# LYSOSOMAL MEMBRANE TRANSPORT
# PHYSIOLOGICAL AND PATHOLOGICAL EVENTS

Front cover: The choir gallery by Donatello, at the Museum of the Opera del Duomo (Firenze), can be imagined as a busy and lively biological membrane.

# LYSOSOMAL MEMBRANE TRANSPORT PHYSIOLOGICAL AND PATHOLOGICAL EVENTS

# LYSOSOMAAL MEMBRAAN TRANSPORT FYSIOLOGISCHE EN PATHOLOGISCHE PROCESSEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. C.J. RIJNVOS
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 18 DECEMBER 1991 OM 13.45 UUR

DOOR

GRAZIA MARIA SIMONETTA MANCINI
geboren te San Severo

PROMOTIECOMMISSIE

| | |
|---|---|
| Promotor: | Prof. Dr. H. Galjaard |
| Co-promotor: | Dr. F.W. Verheijen |
| Overige leden: | Prof. Dr. P.P. Aula<br>Prof. Dr. H.R. Scholte<br>Prof. Dr. H.K.A. Visser |

Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam.
Het onderzoek werd financieel gesteund door de Stichting Klinische Genetica regio Rotterdam.

# LYSOSOMAL MEMBRANE TRANSPORT PHYSIOLOGICAL AND PATHOLOGICAL EVENTS

THESIS

TO OBTAIN THE DEGREE OF DOCTOR
AT THE ERASMUS UNIVERSITY OF ROTTERDAM
BY AUTHORIZATION OF
THE RECTOR MAGNIFICUS PROF. DR. C.J. RIJNVOS
AND BY THE DECISION OF THE COLLEGE OF DEANS.

THE PUBLIC DEFENSE WILL TAKE PLACE
ON WEDNESDAY, DECEMBER 18th 1991 AT 13.45 p.m.

BY

GRAZIA MARIA SIMONETTA MANCINI
born in San Severo

*"Felix qui potuit rerum cognoscere causas."*
(P. Vergilius Maro, Georgica)

To all the children who cannot be cured
by a few difficult words.

CONTENTS:

# Introduction

Lysosomes are intracellular acid organelles containing a wide variety of hydrolases and surrounded by a single membrane. Compared with the amount of knowledge available about the function and the genetic defects of the lysosomal hydrolases, the understanding of the function of the lysosome membrane in physiological and pathological processes is only at the beginning. All the information available has been gained in the last decade. Before that, the lysosome membrane has been considered to be only a mechanical border limiting the access of the acidic hydrolases to the surrounding cytoplasmic substrates. This finds its explanation in the assumption that lysosomes represent essentially the "terminal degradative compartment" of the cell (Kornfeld and Mellman, 1989), and that their function is strictly linked to cellular catabolism.

Recent work has shown that efflux and bioavailability of lysosomal degradation products is controlled by specific transport proteins. In addition, in this organelle, *active* metabolites (vitamines, antigens, hormones like tyroxine) are produced and released via specific carriers, before reaching their cytoplasmic target (Gahl, 1989; Harding et al., 1991; Tietze et al., 1989). Lysosomes can also represent a storage compartment for neurotransmitters (biogenic amines, in lysosome-like specialized organelles) or for substances (like $Ca^{++}$ ions) (Lemons and Thoene, 1991; Nemere and Norman,1986), which are released under humoral control (Nemere and Norman, 1989; Nelson, 1991). Moreover, not only the efflux, but also the influx of low molecular weight metabolites into the lysosomes can represent a mechanism of regulation of metabolic functions. For example, lysosomal uptake

of cytosolic amino acids is thought to regulate autophagic proteolysis during starvation (Mortimore et al., 1988; Christensen, 1990).

All these processes are under control of specific transporters in the lysosome membrane. Information about lysosomal membrane transport proteins, as described in this thesis, has contributed to new concepts about the availability of metabolites, which in turn are essential for the metabolism in the intracellular compartments.

On the one hand, the study of cell material derived from patients with a single gene defect has enabled the identification of new transport mechanisms (cystinosis, sialic acid storage diseases, etc.). On the other hand, biochemical studies have revealed an unexpectedly large number of different types of transport systems which in some instances have formed the basis for the elucidation of the molecular defect(s) in hitherto unexplained genetic disorders.

This thesis deals with the identification of a lysosomal transport mechanism for sialic acid. A primary genetic defect of this transport mechanism is demonstrated in patients affected by lysosomal storage of free sialic acid and excessive sialuria (sialic acid storage diseases, SASD). A new patient is described, who presents with a clinical form of the disease slightly different from that of the other two well defined phenotypes (Salla disease and the infantile sialic acid storage disease, respectively). The lysosomal sialic acid carrier works as a unique $H^+$-cotransport system. Unexpectedly, this carrier shows a wide substrate specificity for acidic monosaccharides, including, for example, glucuronic acid. The demonstration of a transport defect for both sialic acid and glucuronic acid in lysosomes of patients with SASD introduced the concept of multiple transport defect in lysosomal disorders. In addition, a distinct carrier for neutral hexoses has been characterized, and its normal function in patients with SASD has been demonstrated. The first successful functional reconstitution of the lysosomal acidic sugar transporter, achieved in the course of our research, represents an important step to enable its purification and detailed molecular characterization.

REFERENCES


* Christensen, H.N. (1990) Role of amino acid transport and countertransport in nutrition and metabolism. *Physiol. Rev.* 70:43-77

* Gahl, W.A. (1989) Lysosomal membrane transport in cellular nutrition. *Annu. Rev. Nutr.* 9:39-61

* Harding, C.V., Collins, D.S., Slot, J.W., Geuze, H.J. and Unanue, E.R. (1991) Liposome-encapsuled antigens are processed in lysosomes, recycled and presented to T cells. *Cell* 64:393-401

* Kornfeld, S. and I. Mellman (1989) The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483-525

* Lemons, R.M. and Thoene, J.G. (1991) Mediated calcium transport by isolated human fibroblast lysosomes. *J. Biol. Chem.* 266:14378-14382

* Mortimore, G.E., Wert, J.J. and Adams, C.E. (1988) Regulation of microautophagy and basal protein turnover in rat liver. Effects of short term starvation. *J. Biol. Chem.* 263:19545-19551

* Nelson, N. (1991) Structure and pharmacology of the proton-ATPases. *Trends in Physiol. Sci.* 12:71-75

* Nemere,I. and Norman, A.W. (1986) 1,25 Dihydroxyvitamin $D_3$-mediated intestinal calcium transport. Biochemical identification of lysosomes containing $Ca^{++}$ and $Ca^{++}$-binding protein (calbindin-D28K) *J. Biol. Chem.* 261:16106-16114

* Nemere, I. and Norman, A.W. (1989) 1,25-Dihydroxyvitamin $D_3$-mediated vesicular calcium transport in intestine: dose-response studies. *Mol. Cell. Endocrinol.* 67:47-53.

* Tietze, F., Kohn, L.D., Kohn, A.D., Bernardini, I., Andersson, H.C., Adamson, M.D., Harper, G.S. and Gahl, W.A. (1989) Carrier-mediated transport of monoiodotyrosine out of thyroid cell lysosomes. *J. Biol. Chem.* 264:4762-4765

Chapter 1

# THE LYSOSOMAL MEMBRANE

**Chemical structure: lipid composition**

The lysosomal membrane is constituted by a phospholipid bilayer, containing a large number of membrane-associated and integral-membrane proteins. The protein/lipid ratio, in the rat liver, is the highest among the intracellular membrane types (Bleistein et al., 1980).

The lipid composition of the different intracellular organelles varies. For example, the plasma membrane has the highest content of cholesterol and contains glycosphingolipids (GLS) which are both absent in the membrane of the endoplasmic reticulum (ER) (Gennis, 1989). Yet, the majority of the lipids is synthesized in the ER, implying that specific sorting mechanisms for lipids are present in the cell.

The lysosomal membrane has a composition which resembles that of the plasma membrane, with high levels of cholesterol, sphingomyelin (SPH) and GLS. However, the level of phosphatidylserine (PS) is very low and that of phosphatidylcholine (PC) is the highest. The lysosomal membrane contains lysobisphosphatidic acid (*bis*-[monoacylglycero]-phosphate), a phospholipid which is unique to this membrane (Brotherus and Rekonen, 1977).

In lysosomes, like in other intracellular vesicles, PC, SPH and GLS are exclusively present in the lipid bilayer at the luminal (exoplasmic) face of the membrane. It has been hypothesized that cholesterol has the same location (van Meer, 1989). GLS and SPH do not translocate (flip-flop) in the lysosomal membrane. Since glycosphingolipids can translocate in the ER membrane by means of a specific "flippase" (Bishop and Bell, 1988), it was

deduced that the flippase activity is retained in the endoplasmic reticulum (van Meer, 1989).

Since GLS and SPH are restricted to transport vesicles, do not translocate in the membrane and are only present at the exoplasmic leaflets of various organelles, their location must be established during biosynthesis, but the details of this process are still unclear.

Cholesterol is abundant in the lysosomal membrane, where it is delivered from *de novo* synthesis and lipoprotein degradation (van Meer, 1989). Low density lipoprotein (LDL) -derived cholesterol can influence the intracellular metabolism of cholesterol much faster than the plasma membrane inserted cholesterol (Liscum and Faust, 1987). A possible explanation is that LDL-derived cholesterol, produced in the lysosome, can flip-flop in the membrane and rapidly reach the endoplasmic reticulum. The existence of a specific flippase, however is still hypothetical (van Meer, 1989). Studies of the lysosomal storage disease, Niemann-Pick type C, have suggested the presence of a cholesterol transport mechanism that might be defective in these patients (Vanier et al., 1991; Thomas et al., 1989; Liscum and Faust, 1987). It is known that a decrease of the sphingomyelin pool leads to a different cholesterol distribution, while the biosynthesis of sphingolipids is also under control of LDL uptake. This suggests that SPH and cholesterol metabolism are strictly linked to each other and that apparently the sorting of cholesterol to the plasma membrane is regulated by SPH in the endoplasmic reticulum (van Meer, 1989).

Though SPH is present in increased amounts in the lysosome of patients with Niemann-Pick type C disease (Vanier et al., 1991), it appeared unlikely that a primary dysregulation of SPH-cholesterol interaction or a primary SPH distribution defect is responsible for secondary lysosomal cholesterol accumulation (Thomas et al., 1989). Instead, most recent findings support that the primary defect of this disease involves the intracellular mobilization of cholesterol out of the lysosome, with a secondary inhibition of sphingomyelinase activity and consequent abnormality of SPH degradation (Vanier et al., 1991)

**Membrane proteins**

Membrane proteins are classified on the basis of the interaction between the polypeptide and the membrane (for a review, see Gennis,1989) (figure 1). The current classifications arrange the different types according to common biosynthetic pathways.

*Membrane-associated proteins* do not span the lipid bilayer but generally interact via the hydrophobic face of an amphiphilic ß-sheet or $\alpha$-helix segment with the membrane, or are anchored in the membrane through a covalent bond to phospholipids (von Heijne, 1988). Proteins which are *partially inserted* into the lipid bilayer (*monotopic* proteins; Blobel, 1980) are rare in nature (Singer, 1990). Among these, a well characterized example is cytochrome $b_5$.

