenous activity.

- ○ β-galactosidase
- ● β-galactosidase, in the presence of 0.1 mM mannose 6-phosphate
- □ β-glucuronidase
- ■ β-glucuronidase, in the presence of 0.1 mM mannose 6-phosphate.

taken into consideration, the results of Table 2 suggest that hexosaminidase and β-galactosidase are taken up better than β-glucuronidase. This was confirmed for β-galactosidase and β-glucuronidase by application of different doses of rat hepatoma released enzymes to I-cells (Fig. 2); a higher uptake of β-galactosidase was achieved than of β-glucuronidase at comparable doses of exogenous enzyme. Half-maximal uptake from hepatoma cell medium was reached at a β-glucuronidase activity of 11.7 nmoles/ml.hr. and a β-galactosidase activity of 8.2 nmoles/ml.hr. The uptake of rat hepatoma enzymes could be competitively inhibited

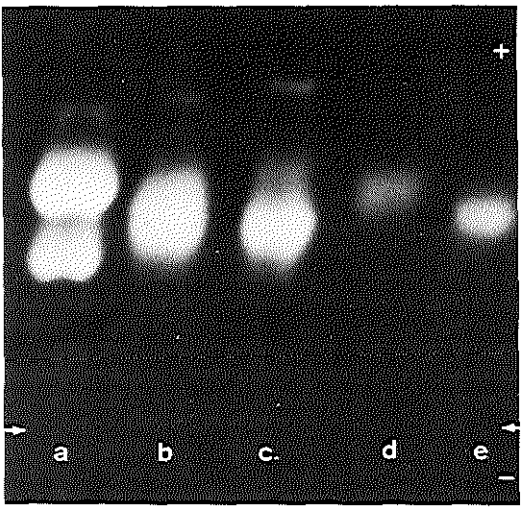

Fig. 3
Uptake by rat cells of normal human hexosaminidase. Cellulose acetate electrophoresis of hexosaminidase after growth of rat fibroblasts and hepatoma cells in medium conditioned by normal human fibroblasts. a = normal human fibroblasts; b = rat hepatoma cells after growth in conditioned medium; c = rat fibroblasts after growth in conditioned medium; d = rat hepatoma cells; e = rat fibroblasts.

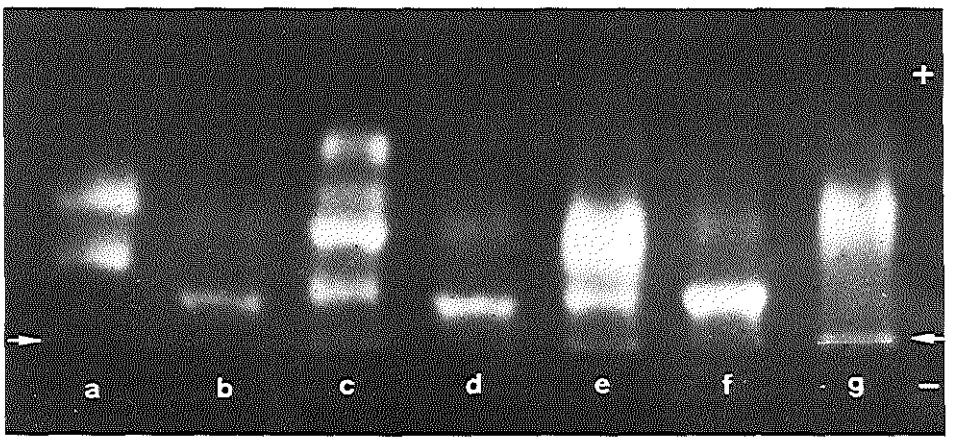

Fig. 4
Uptake by rat cells of human hexosaminidase from normal fibroblasts and "I-cells". Cellulose acetate electrophoresis of hexosaminidase after co-cultivation of rat hepatoma cells and hepatocytes with normal human fibroblasts or "I-cells". a = normal human fibroblasts (3.5 µg protein; b = rat hepatocytes after co-cultivation with normal human fibroblasts (101 µg protein); c = "I-cells" (20 µg); d = rat hepatocytes after co-cultivation with "I-cells" (93 µg); e = rat hepatoma cells after co-cultivation with "I-cells" (7 µg); f = rat hepatocytes (84 µg); 9 = rat hepatoma cells (6 µg).

by 0.1 mM mannose 6-phosphate. The apparent K_i values calculated for β-glucuronidase and β-galactosidase were 0.014 and 0.046 mM respectively.