*Integral membrane proteins*, spanning the lipid bilayer, are structurally classified in four types (Singer, 1990). Type I and type II consist of a single transmembrane domain, with an exoplasmic N-terminal (type I) or C-terminal (type II) end, mostly arranged in a

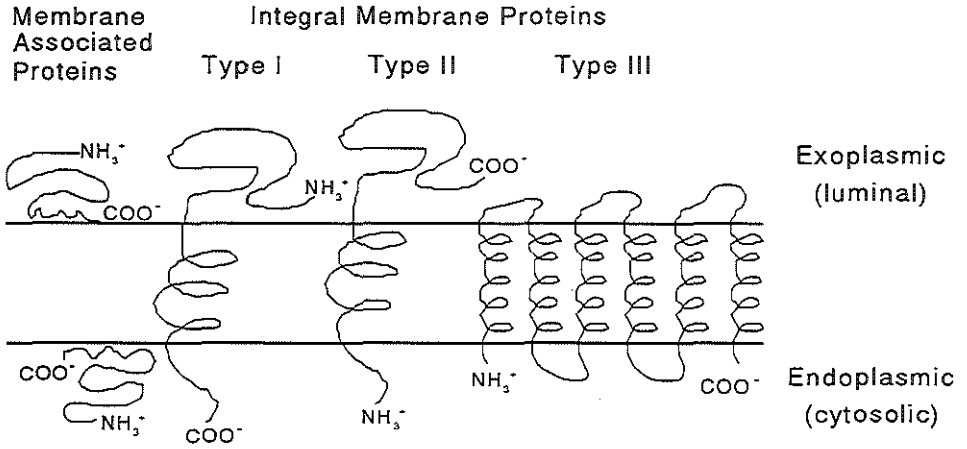


**Figure 1.** Classification of membrane proteins on the basis of the interaction between the polypeptide and the lipid bilayer.

hydrophobic α-helix (*bitopic* membrane proteins; Blobel, 1980). Most of these proteins are functioning as enzymes or receptors, but not as transporters.

Type III proteins contain multiple transmembrane domains alternated with short hydrophilic stretches (*polytopic* proteins according to Blobel). Polypeptide portions of about 20-22 nonpolar amino acids, arranged in hydrophobic α-helices, are supposed to represent the transmembrane domains, being long enough to span the lipid bilayer. But, it has been hypothesized that they may be shorter (Lodish, 1988)

Type IV integral membrane proteins are aggregates of multiple (identical) type III subunits containing α-helical transmembrane domains. An important exception to this rule and so far a unique example among type IV proteins is represented by the mitochondrial and bacterial porins. In this case, the large transmembrane domains are not made by stretches of hydrophobic amino acids arranged in α-helices, but by sequences of alternated charged residues which are compatible with an amphiphilic ß-sheet. This results in a large barrel-shaped structure (pore) through the lipid leaflet, which allows relatively indiscriminated passage of large hydrophilic molecules (Jap, 1989).

All known transport proteins have structural features of type III or type IV membrane proteins. The correlation seems so close that a transport function can be forecasted for a new gene product on the basis of the structure predicted by the hydropathy plots (Kyte and

Doolittle, 1982), like in the cases of the major histocompatibility complex type II (MHC II) and the cystic fibrosis transmembrane conductance regulator (CFTR) gene products (Parham, 1990; Riordan et al., 1989).

Proteins associated with the lysosome membrane can be classified as membrane-associated, which contain hydrophobic domains, and integral membrane proteins which span the lipid leaflet; both types are recovered in the membrane pellet if lysosomes are disrupted.

Some membrane-associated proteins are acid hydrolases like ß-glucocerebrosidase (Rijnbout et al., 1991), α-neuraminidase (Verheijen et al., 1985) present at the inner face of the organelle, or, at the outer face, the two recently identified regulatory G proteins (Philips et al., 1991; Balch, 1990) and the 50 kDa immotilins which mediate ATP-dependent stable association of lysosomes to microtubules (Mithieux and Rousset, 1989).

In the following, some well studied integral lysosomal membrane glycoproteins of unknown function and integral membrane enzymes that have been characterized to date will be discussed. Integral membrane proteins with a transport function will be reviewed in chapter 2.

**Integral lysosomal membrane proteins of unknown function**

Based on purification procedures for abundant membrane proteins, a number of integral membrane proteins of unknown function has been purified to homogeneity from lysosomes. These proteins have an apparent molecular weight between 100 and 120 kDa and account for about 50% of the total content of integral membrane protein from highly purified lysosomes (Marsh et al., 1987). Distinct types have been identified within the same species. The major are: LAMP-1[1] and LAMP-2, in mouse (Chen et al., 1985) and humans (Carlsson et al., 1988; Mane et al., 1989); rat lgp 120, lgp 110, LIMP III (Lewis et al., 1986; Barriocanal et al., 1986); chicken LEP100 (Lippincott-Schwartz and Fambrough, 1986).

Cloning of the respective genes has revealed a great degree of structural homology among the different types in the same species and among different species (Kornfeld and Mellman, 1989). Analysis of genomic DNA has shown that some of these proteins are encoded by house-keeping genes (Zot and Fambrough, 1990).

Sequence analysis predicts in all of them a single membrane spanning domain, a short C-terminal cytoplasmic tail and a large, heavily glycosylated part at the luminal side. This structure warrants a classification as type I integral membrane proteins. The general structure of LAMPs, the best characterized glycoproteins, include a protein core of about 40-kDa, a precursor of 90-kDa with 17 to 20 N-linked high mannose oligosaccharides (OGS),

[1] The abbreviations stand, in general, for *lysosomal-associated (or integral) membrane (glyco)proteins.* Each of them, however, is immunologically distinct. LEP100 stands for *lysosomal, endosomal, plasma membrane protein Mr=100·000*

later processed to complex type OGS (Fukuda et al., 1988; for review see Cha et al., 1990).The oligosaccharide chains seem to be particularly rich in sialic acids and in peculiar polylactosamines (Carlsson et al., 1988). The sialic acid residues contribute to a net negative surface charge (acid pI) and possibly fulfil a protective function against protease degradation.

The biosynthetic processing of these proteins includes translocation into the endoplasmic reticulum via a signal sequence. The cytoplasmic tail is responsible for targeting to the late endosomes and then directly to the lysosomes, essentially without routing via the plasma membrane and the endocytic vesicles (early endosomes) (Kornfeld and Mellman, 1989). A tyrosine residue (*tyr*) in the short cytosolic tail is essential for lysosomal targeting (Williams and Fukuda, 1990). A minor part of LEP100 is routed via the plasma membrane. This protein exchanges continuously with plasma and endosome membranes, although 91% normally resides in the lysosome (Lippincott-Schwartz and Fambrough, 1987). Chloroquine treatment increases the plasma membrane pool of LAMP-1 and a mutation of the *tyr* in the C-terminal tail of human LAMP-1 leads to localization in the plasma membrane. Thus, this *tyr* seems to be essential as a sorting signal both in the trans-Golgi network and at the cell surface (Williams and Fukuda, 1990). Unlike soluble lysosomal hydrolases, the sugar chains do not contain phosphomannosyl oligosaccharides, which, therefore, are not a prerequisite for the lysosomal localization (Barriocanal et al., 1986). A similar mannose-6-phosphate-independent targeting to lysosomes has been observed for some membrane-associated hydrolases like ß-glucocerebrosidase and acid phosphatase (see further).

Attempts have been made to correlate the peculiar structure of these glycoproteins to a specific function. The (faulty) expression of these proteins at the cell surface has been correlated with metastatic tumor transformation, and accounts for increased cell binding to extracellular matrix components, which envisages a role in tumor invasiveness (Metzelaar et al., 1991).

However, these glycoproteins are also expressed at the cell surface of thrombin activated platelets (Febbraio and Silverstein, 1990) and after cell exposure to weak bases (chloroquine or methylamine). Cell surface expression seems also to correlate with low levels of cellular differentiation (Mane et al., 1989). It is possible that discrepancies between the routing of different lysosome membrane proteins (Lippincott-Schwartz and Fambrough, 1987; Green et al., 1987) are partially related to the differentiation stage of the cell types used to perform processing studies *in vitro*.

Sequence analysis suggests that the luminal part of LAMPs can be arranged in four loops, with each loop formed by a disulfide bond. The size of the loops and the *tyr-X-cys* consensus sequence found at the C-terminal end is the same as in the immunoglobulin superfamily. This suggested the possibility of a receptor function for LAMP molecules (Fukuda et al., 1988). It is not known whether the cytoplasmic domain has an enzymatic activity, like many type I integral membrane proteins (Singer, 1990).

Recently, the gene encoding the CD 63[2] antigen has been cloned. The gene product was found to be a protein with the structural features of an integral membrane protein, normally localized in the lysosome membrane (Metzelaar et al., 1991). Its structure includes four transmembrane loops, and three N-glycosylation sites, with a predicted protein mass of 25 kDa. There are no sequence homologies with human LAMP-1 and LAMP-2, nor with other lysosome integral membrane glycoproteins. The CD63 antigen is found to be identical to the ME 491, a melanoma associated antigen, while its function is still unknown.

**Integral lysosomal membrane enzymes**

Two enzymes are known so far to be integral proteins of the lysosome membrane: acid phosphatase and acetyl-CoA: α-glucosaminide-N-acetyltransferase. For the former, this has been demonstrated by molecular studies of the protein structure and biosynthesis. For the latter, details of the catalytic mechanism and subcellular localization studies warrant the definition of integral membrane protein.

Acid phosphatase is synthesized as a mannose-6-phosphate independent (Gottschalk et al., 1989a) integral membrane precursor (Waheed et al., 1988). It contains 7 to 8 N-linked oligosaccharide chains. The delivery to the lysosome is dependent on an essential tyrosine at position 413 of the cytoplasmic tail (Peters et al., 1990). In the lysosome, the cytosolic tail is first cleaved by an acid protease (the reaction depends upon the acidification of lysosomes) and then the intraluminal part is released from the membrane spanning domain by an acid aspartyl protease (Gottschalk et al., 1989b). The soluble phosphatase is then further processed (Tanaka et al., 1990). The transport to the lysosome also involves a rapid recycling of the membrane bound form through the plasma membrane, from where endocytosis follows (Braun et al., 1989).

Features in common with other lysosomal integral membrane proteins are: a single transmembrane domain, a short C-terminal cytoplasmic tail of 19 aminoacids with a *tyr* residue essential for the targeting, which is similarly independent of the mannose-6-phosphate. Lysosomal targeting is also independent of oligosaccharide processing and vesicular acidification (Gottschalk et al., 1989a). While ammonium chloride treatment increases the plasma membrane pool of LEP100 (Lippincott-Schwartz and Fambrough, 1987), it decreases the plasma membrane pool of acid phosphatase (Braun et al., 1989), which instead increases when the essential *tyr* is mutated.

ß-glucocerebrosidase is a membrane-associated lysosomal hydrolase which shares some similarities in the biosynthetic process with acid phosphatase. Its lysosomal targeting is independent of mannose-6-phosphate residues (Rijnbout et al., 1991). Its structure does not include a transmembrane domain, but only hydrophobic regions, which may act as membrane anchor sites (Sorge et al., 1985).

Acetyl-CoA: α-glucosaminide N-acetyltransferase is the enzyme genetically deficient

[2] Identified by use of the CD63 monoclonal antibodies, against platelet membrane antigens.

in Sanfilippo disease type C. It catalyzes the transfer of an acetyl group from cytoplasmic acetyl-CoA to terminal α-glucosamine residues of heparansulfate in the lysosome. Studies with purified lysosomal membranes have shown that the reaction can be divided in two half-reactions. First the enzyme is covalently acetylated at a histidine residue, exposed at the cytosolic side of the membrane, then the acetyl group is transferred to the inside of the lysosomal membrane, where the acetyl-histidine is protonated (Bame and Rome, 1986a). Afterwards, the glucosamine residue of heparan sulfate binds to a separate site on the enzyme inside the lysosome and the acetyl group is transferred to form N-acetylglucosamine. A final conformational shift warrants return of the enzyme to the starting configuration (Bame and Rome, 1985). The molecular structure of the enzyme is unknown, although different mutations in Sanfilippo type C disease can produce distinct defects of each half of the reaction (Bame and Rome, 1986b). It is not known whether the mutations belong to separate complementation groups, i.e. whether the enzyme consists of distinct subunits, each accomplishing part of the reaction.

Other enzymes involved in heparansulfate degradation are thought to exist in a membrane associated complex , but it is not known whether any other enzyme is present as an integral membrane protein (Hopwood, 1989). A well characterized enzyme complex associated with the lysosomal membrane is formed by acid ß-galactosidase, α-neuraminidase and the "protective protein" (Verheijen et al., 1982; D'Azzo et al., 1982; Hoogeveen et al., 1983; for review see Galjaard et al., 1987) but the exact membrane association is still unknown.

REFERENCES


* Balch, W.E. (1990) Small GTP-binding proteins in vesicular transport. *Trends in Biochem. Sci.* 15:473-477

* Bame, K.J. and Rome, L.H. (1985) Acetyl coenzyme A:α-glucosaminide N-Acetyltransferase. Evidence for a transmembrane acetylation mechanism. *J. Biol. Chem.*260:11293-11299

* Bame, K.J. and Rome, L.H. (1986a) Acetyl-coenzyme A:α-glucosaminide N-Acetyltransferase. Evidence for an active site histidine residue.*J. Biol. Chem.* 261:10127-10132

* Bame, K.J. and Rome, L.H. (1986b) Genetic evidence for transmembrane acetylation by lysosomes. *Science* 233:1087-1089

* Barriocanal, J.G., Bonifacino, J.S., Yuan, L., Sandoval, I.V. (1986) Biosynthesis, glycosylation, movement through the Golgi system and transport to lysosomes by a N-linked carbohydrate independent mechanism of three lysosomal integral membrane proteins. *J. Biol. Chem.* 261:1604-1607

* Bishop, W.R. and Bell, R.M. (1988) Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movements and intracellular translocation. *Annu. Rev. Cell Biol.* 4:579-610

* Bleistein, J., Heidrich, H.G., and Debuch, H. (1980) The phospholipids of liver lysosomes from untreated rats. *Hoppe-Seyler's Z. Physiol. Chem.* 361:595-597

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

* Braun, M., Waheed, A. and von Figura, K. (1989) Lysosomal acid phosphatase is transported to lysosomes via the cell surface. *EMBO J.* 8:3633-3640

* Brotherus, J. and Rekonen, O. (1977) Phospholipids of subcellular organelles isolated from cultured BHK cells. *Biochim. Biophys. Acta* 486:243-253
* Carlsson, S.R., Roth, J., Piller, F., and Fukuda, M. (1988) Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. *J. Biol. Chem.* 263:18911-18919
* Cha, Y., Holland, S.M. and August, J.T. (1990) The cDNA sequence of mouse LAMP-2. Evidence for two classes of lysosomal membrane glycoproteins. *J. Biol. Chem.* 265:5008-5013
* Chen, J.W., Murphy, T.L., Willingham, M.C., Pastan, I. and August, J.T. (1985) Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* 101:85-95
* D'Azzo, A., Hoogeveen, A.T., Reuser, A.J.J., Robinson, D. and Galjaard, H. (1982) Molecular defect in combined ß-galactosidase and neuraminidase deficiency in man. *Proc. Natl. Acad. Sci. USA* 79:4535-4539
* Febbraio, M. and Silverstein, R.L. (1990) Identification and characterization of LAMP-1 as an activation-dependent platelet surface glycoprotein. *J. Biol. Chem.* 265:18531-18537
* Fukuda, M., Viitala, J., Matteson, J. and Carlsson, S.R. (1988) Cloning cDNAs encoding human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. *J. Biol. Chem.* 263:18920-18928
* Galjaard, H., Willemsen, R., Hoogeveen, A.T., Mancini, G.M.S., Palmeri, S., Verheijen, F.W. and D'Azzo, A. (1987) Molecular heterogeneity in human ß-galactosidase and neuraminidase deficiency. *Enzyme* 38:132-143
* Gennis, R.B. (1989) Biomembranes. Molecular structure and function. Springer Verlag, New York.
* Gottschalk, S., Waheed, A. and von Figura, K. (1989a) Targeting of lysosomal acid phosphatase with altered carbohydrate. *Biol. Chem. Hoppe-Seyler* 370:75-80
* Gottschalk, S., Waheed, A., Schmidt, B., Laidler, P. and von Figura, K. (1989b) Sequential processing of lysosomal acid phosphatase by a cytoplasmic thiol proteinase and a lysosomal aspartyl proteinase. *EMBO J.* 8:3215-3219
* Green, S.A., Zimmer, K.P., Griffith, G. and Mellman, I. (1987) Kinetics of intracellular transport and sorting of lysosomal membrane and plasma membrane proteins. *J. Cell Biol.* 105:1227-1240
* von Heijne, G. (1988) Transcending the impenetrable: how proteins come to terms with membranes. *Biochim. Biophys. Acta* 947:307-333.
* Hoogeveen, A.T., Verheijen, F.W. and Galjaard, H. (1983) The relation between human lysosomal ß-galactosidase and its protective protein. *J. Biol. Chem.* 258:12143-12146
* Hopwood, J.J. (1989) Enzymes that degrade heparin and heparansulfate. In "Heparin: chemical and biological properties and clinical applications". Lane, D.A. and Lindhall,U. eds. Edward Arnold publisher, London.
* Jap, B.K. (1989) Molecular design of PhoE porin and its functional consequences. *J. Mol. Biol.* 205:407-419.
* Kornfeld, S. and I. Mellman (1989) The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483-525
* Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105-120
* Lewis, V.A., Green, S.A., Marsh, M., Vihko, P., Helenius, A. and Mellman, I. (1985) Glycoproteins of the lysosomal membrane. *J. Cell Biol.* 100:1839-1847
* Lippincott-Schwartz, J. and Fambrough, D.M. (1986) Lysosomal membrane dynamics: structure and interorganellar movement of a major lysosomal membrane glycoprotein. *J. Cell Biol.* 102:1593-1605
* Lippincott-Schwartz, J. and Fambrough, D.M. (1987) Cycling of the integral membrane glycoprotein, LEP100, between plasma membrane and lysosomes: kinetic and morphological analysis. *Cell* 49:669-677

* Liscum, L. and Faust, J.R. (1987) Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Niemann-Pick type C fibroblasts. *J. Biol. Chem.* 262;17002-17008

* Lodish, H.F. (1988) Multi-spanning membrane proteins: how accurate are the models? *Trends in Biochem. Sci.* 13:332-334

* Mane, S.M., Marzella, L., Bainton, D.F., Holt, V.K., Cha, Y., Hildreth, J.E.K., and August, J.T. (1989) Purification and characterization of human lysosomal membrane glycoproteins. *Archiv. Biochem. Biophys.* 268:360-378

* Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I. and Helenius, A. (1987) Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol.* 104:875-886

* van Meer, G. (1989) Lipid traffic in animal cells. *Ann. Rev. Cell Biol.* 5:247-275

* Metzelaar, M.J., Wijngaard, P.L.J., Peters, P.J., Sixma, J.J., Nieuwenhuis, H.K. and Clevers, H.C. (1991) CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. *J. Biol. Chem.* 266:3239-3245

* Mithieux, G. and Rousset, B. (1989) Identification of a lysosome membrane protein which could mediate ATP-dependent stable association of lysosomes to microtubules. *J. Biol. Chem.* 264:4664-4668

* Parham, P. (1990) Transporters of delight. *Nature* 348:674-675

* Peters, C., Braun, M., Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A. and von Figura, K. (1990) Targeting of a lysosomal membrane protein: a tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes. *EMBO J.* 9:3497-3506

* Philips, M.R., Abramson, S.B., Kolasinski, S.L., Haines, K.A., Weissmann, G. and Rosenfeld, M.G. (1991) Low molecular weight GTP-binding proteins in human neutrophil granule membrane. *J. Biol. Chem.* 266:1289-1298

* Rijnbout S., Aerts, H.M., Geuze, H.J., Tager J.M. and Strous, G.J. (1991) Mannose 6-phosphate-independent membrane association of cathepsin D, glucocerebrosidase and sphingolipid-activating protein in HepG2 cells. *J. Biol. Chem.* 266: 4862-4868.

* Riordan, J.R., Rommens, J.M., Kerem, B.S., Alon, N., Rozmahel, R., Grzelczack, Z., Zielenski,J., Lok,S., Plavsic, N., Chou, J.L., Drumm, M.L., Iannuzzi, M.C., Collins, F.S. and Tsui, L.C. (1989) Identification of the cystic fibrosis gene: cloning and characterization of the complementary DNA. *Science* 245:1066-1073

* Singer, S.J. (1990) The structure and insertion of integral proteins in membranes. *Annu. Rev. Cell Biol.* 6:247-296.

* Sorge, J., West, C., Westwood, B. and Beutler, E. (1985) Molecular cloning and nucleotide sequence of human glucocerebrosidase cDNA. *Proc. Natl. Acad. Sci. USA* 82:7289-7293

* Tanaka, Y., Himeno, M. and Kato, K. (1990) Release of acid phosphatase from lysosomal membranes by cathepsin D. *J. Biochem.* 108:287-291

* Thomas, G.H., Tuck-Miller, C.M., Miller, C.S. and Reynolds, L.W. (1989) Correction of sphingomyelinase deficiency in Niemann-Pick type C fibroblasts by removal of lipoprotein fraction from culture media. *J. Inher. Metab. Dis.* 12:139-151

* Vanier, M.T., Rodriguez-Lafrasse, C., Rousson, R., Gazzah, N., Juge, M.C., Pentchev, P.G., Revol, A. and Luisot, P. (1991) Type C Niemann-Pick disease: spectrum of phenotypic variation in disruption of intracellular LDL-derived cholesterol processing. *Biochim. Biophys. Acta* 1096:328-337

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

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

* Waheed, A., Gottschalk, S., Hille, A., Krentler, C., Pohlmann, R., Braulke, T., Hauser, H., Geuze, H. and von Figura, K. (1988) Human lysosomal acid phosphatase is transported as a transmembrane protein to lysosomes in transfected baby hamster kidney cells. *EMBO J.* 7:2351-2358

* Williams, M.A. and Fukuda, M. (1990) Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J. Cell Biol.* 111:955-966

* Zot, A. and Fambrough, D.M. (1990) Structure of a gene for a lysosomal membrane glycoprotein (LEP100). Housekeeping gene with unexpected exon organization. *J. Biol. Chem.* 265:20988-20995

Chapter 2

# LYSOSOMAL MEMBRANE TRANSPORTERS

**Transport mechanisms**

Before analyzing the transporters which have been detected and characterized in the lysosome membrane, the different mechanisms of membrane transport will be summarized. However, recent discoveries indicate that the functional discrimination does not always correlate with structural differences among transport proteins (Dierks et al., 1990; Henderson and Maiden, 1990; Murakami et al., 1990; Simon and Blobel, 1991; Fischbarg et al., 1989; Higgins and Hyde, 1991). More knowledge about the actual structure of transport proteins will help to understand these discrepancies, but so far the three-dimensional protein structure is known only for three transmembrane proteins, at a resolution of 10 Å: the photoreaction center of photosynthetic bacteria, bacteriorhodopsin and porin.