In addition to the uptake of enzymes by human (mutant) fibroblasts we have also studied the uptake of human hexosaminidases by rat cells. This was possible because of the different electrophoretic mobility of these isoenzymes in human and rodent cells. The results in Fig. 3 illustrate that hexosaminidase released by normal human fibroblasts is taken up by both rat fibroblasts and rat hepatoma cells. These experiments were performed by growing rat cells in medium previously conditioned by human cells. The electrophoretic studies shown in Fig. 4 represent co-cultivations of human I-cells with different types of rat cells. It is shown that rat hepatocytes do not take up human hexosaminidases whereas hepatoma cells ingest hexosaminidases released by normal human fibroblasts and, more surprisingly, enzyme that is released by I-cells.

DISCUSSION

Co-cultivation experiments indicated differences in the ability of cultured fibroblasts, hepatocytes and hepatoma cells from the rat to supply human deficient fibroblasts with hexosaminidase. In addition, electrophoretic studies showed that analogous differences may exist in the capacity of these different types of rat cells to ingest human fibroblast hexosaminidase. In co-cultivation experiments lack of intercellular transfer from rat hepatocytes to deficient fibroblasts was found for both hexosaminidase and β-galactosidase. A comparison of the uptake of hexosaminidase, β-glucuronidase and β-galactosidase from rat

fibroblasts and hepatoma cells was made using media conditioned by these cells. The mannose 6-phosphate inhibitable uptake of the lysosomal hydrolases released by hepatoma cells appeared to be more efficient than the uptake of fibroblast-released hydrolases. The results were suggestive for heterogeneity in the uptake properties among the lysosomal enzymes from rat hepatoma cells. Electrophoretic studies showed that rat hepatoma cells may internalize normal human fibroblast enzyme but also partly processed form(s) released by I-cell fibroblasts.

The efficiency of uptake of purified lysosomal enzymes by cultured human fibroblasts varies widely with the source of enzyme (6, 14). ß-Glucuronidase purified from blood platelets, for instance, was found to be particularly rich in "high uptake" enzyme, compared with enzyme from placenta, liver or urine, which was taken up much less efficiently (15). Hexosaminidases from the latter sources were likewise poorly ingested by human fibroblasts (16-17). "In vivo" studies have shown that "low-uptake" forms for cultured fibroblasts are rapidly cleared from the circulation by reticuloendothelial cells (18-20). This indicates that the same enzyme may be taken up with different efficiency by different cell types.

The possibility of species specific differences in the uptake of lysosomal enzymes was investigated by Frankel et al. (21). They found a tenfold difference in the uptake of human platelet ß-glucuronidase between human and mouse fibroblasts. Rat fibroblasts were reported to be more similar to human fibroblasts, as they internalized 15% of the added activity, compared with 24% for deficient human fibroblasts. In our own experiments we did not find clear differences in the uptake of hexosaminidase released by

rat or human fibroblasts, when both human recipient strains are considered. We did, however, observe a clear difference in release and uptake among different types of rat cells. In co-cultivations, rat hepatocytes release considerable amounts of hexosaminidase, β-galactosidase and β-glucuronidase, but only β-glucuronidase was taken up by human fibroblasts. On the other hand, both co-cultivation studies and analyses of deficient human cells grown in conditioned media revealed that growth in the presence of lysosomal enzymes released by rat hepatoma cells yields high activities in deficient human fibroblasts. These activities are higher than after exposure to fibroblast enzymes, which is not related to differences in stability of the enzymes in the medium, since we found identical degrees of inactivation irrespective of the source of the enzyme. Degradation of ingested enzyme in recipient cells may be expected to occur at a lower rate in I-cells than in other fibroblasts, as a result of the simultaneous deficiency of a large number of enzymes normally involved in the degradation of glycoproteins. In agreement with this, Hickman et al. (17) found that ingested bovine liver β-glucuronidase had a much longer half-life in I-cells than in either normal fibroblasts or in β-glucuronidase deficient cells. This may explain why, in our experiments, the activities in I-cells after enzyme uptake were consistently higher than in fibroblasts with a single lysosomal enzyme deficiency (see Table 2). The differences between hepatoma cells and other cells as sources of exogenous enzyme were expressed both in I-cells and in the other recipient cells. It is therefore likely that different degrees of intracellular degradation do not interfere and that lysosomal enzymes released by hepatoma cells are taken up more efficiently

than those from the other sources tested. The suggestion that rat hepatoma cells release enzymes that are taken up with high efficiency by human fibroblasts is supported by the estimated K_i values for the inhibition of uptake by mannose 6-phosphate. These values are of the same order of magnitude as those reported for purified "high uptake" forms of α-L-iduronidase (2) and β-glucuronidase (1). The different efficiencies of uptake of hexosaminidase, β-galactosidase and β-glucuronidase indicate that heterogeneity of uptake properties may exist between the enzymes. (Table 2 and Fig. 2).

It is interesting to note that, in contrast to rat hepatocytes, rat hepatoma cells are able to ingest lysosomal hydrolases released by human fibroblasts. As was shown by our electrophoretic studies, hepatoma cells not only ingested hexosaminidases from normal human fibroblasts but also from I-cell fibroblasts. The latter is surprising in view of the fact that the defective processing of lysosomal hydrolases (4, 5) is thought to interfere with their correct intracellular compartmentalization and also with their recognition and uptake by other fibroblasts (22). Apparently the membrane properties of hepatoma cells are different from both cultured hepatocytes and fibroblasts.

The properties of the membrane, the release of "high uptake" lysosomal hydrolases, and the possibility to study heterogeneity in the uptake properties of lysosomal enzymes from one cellular source, make rat hepatoma cells an interesting model for the study of recognition and uptake of lysosomal enzymes.

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paper IV

Comparison of the α-Mannosidases in Fibroblast Cultures from Patients with Mannosidosis and Mucolipidosis II and from Controls

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The intracellular and extracellular acidic α-mannosidase in cultures of fibroblasts from mucolipidosis-II patients has normal kinetics. The extracellular activity in cultures of cells from mannosidosis patients is normal, but a mutant enzyme is associated with the cell surface and intracellular fraction. The results support the involvement of membrane cycling and recognition markers in lysosomal enzyme localization.

Several forms of α-mannosidase with different subcellular localizations occur in mammalian tissues. The lysosomal forms (Carroll *et al.*, 1972; Chester *et al.*, 1975) have an acidic pH optimum, and the cytosol contains α-mannosidase activity with an optimum at neutral pH (Marsh & Gourlay, 1971). A third type of activity with an intermediate pH optimum (Snaith, 1973; Phillips *et al.*, 1974) has been purified from Golgi membranes (Tulsiani *et al.*, 1977). This intermediate α-mannosidase activity has also been detected in body fluids such as cerebrospinal fluid and urine(Chéron *et al.*, 1975), and in serum (Chéron *et al.*, 1975; Hirani *et al.*, 1977), where it is quantitatively the major form of α-mannosidase. Apart from their pH optima, the three forms can be distinguished by their characteristic responses to Co^{2+} and Zn^{2+} ions (Hirani *et al.*, 1977). The acidic form is activated by Zn^{2+} and inhibited by Co^{2+}. The intermediate form is activated by both ions, whereas the neutral form is activated by Co^{2+} and slightly inhibited by Zn^{2+}. As the Zn^{2+} activation is pH-dependent, the presence or absence of Zn^{2+} affects the apparent pH optimum.

The human lysosomal storage disease mucolipidosis II is characterized by a multiple intracellular deficiency of lysosomal hydrolases, including acidic α-mannosidase, in cultured fibroblasts and lymphocytes, and by elevated activities of the same enzymes in extracellular fluids (Wiessman *et al.*, 1971). It has been suggested that the faulty distribution of hydrolases between the extracellular and lysosomal compartments in mucolipidosis II is due to a defect in a common recognition marker on the enzymes (Hickman & Neufeld, 1972).