Transporters are functionally classified as: a) channels, b) pumps and c) carriers.

a) **Channels** (or pores) are known to allow substrate ions (often inorganic ions) flow along an electrochemical gradient towards equilibrium, generating a membrane potential defined by the Nernst equation ( $\Delta E = RT/zF.\ln\{[X]_o/[X]_i\}$ ). This equation defines the potential difference of solutions as a function of the electrical charge (z) of the ions present and the concentration of ions. Channels can be activated in their function by electrical forces (voltage-gated channels), or by chemicals (ligand- or nucleotide-gated) (Stanfield, 1987). Channels possess several binding sites which are accessible to ions from both sides of the membrane at the same time (and not alternatively), but do not undergo conformational

modifications during each transport event. Conformational changes are usually related to activation of their function (opening of the channel). Single channels are studied by patch-clamp electrophysiological registrations (Sakmann and Neher, 1983) performed on (sub)cellular membrane fragments (Quinton and Bijman, 1983) or after reconstitution of the channel in planar lipid bilayers (Bridges and Benos, 1990). Their properties are defined on the basis of their ion selectivity and the current-voltage (I-V) relationship characteristics. The essential characteristic of a channel compared with other types of transporters is the extremely fast rate of transport (in the orders of $10^6$-$10^8$ ions/sec) (Stein, 1986) which leads *per se* to a rapid change of the electrical property of the membrane. Channels can be switched "on" and "off" and when the channel is closed the ion permeability is zero.

Recent studies demonstrate that high conductance channels can at the same time act as transporters for complete proteins *in vivo* (Simon and Blobel, 1991). An example is the channel portion of the ribosome-nascent protein signal recognition complex, which allows secretory proteins to cross the endoplasmic reticulum membrane and enter the secretory pathway. The described channel (115 pS[3]) is selective for anions like chloride and glutamate, but also for complete nascent polypeptides. To stress that functional differences between transporters do not represent definite structural differences, it has been shown that a channel needed for the import of proteins in mitochondria is identical to the mitochondrial phosphate carrier (Murakami et al., 1990).

b) **Pumps** are *primary active* transporters, driven by energy directly provided by nucleotide (ATP) hydrolysis or redox reactions; they possess binding sites and undergo conformational changes during transport. They may also be involved in ATP synthesis. The process is usually unidirectional and proceeds against an electrochemical gradient. Their experimental demonstration is based on the absolute energy dependence of the transport function. Also the proton pumps of oxidative phosphorylation and the light-driven ion pumps of certain halophilic bacteria (like bacteriorhodopsin) are considered primary active transport systems (Gennis, 1989; Forgac, 1989).

c) **Carriers** (or permeases, translocases) facilitate the diffusion of hydrophilic substrates. The solutes interact with a finite number of binding sites, therefore transport function is saturable and the conformational modifications needed for transport fulfil the hypothesis of the mobile carrier (Scarborough, 1985; West, 1983; Stein, 1986). The direction of transport follows an electrochemical gradient and sometimes can be coupled to binding of another species (ions), which associates in a *cotransport* mechanism. In this case transport is activated as long as the ion gradient holds. Other cotransporters can function as symporters or antiporters, allowing movement of the different substrates in opposite directions. As a consequence, the chemical gradient of one of the substrates can influence the direction of

[3] pS, picoSiemens, is the unit for $g$, single channel conductance.

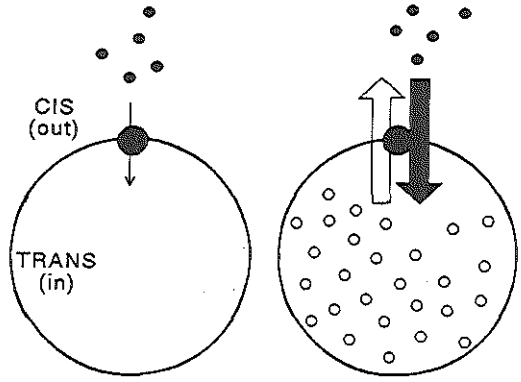


**Figure 2.** Countertransport or *trans*-stimulation. In the preloaded vesicle (right) the influx is much faster than in the not preloaded one (left). (●) labeled substrate; (○) unlabeled substrate.

transport of the other substrate, which in turn can be transported against its own concentration gradient. This mechanism is also called *secondary active* transport as the gradient of the driving ions is usually maintained by an independent, primary active pump. (Geck and Heinz, 1981). The kinetics of carrier-mediated transport can be compared with that of an enzyme, expressed by the Michaelis-Menten equation ($v=SV_{max}/[K_m+S]$). Saturability of the transport process will show a definite affinity constant ($K_m$, often called $K_t$ for transport systems) and a maximal velocity rate ($V_{max}$), depending on the number of substrate molecules transported in a time interval, at a given substrate concentration (S). Transport is sensitive to substrate inhibition in a competitive or non-competitive mode.

Countertransport and/or *trans*-stimulation are experimental properties which directly demonstrate the existence of a carrier and are dependent on the symmetrical function of such proteins (figure 2). They are based on the fact that the binding sites are alternatively available at each side of the membrane. Thus, the substrate at one side is not in competition with that at the other side. The conformational changes necessary to transport the substrate from the *cis*- to the *trans*-side of the membrane, enable the carrier to exchange the substrate, released at the *trans*-side, with another substrate molecule. Supposing that, at the start of the turnover cycle, the substrate concentration at the *trans*-side is higher than at the *cis*-side, the net substrate flux will be from *trans* to *cis*. Consequently, the velocity of the process will be determined by the higher concentration present at the *trans*-side. Experimentally, if in such a case only the molecules at the *cis*-side are radiolabeled, thus chemically detectable, we will measure a faster rate of transport from *cis* to *trans* than it would be expected in the absence of substrate at the *trans* side, with the apparent paradox of substrate moving against its own

concentration gradient. This phenomenon, which sometimes can be measured in a test tube, is called countertransport when provoked by the same molecular species at both sides of the membrane, or *trans*-stimulation when the molecule at the opposite side of the membrane is a substrate analog, recognized and transported by the carrier. Countertransport or *trans*-stimulation not only depends on the properties of the transport protein involved, but also on the ability to create test tube conditions to assay it, i.e. to have a vesicular system where the internal substrate concentration can be higher than the $K_m$, without significant leakage, and efflux of the radiolabeled species is competed by a high amount of unlabeled substrate.

Transport of a metabolite which is driven by the gradient of another molecular species (countertransport, *trans*-stimulation or cotransport with a coupled ion gradient) can experimentally manifest itself by an *overshoot* of uptake in a vesicular system, where the substrate concentration into a vesicular compartment can transiently arise above its equilibrium level, as long as the gradient of the other molecular species holds (Turner, 1983; Gennis, 1989).

Notwithstanding the biochemical and physiological heterogeneity, structural information about many transport proteins indicate that all transporters in living organisms show a surprising level of structural homology. This concept has recently been reviewed by Henderson and Maiden (1990), and Maloney (1990) and it has led to the assumption that during evolution all transport proteins, from prokaryotes on, have evolved from a common ancestor containing a paired number of membrane spanning domains, often six, arranged in $\alpha$-helix forms, which can interact at the endoplasmic side (intracellular or extraluminal) with an hydrophilic, regulatory (nucleotide-binding) protein. During evolution, the regulatory protein could have become a regulatory domain of the transporting polypeptide and the membrane spanning domain has duplicated in a paired way, as well as the regulatory hydrophilic domain. This way, the ABC (from ATP Binding Cassette) superfamily has been defined (Hyde et al., 1990). The ATP binding domains of the bacterial ABCs (the so called "periplasmic transport systems") show extensive sequence similarity among one another, much more than required for ATP recognition, for example, by other sorts of ATP-binding proteins. It seems that these proteins are evolutionary related to archetypes of a family of structurally related proteins which couple ATP hydrolysis to different cellular functions in eukaryotes and prokaryotes. (Higgins et al., 1990). Comparison of sequence homology between six different sugar carriers from prokaryotes and eukaryotes up to humans showed overall homology of 150 amino acids (circa 30%) (Henderson and Maiden, 1990). Recent developments also indicate that the structural homology of carriers and pumps can be extended to channels as well. The cystic fibrosis transmembrane conductance regulator (CFTR) gene product, which is mutated in a human genetic disorder, cystic fibrosis, shares high levels of sequence homology with pumps like the transporters providing multi drug resistence (MDRs) and ABC family members (Riordan, et al. 1989; Hyde et al., 1990). It's function as a chloride channel is no more in doubt (Anderson et al., 1991; Rich et al., 1991).

As mentioned, at least two other transport proteins seem to function both as a channel and as a carrier, the phosphate carrier/protein import system of mitochondria and the anion channel/protein transporter of the endoplasmic reticulum. The clarification of the apparent discrepancy between similar structures and different functions of transport proteins will be a major challenge for the near future (Higgins and Hyde, 1991)

**Channels and pumps in the lysosome**

A recent review about the permeability properties of the lysosome membrane has accurately listed all the categories of compounds which are able, or predicted to cross the membrane *in vivo* (Forster and Lloyd, 1988).

Essentially, two types of mechanisms have been considered: unassisted simple diffusion across the lipid bilayer and transport mediated by a specific transport protein. The role of the simple diffusion is still debated, especially for those compounds, which could theoretically freely diffuse and are demonstrated to be transported by a specific carrier (glucose, amino acids). Since it is very difficult to judge the physiological role of simple diffusion mechanisms with "in vitro" detection systems affected by artificial leakage, only the mechanisms of transport which have been shown to involve a specific transport protein will be reviewed here.

Up to now, the structure of only one transporter, the lysosomal proton pump, has been partially unraveled. This means that all our knowledge about lysosomal transport proteins is based on the biochemical characteristics of transport, as assayed in the native membrane. The correspondence of transport events to theoretical kinetic models sustains the distinction between lysosomal carriers, pumps and channels. From the data reported in the literature, a functional classification of lysosomal membrane transport systems has been attempted in table I.

The interior of the lysosome, like that of other organelles of the endo- and exocytic pathway, is acidic compared with the cytosol, as first observed by Metchnikoff (1893), long before the lysosomes were identified by de Duve (1963). The pH is about 4.7, as measured by fluorescein-labeled dextran (Ohkuma and Poole, 1978; for review, Ohkuma, 1987). The pH of the lysosome is the lowest among all the acidic intracellular organelles (Tycko and Maxfield, 1982; for review see Anderson and Orci, 1988).

The acidification of the interior is maintained by an $H^+$-translocating ATPase (Dell'Antone, 1979; Schneider, 1981; Ohkuma and Poole,1978). The $Mg^{++}$-ATPase function was first demonstrated to be membrane bound (Schneider et al., 1978) and later the $H^+$ translocating function was proven to be associated with ATP hydrolysis (Schneider, 1979). The pump is N-ethylmaleimide sensitive and insensitive to ouabain, oligomycin and vanadate (Ohkuma, 1987). These properties can be used to discriminate it from other cellular proton pumps. The process of proton translocation is electrogenic (Harikumar and Reeves, 1983; Dell'Antone, 1979), i.e., in the absence of permeable compensating anions, it produces an

TABLE I

Lysosomal membrane transport systems
(functional classification)

| Type | Substrate | Source | Ref. |
|---|---|---|---|
| **Channels** | | | |
| *Chemically regulated*: | chloride channel | rat liver | Tilly,1991 |
| **Transporters** | | | |
| *Passive*: | | | |
| | neutral small amino acids (2) | fibroblasts | Pisoni,1987b |
| | proline, 3,4-dehydroproline | fibroblasts | Pisoni,1987b |
| | neutral bulk amino acids | rat thyroid cells | Bernar,1986 |
| | branched and aromatic amino acids | fibroblasts | Stewart,1989 |
| | anionic amino acids | fibroblasts | Collarini,1989 |
| | N-acetylated hexoses | rat liver | Jonas,1989 |
| | neutral monosaccharides | rat liver,fibroblasts | publ.IV,V |
| | | rat liver | Jonas,1990 |
| | nucleosides | fibroblasts | Pisoni,1989 |
| | phosphate ion | fibroblasts | Pisoni,1991 |
| | calcium ions | fibroblasts | Lemons,1991 |
| | folylpolyglutamates | S180 cells | Barrueco,1991 |
| *Active*: | | | |
| *primary active* | | | |
| | $H^+$ pump/ATPase | rat liver | Schneider,1979 |
| | | chromaffin granules | Moriyama,1989a |
| | | coated vesicles etc. | Forgac,1983 |
| *secondary active* | | | |
| | acidic sugars | rat liver,fibroblasts lymphoblasts | publ.III,V |
| *probably secondary active* | | | |
| | cystine | leucocytes | Gahl,1982 |
| | | rat liver | Jonas,1982 |
| | cysteine | fibroblasts | Pisoni,1990 |
| | sulfate ions | rat liver | Jonas,1990 |
| | vitamin $B_{12}$ | rat liver | Jonas,1991 |
| | cationic amino acids | fibroblasts | Pisoni,1985 |
| | taurine | rat liver | Vadgama,1991 |
| **Unknown mechanism** | | | |
| | cholesterol | fibroblasts | Liscum,1989 |
| | small dipeptides | rat liver | Bird,1990 |

increase of the membrane potential which limits the acidification process.

A similar type of proton translocating ATPase has been identified in several other acidic organelles of eukaryotes, like clathrin-coated vesicles (Stone et al., 1983; Forgac, 1983), endosomes (Galloway et al., 1983), Golgi vesicles (Glickman et al., 1983; Zhang and Schneider, 1983), multivesicular bodies (Van Dyke et al., 1985) and chromaffin granules (Johnson et al., 1982; Cidon and Welson, 1983; Dean et al., 1984). They all have similar characteristics and are called in general *vacuolar* proton pumps. In kidney and macrophages, a vacuolar type of pump is present on the plasma membrane (Gluck et al., 1982; Swallow et al., 1990). A similar set of proton pumps is also present in plants (Manolson et al., 1985), archaebacteria (Südhof et al., 1989), lower eukaryotes as Neurospora (Bowman, 1983) and yeast (Uchida et al., 1985). Recently it has been suggested, on the basis of structural comparison that the mitochondrial ATP synthetase ($F_1F_0$, complex V) and the vacuolar classes of proton pumps have common ancestor genes (Moriyama et al., 1986; Forgac, 1989; Nelson, 1987; Mandel et al., 1988).

The reconstitution of the lysosomal proton pump after solubilization of the lysosome membrane proteins has definitively proven that the proton translocation is not intrinsecally coupled to another ionic species (D'Souza et al., 1987). Acidification of artificial liposomes containing the reconstituted proton pump was in fact accomplished in the total absence of permeant anions, when the membrane potential was compensated by free flow of $K^+$ in the presence of the ionophore valinomycin.

Vacuolar proton pumps from clathrin-coated vesicles and chromaffin granules have been purified thanks to the availability of specific inhibitors. These pumps appear as heterologous multimeric complexes of integral membrane components and membrane associated polypeptides: the proton translocating function and the ATPase function are apparently associated with separate polypeptides (Forgac, 1989). Recently, Moriyama and Nelson (1989a) have shown cross-reactivity of antibodies against different subunits of the proton pump from chromaffine granules with lysosomal membrane associated polypeptides. These experiments were performed using membrane associated hydrophilic subunits of the $H^+$-ATPase released by treatment of lysosomal membranes with MgATP at 0° C (Moriyama and Nelson, 1989b). The lysosomal $H^+$-ATPase showed at least five different hydrophilic subunits, immunologically and electrophoretically identical to the pump from chromaffine granules.

In total, there are at least eight major polypeptides associated with the vacuolar $H^+$-ATPase function, with molecular weights 116-, 70-, 58-, 40-, 38-, 34-, 33- and 17-kDa. To date, four out of the eight subunits have been sequenced after cloning the respective genes. Most of the information was obtained from chlatrin-coated vesicles and chromaffin granules. Based on the predicted amino acid sequence, the 17-kDa subunit possesses four transmembrane domains which should directly be involved in $H^+$ channeling (Mandel et al., 1988). It has been recently shown that this subunit specifically binds the transforming protein E5 of bovine papilloma virus type I. This suggests a role for a disturbed lysosomal

acidification in the transformation process produced by this oncoprotein (Goldstein etal., 1991).

The subunits 70-, 58-, 40- and 33-kDa are all concomitantly needed for ATP hydrolysis (Xie and Stone, 1988). Deduced amino acid sequence of the 58- and 70-kDa subunit predicts ATP and N-ethylmaleimide recognition sites (Südhof et al., 1989), along with an extraordinary degree of homology with $H^+$ pumps from archaebacteria, plants and fungi as well as with the mitochondrial ATP synthetase (ibidem). The 116-kDa polypeptide has also recently been purified and the gene cloned (Perin et al., 1991). Analysis of the deduced aminoacid sequence suggests that it consists of two fundamental domains: a hydrophilic amino-terminal half containing many charged residues and a hydrophobic carboxyl terminal with at least six transmembrane spanning regions. This finding supports the proposed function of the 116-kDa protein of coupling ATP hydrolysis, accomplished by the cytoplasmic 70-, 58-, 40- and 33-kDa subunits, with proton translocation, carried out together with the 17-kDa subunit. Former biochemical experiments had shown that loss of the 116-kDa and 38-kDa subunits leads to loss of $Mg^{++}$ dependence for the ATPase and loss of proton pumping function (Xie and Stone, 1988).

The gene for a 31-kDa subunit of the proton pump from bovine kidney medulla has also been cloned (Hirsch et al., 1988). Since this gene is highly expressed in the kidney, it has been assumed that it encodes for a subunit of the plasma membrane proton pump which in kidney is of the vacuolar type. A large hydrophilic subunit without transmembrane domains and with a function in the regulation of proton access to the translocating channel has been predicted for this subunit.

A recent observation by Gürich et al. (1991) exploits the activation of a mammalian endosomal $H^+$ pump by GTP and the non-hydrolyzable derivative GTP$\gamma$S, suggesting that G proteins are involved in the direct modulation of vacuolar acidification. The mechanism is not known.

For a simple uniport pump like the lysosomal proton pump, anion conductance through the membrane is required for accumulation of acid into the lysosome. Consequently, a physiological counter-ion which performs *in vivo* this task could be identified in the $Cl^-$ anion. Halides seem to support $H^+$ pump activity by serving as a counter-ion in clathrin-coated vesicles (Xie et al., 1983), chromaffin granules (Moriyama and Nelson, 1987), Golgi membranes (Forgac, 1989), endosomes (Fuchs et al., 1989), multivesicular bodies (Van Dyke, 1988) and secretory granules (Barasch et al., 1988).

In rat liver lysosomes, chloride was mostly efficient in providing an adequate conductance for proton transport (Cuppoletti et al., 1987). The capacity of the chloride gradient was enough to generate the proton gradient, independently of the $H^+$ pump activation (by means of tetrachlorosalicylanilide[4] in the absence of ATP). On the basis of

[4] A $K^+/H^+$ exchanger

these findings, the nature of the chloride conductance can be speculated. Forgac (1989) and Glickman et al. (1983) hypothesize, without experimental proof, a conductive chloride channel. Recently Jonas and Jobe (1990) have suggested that the lysosomal carrier for sulfate can function also as an antiporter exchanging sulfate with chloride. We have recently demonstrated a chloride specific anion channel in the lysosomal membrane of rat liver. This channel is gated by nucleotide (ATP, ATP-γS, AMP-PNP) binding, independently of kinase activities (Tilly et al., submitted). These results were obtained after electrophysiological studies performed with purified rat liver lysosome membranes, reconstituted in an artificial lipid bilayer. This is a new example of an ion channel regulated by nucleotide binding (Stanfield, 1987) and provides the physiological basis for a compensation mechanism of the membrane potential generated during lysosomal acidification. Synergically, ATP is useful to activate $H^+$ pumping and $Cl^-$ flux.

These are to our knowledge the first electrophysiological studies leading to the definition of an ion channel in the lysosomal membrane. In view of the recent demonstration that the CFTR gene product is a nucleotide-regulated chloride channel (Rich et al., 1991; Anderson et al., 1991) and that the endosomal acidification is defective in cystic fibrosis cells (mutated in CFTR) (Barasch et al., 1991), it will be interesting to investigate the structure of the lysosomal chloride channel and its function in cystic fibrosis patients.

**Lysosomal membrane carriers**

All the carriers of which the primary structure is known, are derived from eukaryotic plasma membranes, bacterial membranes or phylogenetically related mitochondria. In general, the structural homology is high, but it is not possible at the moment to conclude that the similarity can be extended to the transporters in membranes of other intracellular organelles.

Among lysosomal integral membrane proteins, carriers are best characterized biochemically, but none of them has as yet been purified to homogeneity.

Initially it was thought that the lysosome membrane works only as an aspecific molecular sieve, allowing the free diffusion of molecules of molecular weight up to 200-kDa, and excluding any compound of larger size (Lloyd, 1969; Reijngoud and Tager, 1977; Ehrenreich and Cohn, 1969). When it was found that also larger molecules could pass the lysosome membrane, it was assumed that the permeability rate would better correlate with the hydrogen bonding capacity (hydrophilicity) of the solute, with the prediction that large hydrophobic molecules are more permeable than large hydrophilic ones (Bird et al., 1987; Iveson et al., 1989). The presence of specific carriers for the efflux of intralysosomal degradation products was hypothesized by Hales (1978) and Docherty et al (1979). Even if nowadays many specific carriers for solutes have been described, the role of passive diffusion of hydrophobic compounds is still actively investigated (Iveson et al., 1989).

The different lysosomal carriers will be discussed according to their substrate specificity for amino acids, sugars or miscellaneous compounds.

*Amino acids*

The role of amino acid transport in cellular nutrition and in the regulation of several processes has been recently reviewed. Lysosomal uptake and efflux systems appear to participate actively in the regulation of proteolysis (Christensen, 1990; Mortimore et al., 1988; Ohkuma, 1987).

The first kinetic demonstration of a lysosomal carrier came from the work done on cystinosis, a genetic disorder where the disulfide cystine accumulates in the lysosome. In 1982, Gahl et al. demonstrated saturation kinetics for cystine lysosomal countertransport, using leucocyte granular (lysosome-rich) subcellular fractions. This work both discovered the genetic defect of cystinosis as an inborn error of the cystine transport system and unequivocally demonstrated that solute flux across the lysosome membrane is strictly regulated by highly specific transport proteins (Gahl et al., 1982; Jonas et al., 1982). The lysosomal cystine carrier is highly specific for this amino acid and for the related cystathionine. Recently, a $K_t$ of 0.27 mM for zero-trans flux has been reported for the carrier of mouse L-929 fibroblasts (Greene et al., 1990). The system is sensitive to changes of the membrane potential and of the pH gradient across the membrane (Smith et al., 1987). Although no definitive proof was given (Turner, 1983), this system has been classified in table I among the probably secondary active carriers.

After the discovery of the cystine carrier, using different techniques, several investigators have demonstrated many more saturable transport systems: the work from Pisoni's and Gahl's group has practically covered the complete field of amino acid transport in lysosomes (see table I for references).

In rat thyroid lysosomes a carrier recognizes bulk neutral amino acids like tyrosine, leucine, histidine, phenylalanine and tryptophan (Bernar et al., 1986). Lysosomal tyrosine countertransport is stimulated by TSH, probably through a cAMP-dependent pathway (Harper et al., 1988). TSH-regulated transport of monoiodo- and diiodotyrosine is apparently carried out by the same transporter (Andersson et al., 1990). In human fibroblasts, instead, two separate transport systems for aromatic (system *t*) and bulky dipolar (system *l*) amino acids carry out transport of the larger neutral amino acids (Stewart et al., 1989). Nothwithstanding the extensive biochemical similarity between the latter transport mechanisms and that of transformed thyroid cells, the authors could not come to a definitive conclusion about the identity of them.