A specific deficiency of acidic α-mannosidase is associated with another human storage disease, mannosidosis (Öckerman, 1969), and a mutation in the structural gene has been proposed as the primary defect in the disease (Beaudet & Nichols, 1976). A recognition defect, similar to that in mucolipidosis II, but affecting only α-mannosidase, has also been postulated (Hultberg & Masson, 1977).

In the present study some characteristics of the intra- and extra-cellular α-mannosidase activities in fibroblast cultures from patients with human mannosidosis and mucolipidosis II are compared with the corresponding activity in normal controls. In addition, we have attempted to separate that fraction of the activity that is associated with the external surface of the fibroblasts to ascertain whether it originates by re-adsorption of exocytosed enzyme from the medium or by exteriorization of intracellular activity.

Materials and Methods

Cell culture

Fibroblasts were cultured from three normal adults, a patient with mannosidosis and a patient with mucolipidosis II, in Ham's F10 medium (Flow Laboratories, Irvine, Scotland, U.K.) supplemented with 10% (v/v) pH-inactivated foetal-calf serum (Flow Laboratories), penicillin (100 units/ml of medium) and streptomycin (0.1 mg/ml of medium) (both from Gist-Brocades, Delft, The Netherlands). Fresh medium was left in contact with the resuspended cells from confluent cultures of fibroblasts for 5 days (1.5 ml of medium/3×10^5–5×10^5 cells), after which time it was poured off, centrifuged (90*g* for 10 min) and the cell-free supernatant assayed for enzyme activity. This represents the extracellular activity. The pericellular fraction was

prepared by 10 min treatment with 0.25% (w/v) trypsin (ICN Pharmaceuticals, Cleveland, OH, U.S.A.), followed by 1% soya-bean trypsin inhibitor (Worthington, Freehold, NJ, U.S.A.) in saline (0.9% NaCl) and centrifugation (140 *g* for 10 min). This supernatant represented the pericellular activity and the pellet the intracellular fraction. Cell homogenates were prepared by five cycles of freezing and thawing by using liquid nitrogen. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Enzyme assays

α-Mannosidase activities were assayed by incubation of a 10 μl sample with 30 μl of 4-methylumbelliferyl α-D-mannopyranoside in Sorensen's citrate buffer (0.1 M-citric acid in 0.2 M-NaOH adjusted to the appropriate pH with 0.1 M-HCl or -NaOH) for 3 h at 37°C. The final substrate concentration was 1 mM. The reaction was stopped by the addition of 500 μl of 0.5 M-Na_2CO_3 adjusted to pH 10.7 with NaOH, and fluorescence was read in a Perkin–Elmer fluorimeter (emission wavelength 448 nm, excitation at 365 nm). Kinetic studies were performed with final substrate concentrations between 0.5 and 20 mM. The influence of pH was tested between pH 2.6 and 7.2 at a protein concentration of 2.5 μg/10 μl. After inactivation at pH 10, the foetal-calf serum contained less than 1% of the original acidic α-mannosidase activity, which was slightly re-activated by incubation at 37°C. All extracellular activities were therefore corrected for the residual calf α-mannosidase activity present in the serum. Where indicated, $ZnSO_4$ or $CoCl_4$ was included in the reaction mixture at 1 mM final concentration, which did not influence the pH of the assay mixture.

Results and Discussion

The activity–pH profiles for the intra- and extracellular α-D-mannosidase in control fibroblasts showed the expected acidic optimum, pH 4.7, corresponding to acidic α-D-mannosidase and a shoulder of activity between pH 6.0 and 7.0 corresponding to neutral α-D-mannosidase. The pH optimum of the neutral α-D-mannosidase, pH 6.3–6.4, was clearly seen in the intracellular activities from cells from mannosidosis and mucolipidosis-II patients, in which there was a deficiency of acidic α-D-mannosidase. Table 1 shows the relative intra- and extra-cellular activities of acidic and neutral α-D-mannosidase in the strains from mannosidosis and mucolipidosis II patients and in three control strains. The activity and distribution of the neutral enzyme are clearly not affected in either of the mutant strains, both of which are characterized by a profound intracellular deficiency

Table 1. *Acidic and neutral α-mannosidase activities in fibroblast cultures from three controls and from a mannosidosis and a mucolipidosis-II patient*

The activities are the means of four cultures of each cell strain, with the ranges in parentheses, unrelated to the intracelular protein.