These properties were exploited studying countertransport of radiolabeled amino acids in lysosomes preloaded by exposure to amino acids methylesters. The possibility to load specifically lysosomes with amino acids by exposure of non purified preparations to methylesters, which as weak bases accumulate in acidic organelles, had been shown by early work of Goldman and Kaplan (1973) and has been extensively used to demonstrate specific carrier-mediated countertransport.

With the same technique, another carrier specific for the class of cationic amino acids arginine, lysine, ornithine, histidine, diaminobutyrate was found in fibroblast lysosomes

(Pisoni et al., 1985). This carrier, called system *c*, performed maximal countertransport at extralysosomal alkaline pH. It accomodated also the mixed cysteine-cysteamine disulphide, formed by the reaction of a cysteine with a cysteamine molecule, providing the explanation for the success of cysteamine in the therapy of cystinosis. The understanding of the physiological way thiols intervene in lysosomal proteolysis was completed by the detection of a carrier for cysteine (Pisoni et al., 1990). The model predicts that cysteine enters the lysosome via a highly specific import mechanism; there it participates into a disulfide exchange with the thiol groups of proteins and it is released as the disulfide cystine by thiol proteases. Cystine then effluxes out of the lysosome via another specific carrier, which does not recognize cysteine. As mentioned, the cysteine carrier instead recognizes also cysteamine, which can also participate into the disulfide exchange mechanism.

Later, other thiols were found to be as effective as cysteamine to prevent formation of cystinotic lysosomes, such as thiocholine (Pisoni et al., 1987a) which, bound to cysteine, transits via the cationic amino acid carrier, and mercaptoethylgluconamide (MEG) (Pisoni et al., 1989a) which might enter the lysosomal system by the acid sugar carrier (Christensen, 1990).

The substrate specificity and the lack of $Na^+$ dependence of the cationic amino acid carrier resembles that of the system $y^+$ of the plasma membrane (Pisoni et al., 1987a). However, uptake studies of radiolabeled arginine revealed that the lysosomal carrier is highly pH sensitive, with maximum uptake at extralysosomal alkaline pH, it has an eight times higher $K_t$ (0.32 mM) compared with the system $y^+$ and presents a substrate specificity which does not completely overlap with the plasma membrane system (Pisoni et al., 1987a).

Interestingly, while the physiological transmembrane pH should support arginine influx *in vivo*, in the presence of MgATP, arginine efflux is rather favoured and uptake is retarded. Conclusions were not definitively drawn about this system, which probably functions as a $H^+$/arginine antiporter. Yeast vacuoles, phylogenetically related to lysosomes, contain a very similar $H^+$/arginine antiporter with a similar $K_t$ (0.26 mM) (Ohsumi and Anraku, 1981).

Subsequently, three distinct carriers for small neutral amino acids were identified by uptake studies in lysosomes purified on a continuous gradient of silica (Percoll). They are respectively called system *e*, *f* and *p*. System *p* is specific for proline and 3,4-dehydroproline only, but with a relatively high $K_t$ (0.07 mM), compared with the other two (about 0.01 mM). System *f* has high affinity for proline, N-methylated amino acids but also alanine, serine and threonine. Proline uptake is maximal at extralysosomal acidic pH. System *e* recognizes the small amino acids alanine, serine and threonine, but does not accomodate N-methylated forms (Pisoni et al., 1987b). The methods used here allowed for the first time calculation and comparison of affinity constants. In a similar way, Collarini et al (1989) characterized a carrier specific for anionic amino acids (system *d*), less restrictive and with a higher affinity than the corresponding $X^-_{AG}$ (for aspartate and glutamate) plasma membrane carrier.

Movements of taurine, a unique sulfur amino acid derivative, across the lysosomal membrane seem also to be mediated by a transporter (Vadgama et al., 1991).

None of the lysosomal amino acid carriers works as $Na^+$-cotransporter, unlike many plasma membrane carriers, and all have relatively high $K_t$.

An interesting complementation to these studies on amino acid transport is the recent investigation about lysosomal transport of (di)peptides. Early studies by Lloyd (1971) showed that dipeptides cross the lysosome membrane faster than single amino acids. Recently, the hypothesis has been forewarded that a carrier-mediated transport mechanism might sustain dipeptide influx (Bird and Lloyd, 1990). Though the physiological implications of such a transporter are unclear at the moment, it will be very interesting to investigate whether this hypothetical transporter is homologous to the new class of channel-like peptide porters recently identified in other subcellular membranes (Simon and Blobel, 1991; Parham, 1990; Sambrook, 1991).

*Sugars*

Monosaccharides represent major lysosomal products of glycan catabolism, which are assumed to efflux the lysosome and to be recycled by the cell. Disaccharide production is irrelevant and these compounds are thought to be impermeable (Lloyd, 1969). As mentioned, early studies of sugar permeability in lysosomes contributed to the postulation of the existence of lysosomal transport proteins. The work from Hales and coworkers demonstrated that the uptake of neutral hexoses in tritosomes is sensitive to temperature, pH changes and ionic composition (Docherty and Hales, 1979), is inhibitable by cytochalasin B and other candidate substrates (Maguire et al., 1983) and is saturable ($K_t$: $48 \pm 18$ mM). The system showed some stereospecificity and seemed to recognize also pentoses.

Subsequent experiments performed with the osmotic protection method, an indirect method to evaluate lysosomal permeability to solutes (Lloyd, 1969), indicated instead that the lysosomal influx of neutral hexoses mainly depends on their hydrogen bonding capacity, which is approximately correlated to the number of hydroxyl groups per molecule (Stein, 1967). Matching with this hypothesis, it was observed that hexoses (namely glucose) penetrate slower than pentoses and consequently it seemed unlikely that they would share a common carrier, but the flux of both monosaccharides could be explained by simple diffusion through the lipid bilayer (Bird et al., 1987). When a larger series of compounds was compared with glucose for the respective lysosomal permeability, the perfect correlation between the molecular structure (in terms of hydrogen bonding capacity) and the permeability observed, seemed to support the assumption that simple diffusion was the major mechanism of transport for the investigated compounds (Iveson et al., 1989).

We decided to address the question about the existence of an hexose carrier, using highly purified lysosomal membrane vesicles from rat liver. These studies allowed the demonstration of carrier-mediated transport of neutral hexoses in the lysosomal membrane. Unequivocal proof came from the demonstration of countertransport and saturation kinetics

(publication IV). In our system, the contribution of an aspecific diffusion component was undetectable, and the carrier did not react with pentoses. Parallel studies in intact rat liver lysosomes by others confirmed saturability of glucose uptake (Jonas et al., 1990).

Transport of the sugars N-acetylglucosamine and N-acetylgalactosamine, which are degradation products of glycoproteins and glycosaminoglycans, is mediated by a distinct transport system (Jonas et al., 1989). Recycling of these sugars from the lysosome has been suggested (Rome and Hill, 1986; Trinchera et al., 1990). The properties of this carrier were investigated in intact lysosomes from rat liver. Transport showed an affinity constant of 4.4 mM for both sugars, specificity for acetylated sugars, lack of effect by pH changes, $Mg^{++}$ ions, ATP, but inhibition by 50 $\mu$M cytochalasin B. Bidirectional transport was demonstrated by *trans*-stimulation. In lysosome membrane vesicles the glucose carrier did not support transport of acetylated sugars (publication IV), confirming the data of Jonas for a specific acetylated sugar carrier.

The next distinct sugar transport protein is the carrier for sialic acid and other acidic monosaccharides (publication III). Its existence and properties were clarified in lysosomal membrane vesicles. The peculiar function of this carrier as a proton cotransport mechanism could be demonstrated (publication III). A genetically determined dysfunction of this transport system is the apparent cause of sialic acid storage diseases, a heterogeneous group of disorders spanning from mental and physical handicaps up to adult age, to early neonatal death. The biochemical details of this transport system and the description of the clinical problems related to it will be discussed in the next chapters. Table II summarizes the main biochemical features of lysosomal sugar carriers.

*Other substrates*

Besides amino acids and sugars, other products of the lysosomal catabolism are released from the organelle. For many of them specific membrane carriers have been described. Considering the degradation of the nucleic acids, nucleotides are hydrolyzed to nucleosides and inorganic phosphate, but no nucleosidase activity is known to be associated with the lysosome. In fact, a carrier-mediated mode of transport has recently been found both for nucleosides and for phosphate (Pisoni and Thoene, 1989b; Pisoni, 1991). Saturation kinetics for the uptake of radiolabeled substrates were demonstrated in both cases. A peculiarity of adenosine uptake in intact fibroblast lysosomes is that, once entered the compartment, adenosine is progressively deaminated to inosine, which is recognized and transported by the same carrier. Purine and pyrimidine nucleosides are substrates with similar affinity (9 and 11 mM). *Trans*-stimulation of nucleoside transport could not be elicited (Pisoni and Thoene, 1989b).

Phosphate is taken up by fibroblast lysosomes with an affinity constant of 5 $\mu$M at 37°C, and is metabolized in the lysosome to trichloroacetic acid-precipitable material, which prevents the release of radioactivity from lysosomes previously exposed to $[^{32}P]KH_2PO_4$. However, also the efflux of phosphate can occur, since time-dependent uptake curves reach

TABLE II

Characteristics of lysosomal sugar carriers

| Source | $K_t$ (mM) | pH optimum | Countertransport/ *Trans*-stimulation | Substrate specificity | Regulation/ Inhibitors | Ref. |
|---|---|---|---|---|---|---|
| Acidic sugars | | | | | | |
| rat liver vesicles | 0.2 | acidic ($H^+$ cotransport) | yes | sialic acid<br>glucuronic acid<br>gluconic acid | ATP<br>NEM | public.III |
| human fibroblast vesicles | 0.2 | acidic ($H^+$ cotransport) | yes | sialic acid<br>glucuronic acid | ? | public. V |
| proteoliposomes (rat liver) | 0.38 | acidic ($H^+$ cotransport) | yes | sialic acid<br>glucuronic acid | NEM<br>DEP<br>PG | public. VI |
| Neutral sugars | | | | | | |
| rat liver "tritosomes" | 48 | neutral | ? | D-,L-glucose<br>D-mannose<br>D-ribose<br>2-deoxy-D-glucose | cytochal.B | Maguire, 1983 |
| rat liver vesicles | 75 | acidic | yes | D-glucose<br>D-galactose<br>D-,L-fucose<br>D-mannose | phloretin<br>cytochal.B | public. IV |
| rat liver lysosomes | 22 | neutral | no | D-glucose<br>D-fucose<br>2-deoxyglucose<br>methyl-α-glucoside | cytochal.B | Jonas, 1990 |
| N-Acetylated sugars | | | | | | |
| rat liver lysosomes | 4.4 | neutral | yes | N-acetylglucosamine<br>N-acetylgalactosamine | cytochal.B | Jonas, 1989 |
| rat liver vesicles | 1.3 | neutral | yes | N-acetylglucosamine<br>N-acetylgalactosamine | cytochal.B<br>FITC | Jonas, 1990 |

steady-state levels indicating equilibrium between influx and efflux. This transporter also recognizes arsenate ions as a substrate. The pH curve for phosphate uptake suggests that monobasic phosphate is the form which is taken up, while the hydrolysis of ATP by the membrane ATPase is coupled to the uptake of the liberated inorganic phosphate via the phosphate carrier. The nucleotides CTP and AMP-PNP, glucose-6-phosphate and pyridoxal phosphate seem to interact with phosphate transport in a non competitive way.

Though phosphate is largely produced by intralysosomal degradation of nucleic acids, it is not clear at the moment whether this ion is leaving the lysosome via this highly specific carrier or it is rather further metabolized, while the carrier would rather be used *in vivo* for the import of inorganic phosphate in a process coupled to the function of the $H^+$ pump/ATPase, as earlier suggested by Schneider (1983).

A distinct carrier for the inorganic ion sulfate is also present in the lysosome membrane (Jonas and Jobe, 1990). The system shows countertransport. A good competitive inhibitor for sulfate uptake, thus a good candidate substrate for this transporter is the molybdate ion, a trace element. Sulfate uptake was stimulated by an inward-directed proton gradient, with a $K_t$ of 160 $\mu$M, and was sensitive to membrane potentials. The protonophore CCCP could increase proton-driven sulfate uptake. Chloride could *trans*-stimulate sulfate uptake in the presence of a pH gradient, at the concentration of 150 mM but not of 10 mM. The model of a proton-driven sulfate-chloride exchanger was proposed. Since DIDS could inhibit sulfate countertransport, a comparison has been made with the anion exchanger of the erythrocyte membrane (band 3).

A preliminary report of a transporter for $Ca^{++}$ has recently appeared (Lemons and Thoene, 1991). Considering that calcium is an essential cation in the regulation of many metabolic and hormonal responses, the participation of lysosomes and their membrane transporters in the regulation of the intracellular homeostasis could be important.

The role of lysosomes in the redistribution of endocytosed material seems particularly important in the case of vitamins. The acute metabolic decompensation observed in individuals with a genetic defect of the lysosomal efflux of cobalamin confirms this assumption (Rosenblatt et al., 1986). Vitamin $B_{12}$ is endocytosed as a complex bound to transcobalamin II (Rosenblatt and Cooper, 1990). The complex is degraded in the lysosome where the vitamin, as cobalamin, is released from the lysosome via a carrier mediated route, recently described (Jonas and Idriss, 1991). In the latter study, the uptake of cyanocobalamin in lysosomal membrane vesicles, in the presence of a pH gradient, generated an *overshoot*. Elimination of the proton gradient by the protonophore FCCP led to inactivation of transport, but definitive conclusions about the system working as a $H^+$-cotransporter were not drawn. *Cis*-inhibition and *trans*-stimulation were possible with the analogs adenosylcobalamin and methylcobalamin, the bioactive forms of the vitamin, but not with cobinamide dicyanide. $Mg^{++}$ dependence was shown by inactivation of the transport in the presence of ethylendiaminotetracetate. The detection of this transport system will allow studies in material from patients with the cobalamin F mutation, where cobalamin is trapped in the lysosome

(Rosenblatt et al., 1985).

Also biotin is metabolized in the lysosome before being bioactively available as a cofactor of many carboxylases. A preliminary report suggests that under physiological pH conditions, free biotin is able to efflux from the lysosome via a carrier-mediated mechanism (Idriss et al., 1991).

The exact mechanism for the lysosomal efflux of cholesterol has not been identified yet. Therefore, a discussion about cholesterol release, more than already developed in chapter 1 or in the next chapter, where cholesterol transport defects will be reviewed, will not find place here.

A new interesting lysosomal import system has been recently described for folyl-polyglutamates (Barrueco and Sirotnak, 1991). Polyglutamates can also combine with the folic acid analog and antitumor agent methotrexate and enter the lysosome to undergo degradation by a specific hydrolase. The transport system is stimulated, in an additive manner, by $Mg^{++}$ and $K^{+}$ ions, and polyglutamates with longer chains seem to be better substrates. This is the first example of a transporter for compounds entering lysosomes *before* degradation. A hypothetical inhibitor of this carrier could be of therapeutical importance to inhibit degradation and enhance cytotoxicity of methotrexate.

At last, a unique example of lysosomal coupling of transmembrane transport to enzymatic catalysis is the transfer of acetyl-CoA and subsequent acetylation of glucosamine by an integral lysosomal membrane enzyme, in an ATP-independent manner (see chapter 1).

**Discussion**

None of the lysosomal carriers has as yet been identified nor purified to homogeneity. It is still not known whether they are products of different genes than those encoding the plasma membrane carriers for the same substrates. Biochemical differences found for the transport of cationic amino acids (Pisoni et al., 1987a), and the occurrence of a human disease where apparently only the plasma membrane transport of lysine is affected (Simell, 1989), would rather point to genetic heterogeneity among these proteins.

As indicated in table I, a number of carriers seem to be candidates for *secondary active* transporters coupled to a pH gradient. Some of them are molecules bearing a net charge at physiological pH, therefore a cotransport system might contribute to keep the membrane potential unaltered. Considering the physiological pH gradient existing across the lysosome membrane, this would be an economical way to regulate solute flux. However, in most of the cases the investigations have been performed in intact organelles where the pH at both sides of the membrane and the lysosomal acidification could not be controlled. For many of the systems listed in table I, conclusions about the mechanism of transport were not drawn. The first evidence for a lysosomal *secondary active*, proton cotransport system was provided by studies with lysosomal membrane vesicles where the pH was controlled with impermeant buffers at both sides of the membrane (publication III). It is possible that similar type of investigations will clarify to which extent pH regulates lysosomal transport for other

candidate substrates.

An interesting aspect of lysosomal transport concerns the role of lysosomal carriers in highly differentiated cell types, where they can be involved in very specialized functions. This is for example the case of the thyroid, where transport of monoiodothyrosine and diiodothyrosine (MIT and DIT), liberated during lysosomal degradation of thyreoglobulin in the follicular cells and destined to intracellular recycling, is mediated by the non specialized carrier for large neutral amino acids (Andersson et al., 1990). Apparently, this TSH-regulated system could also transport tyroxine (Tietze et al., 1989). If the initial evidence that $T_3$ and $T_4$ compete for the transport of MIT and DIT will be confirmed by the demonstration that $T_3$ and $T_4$ are real substrates, the hypothesis that an aspecific transport system serves the excretion of a hormone in a specialized endocrine tissue will be replaced by undoubted evidence. As a consequence, one might expect to find transporters which can accomplish very specialized functions in certain tissues, serving for other "housekeeping" functions in non-specialized tissues.

REFERENCES


* Anderson, M.P., Gregory, R.J., Thompson, S., Souza, D.W., Paul, S., Mulligan, R.C., Smith, A.E. and Welsh, M.J. (1991) Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science* 253:202-205

* Anderson, R.G. and Orci, L. (1988) A view of acidic intracellular compartments. *J. Cell Biol.* 106:539-543

* Andersson, H.C., Kohn, L.D., Bernardini, I., Blom, H.J., Tietze, F., and Gahl, W.A. (1990) Characterization of lysosomal monoiodothyrosine transport in rat thyroid cells. Evidence for transport by system h. *J. Biol. Chem.* 265:10950-10954

* Barasch, J., Gershon, M.D., Nunez, E.A., Jamir, H., Al-Awqati, Q. (1988) Thyrotropin induces the acidification of the secretory granules of parafollicular cells by increasing the chloride conductance of the granular membrane. *J. Cell Biol.* 107:2137-2147

* Barasch, J., Kiss, B., Prince, A., Saiman, L., Gruenert, D. and Al-Awqati, Q. (1991) Defective acidification of intracellular organelles in cystic fibrosis. *Nature* 352:70-73

* Barrueco, J.R. and Sirotnak, F.M. (1991) Evidence for the facilitated transport of methotrexate polyglutamates into lysosomes derived from S180 cells. Basic properties and specificity for polyglutamate chain lenght. *J. Biol. Chem.* 266:11732-11737

* Bernar, J., Tietze, F., Kohn, L.D., Bernardini, I., Harper, G.S., Grollman, E.F. and Gahl, W.A. (1986) Characteristics of a lysosomal membrane transport system for tyrosine and other neutral amino acids in rat thyroid cells. *J. Biol. Chem.* 261:17107-17112

* Bird, S.J., Forster, S. and Lloyd, J.B. (1987) Translocation of sugars into rat liver lysosomes. Evidence against a common carrier for D-glucose and D-ribose. *Biochem. J.* 245:929-931

* Bird, S.J. and Lloyd, J.B. (1990) Evidence for a dipeptide porter in the lysosome membrane. *Biochim. Biophys. Acta* 1024:267-270

* Bowman, E.J. (1983) Comparison of vacuolar membrane ATPase of Neurospora crassa with the mitochondrial and plasma membrane ATPase. *J. Biol. Chem.* 258:15238-15244

* Bridges, R. and Benos, D.J. (1990) "Reconstitution of epithelial chloride channels" in Current topics in membrane and transport., vol.37 pp.283-312.

* Christensen, H.N. (1990) Role of amino acid transport and countertransport in nutrition and metabolism. *Physiol. Rev.* 70:43-77
* Cidon, S. and Welson, N. (1983) A novel ATPase in the chromaffin granule membrane. *J. Biol. Chem.* 258:2892-2898
* Collarini, E.J., Pisoni, R.L. and Christensen, H.N. (1989) Characterization of a transport system for anionic amino acids in human fibroblast lysosomes. *Biochim. Biophys. Acta* 987:139-144
* Cuppoletti, J., Aures-Fisher, D. and Sachs, G. (1987) The lysosomal $H^+$ pump: 8-azido-ATP inhibition and the role of chloride in $H^+$ transport. *Biochim. Biophys. Acta* 899:276-284
* Dean, G.E., Fishkes, H., Nelson, P.J. and Rudnick, G. (1984) The hydrogen ion-pumping adenosine triphosphatase of platelet dense granule membrane. Differences from $F_1F_0$- and phosphoenzyme-type ATPases. *J. Biol. Chem.* 259:9569-9574
* Dell'Antone, P. (1979) Evidence for an ATP-driven "proton pump" in rat liver lysosomes by basic dyes uptake. *Biochem. Biophys. Res. Commun.* 86:180-189
* de Duve, C. (1963) The lysosome concept. In "Lysosomes". Ciba Found. Symp. ed. de Reuck, A.V.S. and Cameron, M.P. pp.1-35. London: Churchill.
* Dierks, T., Salentin, A. and Krämer, R. (1990) Pore-like and carrier-like properties of the mitochondrial aspartate/glutamate carrier after modification by SH-reagents: evidence for a preformed channel as a structural requirement of carrier-mediated transport. *Biochim. Biophys. Acta* 1028:281-288
* Docherty, K., Brenchley, G.V., Hales, C.N. (1979) The permeability of rat liver lysosomes to sugars. Evidence for carrier-mediated facilitated diffusion. *Biochem. J.* 178:361-366
* Docherty, K. and Hales, C.N. (1979) The effects of ions and pH on the transport of sugars into rat liver lysosomes. *FEBS Lett.* 106:145-148
* D'Souza, P., Ambudkar, S.V., August, J.T. and Maloney, P.C. (1987) Reconstitution of the lysosomal proton pump. *Proc. Natl. Acad. Sci. USA* 84:6980-6984
* Ehrenreich, B.A. and Cohn, Z.A. (1969) The fate of peptides pinocytosed by macrophages in vitro. *J. Exp. Med.* 129:227-243
* Fischbarg, J., Kuang, K., Hirsch, J., Lecuona, S., Rogozinski, L., Silverstein, S.C. and Loike, J. (1989) Evidence that the glucose transporter serves as a water channel in J774 macrophages. *Proc. Natl. Acad. Sci. USA* 86:8397-8401
* Forgac, M. (1983) Clathrin-coated vesicles contain an ATP-dependent proton pump. *Proc. Natl. Acad. Sci. USA* 80:1300-1303
* Forgac, M. (1989) Structure and function of vacuolar class of ATP-driven proton pumps. *Physiol. Rev.* 69:765-796
* Forster, S. and Lloyd, J.B. (1988) Solute translocation across the mammalian lysosome membrane. *Biochim. Biophys. Acta* 947:465-491.
* Fuchs, R., Mâle, P. and Mellman, I. (1989) Acidification and ion permeabilities of highly purified rat liver endosomes. *J. Biol. Chem.* 264:2212-2220
* Gahl, W.A., Bashan, N., Tietze, F., Bernardini, I. and Schulman, J.D. (1982) Cystine transport is defective in isolated leukocyte lysosomes from patients with cystinosis. *Science* 217:1263-1265
* Galloway, C.J., Dean, G.E., Marsh, M., Rudnick, G. and Mellman, I. (1983) Acidification of macrophage and fibroblast endocytic vesicles in vitro. *Proc. Natl. Acad. Sci. USA* 80:3334-3338
* Geck, P. and Heinz, E. (1981) in "Membrane transport" (Bonting, S.L. and de Pont J.J.H. eds.) series: New comprehensive biochemistry, vol.2, chapter 11. Elsevier, North Holland biochemical press.
* Gennis, R.B. (1989) Biomembranes. Molecular structure and function. Springer Verlag, New York.
* Glickman, J., Croen, K., Kelly, S. and Al Awqati, Q. (1983) Golgi membranes contain an electrogenic $H^+$ pump in parallel to a chloride conductance. *J. Cell Biol.* 97:1303-1308