	α-Mannosidase (munits/mg of protein)			
	Acidic		Neutral	
	Intra-	Extra-	Intra-	Extra-
Control 1	48 (44–51)	30 (27–34)	22 (21–24)	31 (27–33)
Control 2	62 (56–65)	14 (13–15)	30 (29–31)	15 (14–16)
Control 3	37 (35–40)	19 (17–21)	25 (24–25)	17 (15–19)
Mannosidosis	2 (1.7–2.3)	11 (8–13)	21 (20–23)	20 (19–21)
Mucolipidosis II	2 (2.0–2.3)	85 (72–95)	11 (10–12)	15 (12–20)

of acidic α-D-mannosidase. Although the extracellular acidic α-D-mannosidase of the mucolipidosis-II-patient cells is high, as expected for a defect in the localization process, that for the mannosidosis cells is relatively low. Nevertheless there is a significant amount of the acidic activity in the medium from both of these cells types, despite the intracellular deficiency.

The intra- and extra-cellular acidic α-D-mannosidase activities in normal and mucolipidosis-II-patient cells all have similar K_m values and responses to Co^{2+} and Zn^{2+} (Table 2). This suggests that the defect in processing of lysosomal hydrolases in mucolipidosis II does not affect the kinetic properties of acidic α-D-mannosidase. In contrast, the intracellular acidic activity from cultures of fibroblasts from a patient with mannosidosis was different from the extracellular activity, which appeared to be normal. The higher K_m value and activation by Co^{2+} of the intracellular activity were consistent with it being a mutant enzyme. A difference between the intra- and extra-cellular acidic α-D-mannosidase activities in mannosidosis had previously been reported by Hultberg & Masson (1977) who interpreted their observation as a specific defect in the cellular resorption of acidic α-D-mannosidase in this disorder. Therefore it was decided to examine the nature of the pericellular enzyme that could be released from the surface of mannosidosis cells by treatment with trypsin. This enzyme fraction could represent exocytosed intracellular enzyme that has not been released into the medium, or re-adsorbed extracellular enzyme that for some reason was not endocytosed. This enzyme fraction was found to resemble the residual intra-

Table 2. *Effect of Zn^{2+}, Co^{2+} and substrate concentration on acidic α-mannosidase*

Numbers in parentheses indicate numbers of determinations. Inhibition or activation in the presence of Zn^{2+} or Co^{2+} is expressed as the activity in the presence of the ion as a percentage of that activity in its absence.

	Intracellular			Extracellular		
	K_m (mM)	Zn^{2+} activation at pH 4.0 (%)	Co^{2+} inhibition or activation at pH 4.7 (%)	K_m (mM)	Zn^{2+} activation at pH 4.0 (%)	Co^{2+} inhibition or activation at pH 4.7 (%)
Control	0.64	271	80	0.80	219	66
		($n = 2$)		($n = 4$)		
Mannosidosis	6.42	327	276	1.27	259	37
	($n = 3$)	($n = 2$)	($n = 4$)	($n = 3$)		($n = 2$)
Mucolipidosis II	0.66	254	63	0.80	286	51
	(n 2)			($n = 3$)	(n 2)	

cellular activity in being stimulated 2–2.5-fold by Co^{2+} and having a high apparent K_m value (7.48 mM). Recent preliminary results (A. d'Azzo & D. J. J. Halley, unpublished work) indicate also that there is no intercellular transport of secreted α-D-mannosidase from cells of a mannosidosis patient to those of a mucolipidosis-II patient in co-cultivation studies. Thus we may suppose that two forms of acid α-mannosidase are generated by the cell strain from the mannosidosis patient. A small amount of activity with abnormal kinetic properties remains in the cell and may be found exteriorized on the cell surface. On the other hand, the majority of the enzyme that is synthesized has normal kinetics, but is secreted into the medium and apparently lacks the essential properties for binding to the membrane and uptake into the cells.

These results provide evidence for membrane cycling in the localization of lysosomal enzymes (Lloyd, 1977) and for recognition between cell receptors and markers on lysosomal enzymes (Hickman *et al.*, 1974). The clear difference between the extra- and intra-cellular acidic α-mannosidase in mannosidosis make this disorder a valuable model for studying the mechanism of localization of lysosomal enzymes.

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paper V

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

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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 (β-gal$^-$/neur$^-$) deficient 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 is 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.

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 electorphoresis of the various cell homogenates indicates that the genetic complementation also results in a change of the electrophoretic pattern of 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-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*).

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^{-6} 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. Control value is the mean of assays of four control fibroblast strains. 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.

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, 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.

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* ($\times 10^{-9}$ 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.

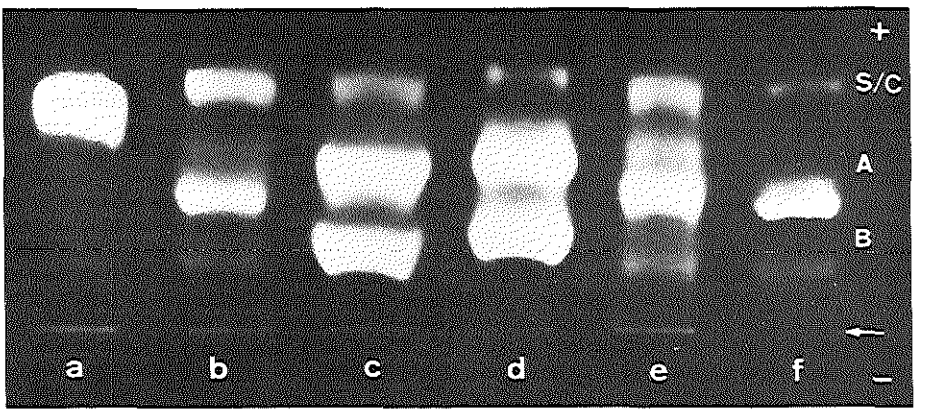

FIG. 1.—Cellulose acetate gel electrophoresis of β-*N*-acetylhexosaminidases after cell hybridization and cocultivation of human mutant fibroblasts. *lane a* = Sandhoff fibroblasts (37 μg protein), *lane b* = I-cell + Sandhoff (37 μg), *lane c* = I-cell × Sandhoff (31 μg), *lane d* = control fibroblasts (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		Experiment II
Controls (no. =3):		$\bar{X}$=53	
I-cell	5	...	6
$I\text{-cell}_2$	...	13	...
Mannosidosis	8	...	3
I-cell + Mannosidosis	7	...	5
$I\text{-cell}_2$ + Mannosidosis	...	11	...
I-cell × Mannosidosis	30	...	29
$I\text{-cell}_2$ × 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 is the mean of assays of three control fibroblast strains.

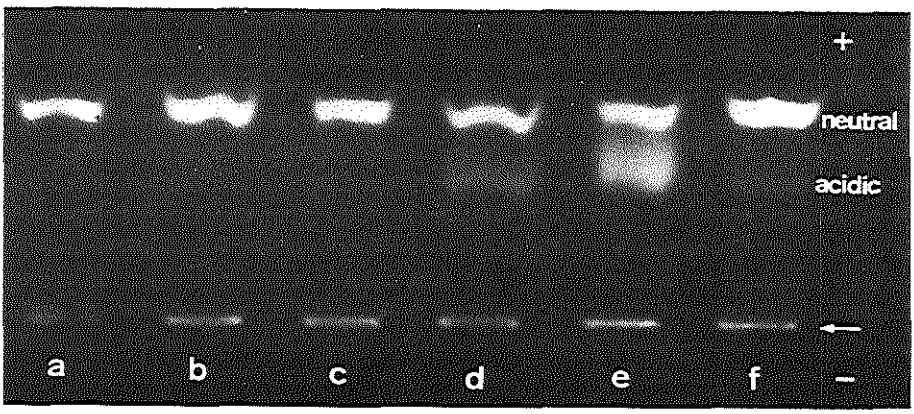

FIG. 2. —Cellulose acetate gel electorphoresis of α-mannosidases after cell hybridization and cocultivation of human mutant fibroblasts. *a* = I-cell × I-cell (34 μg protein), *lane b* = mannosidosis fibroblasts (31 μg), *lane c* = I-cell + mannosidosis (32 μg), *lane d* = I-cell × mannosidosis (24 μg),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

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 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-migration, 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.

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