* Gluck, S., Kelly, S. and Al-Awqati, Q. (1982) The proton-translocating ATPase responsible for urinary acidification. *J. Biol. Chem.* 257:9230-9233
* Goldman, R. and Kaplan, A. (1973) Rupture of rat liver lysosomes mediated by L-amino acid esters. *Biochim. Biophys. Acta* 318:205-216
* Goldstein, D.J., Finbow, M.E., Andresson, T., McLean, P., Smith, K., Bubb, V. and Schlegel, R. (1991) Bovine papillomavirus E5 oncoprotein binds to the 16K component of vacuolar $H^+$-ATPases. *Nature* 352:347-349
* Greene, A.A., Marcusson, E.G., Pintos Morell, G. and Schneider, J.A. (1990) Characterization of the lysosomal cystine transport system in mouse L-929 fibroblasts. *J. Biol. Chem.* 265:9888-9895
* Gürich, R.W., Codina, J., and Du Bose, T.D. Jr. (1991) A potential role for guanine nucleotide-binding protein in the regulation of endosomal proton transport. *J. Clin. Invest.* 87:1547-1552
* Hales, C.N. (1978) Proteolysis and the evolutionary origin of polypeptide hormones. *FEBS Lett.* 94:10-16
* Harikumar, P. and Reeves, J.P. (1983) The lysosomal proton pump is electrogenic. *J. Biol. Chem.* 258:10403-10410
* Harper, G.S., Kohn, L.D., Bernardini, I., Bernar, J., Tietze, F., Andersson, H.C. and Gahl, W.A. (1988) Thyrotropin stimulation of lysosomal tyrosine transport in rat FRTL-5 thyroid cells. *J. Biol. Chem.* 263:9320-9325
* Henderson, P.J.F. and Maiden, M.C.J. (1990) Homologous sugar transport proteins in Escherichia coli and their relatives in both prokaryotes and eukaryotes. *Phil. Trans. R. Soc. London* B 326:391-410
* Higgins, C.F., Gallagher, M.P., Hyde, S.C., Mimmack, M.L. and Pearce, S.R. (1990) Periplasmic binding protein-dependent transport systems: the membrane-associated components. *Phil. Trans. R. Soc. London* 326:353-365
* Higgins, C.F. and Hyde, S.C. (1991) Channelling our thoughts. *Nature* 352:194-195
* Hirsch, S., Strauss, A., Masood, K., Lee, S., Sukhatme, V. and Gluck, S. (1988) Isolation and sequence of a cDNA clone encoding the 31-kDa subunit of bovine kidney vacuolar $H^+$-ATPase. *Proc. Natl. Acad. Sci. USA* 85:3004-3008
* Hyde, S.C., Emsley, P., Hartshorn, M.J., Mimmack, M.L., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E. and Higgins, C.F. (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 346:362-365)
* Idriss, D.S., Koetters, P.J. and Jonas, A.J. (1991) Lysosomal transport of biotin. *Ped. Res.* 29:194A
* Iveson, G.P., Bird, S.J. and Lloyd, J.B. (1989) Passive diffusion of non-electrolytes across the lysosome membrane. *Biochem. J.* 261:451-456
* Johnson, R.G., Beers, M.F. and Scarpa, A. (1982) $H^+$-ATPase of chromaffin granules. Kinetics, regulation and stoichiometry. *J. Biol. Chem.* 257:10701-10707
* Jonas, A.J. and Jobe, H. (1990) Sulfate transport by rat liver lysosomes. *J. Biol. Chem.* 265:17545-17549
* Jonas, A.J., Smith, M.L. and Schneider, J.A. (1982) ATP-dependent lysosomal cystine efflux is defective in cystinosis. *J. Biol. Chem.* 257:13185-13188
* Jonas, A.J., Conrad, P. and Jobe, H. (1990) Neutral-sugar transport by rat liver lysosomes. *Biochem. J.* 272:323-326
* Jonas, A.J., Speller, R.J., Conrad, P.B. and Dubinsky, W.P. (1989) Transport of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine by rat liver lysosomes. *J. Biol. Chem.* 264:4953-4956
* Jonas, A.J. and Idriss, J.M. (1991) Vitamin $B_{12}$ transport by rat liver lysosomal membrane vesicles. *J. Biol. Chem.* 266:9438-9441
* Lemons, R.M. and Thoene, J.G. (1991) Mediated calcium transport by isolated human fibroblast lysosomes. *J. Biol. Chem.* 266:14378-14382

* Lloyd, J.B. (1969) Studies on the permeability of rat liver lysosomes to carbohydrates. *Biochem. J.* 115:703-707
* Lloyd, J.B. (1971) A study of permeability of lysosomes to amino acids and small peptides. *Biochem. J.* 121:245-248
* Maguire, G.A., Docherty, K. and Hales, C.N. (1983) Sugar transport in rat liver lysosomes. Direct demonstration by using labeled sugars. *Biochem. J.* 212:211-218
* Maloney, P.C. (1990) A consensus structure for membrane transport. *Res. in Microbiol.* 141:374-383
* Mandel, M., Moryiama, Y., Hulmes, J.D., Pan, Y.E., Nelson, H. and Nelson, N.(1988) cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of $H^+$-ATPases. *Proc. Natl. Acad. Sci. USA* 85:5521-5524
* Manolson, M.F., Rea, P.A. and Poole, R.J. (1985) Identification of 3-O-(4-benzoyl)benzoyladenosine 5'-triphosphate- and N,N'-dicyclohexylcarbodiimide-bindingsubunits of a higher plant $H^+$-translocating tonoplast ATPase. *J. Biol. Chem.* 260:12273-12279
* Metchnikoff, E. (1893) in "Lectures on the comparative pathology of inflammation". Kegan, Paul, Trench. Trübuer & co. Ltd, London.
* Mortimore, G. E., Lardeux, B.R., and Adams, C.E. (1988) Regulation of microautophagy and basal protein turnover in rat liver. Effects of short term starvation. *J. Biol. Chem.* 263:2506-2512
* Moriyama, Y., Takano, T. and Ohkuma, S. (1986) Similarity of lysosomal $H^+$-ATPase to mitochondrial $F_1F_0$-ATPase in sensitivity to anions and drugs as revealed by solubilization and reconstitution. *Biochim. Biophys. Acta* 854:102-108
* Moriyama, Y. and Nelson, N. (1987) The purified ATP-ase from chromaffin granule membrane is an anion-dependent proton pump. *J. Biol. Chem.* 262:9175-9180
* Moriyama, Y. and Nelson, N. (1989a) Lysosomal $H^+$-translocating ATPase has a similar subunit structure to chromaffin granule $H^+$-ATPase complex. *Biochim. Biophys. Acta* 980:241-247
* Moriyama, Y. and Nelson, N. (1989b) Cold inactivation of vacuolar proton-ATPases. *J. Biol. Chem.* 264: 3577-3582,.
* Murakami, H., Blobel, G. and Pain, D. (1990) Isolation and characterization of the gene for a yeast mitochondrial import receptor. *Nature* 347:488-491
* Nelson, N. (1987) The vacuolar proton-ATPase of eukaryotic cells. *BioEssays* 7:251-254
* Ohkuma, S. and Poole, B. (1978) Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA* 75:3327-3331.
* Ohkuma, S. (1987) The lysosomal proton pump and its effect on protein breakdown. In "Lysosomes: their role in protein breakdown". H. Glaumann and F.J. Ballard eds. Academic Press, London.
* Ohsumi, Y. and Anraku, Y. (1981) Active transport of basic amino acids driven by a proton motive force in vacuolar membrane vesicles of Saccharomyces cerevisiae. *J. Biol. Chem.* 256:2079-2082
* Parham, P. (1990) Transporters of delight. *Nature* 348:674-675
* Perin, M.S., Fried, V.A., Stone, D.K., Xie, X.S. and Südhof, T.C. (1991) Structure of the 116-kDa polypeptide of the clathrin-coated vesicle/synaptic vesicle proton pump. *J. Biol. Chem.* 266:3877-3881
* Pisoni, R.L., Thoene, J.G. and Christensen, H.N. (1985) Detection and characterization of carrier-mediated cationic amino acid transport in lysosomes of normal and cystinotic human fibroblasts. Role in therapeutic cystine removal? *J. Biol. Chem.* 260:4791-4798
* Pisoni, R.L., Thoene, J.G., Lemons, R.M. and Christensen, H.N. (1987a) Important differences in cationic amino acid transport by lysosomal system c and system $y^+$ of the human fibroblast. *J. Biol. Chem.* 262:15011-15018
* Pisoni, R.L., Flickinger, K.S., Thoene, J.G. and Christensen, H.N. (1987b) Characterization of carrier-mediated transport systems for small neutral amino acids in human fibroblast lysosomes. *J. Biol. Chem.* 262:6010-6017

* Pisoni, R.L., Lisowski, K.M., Lemons, R.M., Thoene, J.G. (1989a) Utilization of mercaptoethylgluconamide for depleting human cystinotic fibroblasts of their accumulated cystine. *Ped. Res.* 26:73-76

* Pisoni, R.L., and Thoene, J.G. (1989b) Detection and characterization of a nucleoside transport system in human fibroblast lysosomes. *J. Biol. Chem.* 264:4850-4856

* Pisoni, R.L., Acker, T.L., Lisowski, K.M., Lemons, R.M. and Thoene, J.G. (1990) A cysteine-specific lysosomal transport system provides a major route for the delivery of thiol to human fibroblast lysosomes: possible role in supporting lysosomal proteolysis. *J. Cell Biol.* 110:327-335

* Pisoni, R.L. (1991) Characterization of a phosphate transport system in human fibroblast lysosomes. *J. Biol. Chem.* 266:979-985

* Quinton, P.M. and Bijman, J. (1983) Higher bioelectric potentials due to decreased chloride absorption in the sweat glands of patients with cystic fibrosis. *N. Engl. J. Med.* 308:1185-1189

* Reijngoud, D.J. and Tager, J.M. (1977) The permeability properties of the lysosomal membrane. *Biochim. Biophys. Acta* 472:419-449

* Rich, D.P., Gregory, R.J., Anderson, M.P., Manavalan, P., Smith, A.E. and Welsh, M.J. (1991) Effect of deleting the R domain on CFTR-generated chloride channels. *Science* 253:205-207

* Riordan, J.R., Rommens, J.M., Kerem, B.S., Alon, N., Rozmahel, R., Grzelczack, Z., Zielenski,J., Lok,S., Plavsic, N., Chou, J.L., Drumm, M.L., Iannuzzi, M.C., Collins, F.S. and Tsui, L.C. (1989) Identification of the cystic fibrosis gene: cloning and characterization of the complementary DNA. *Science* 245:1066-1073

* Rome, L.H., and Hill, D.F. (1986) Lysosomal degradation of glycoproteins and glycosaminoglycans. Efflux and recycling of sulphate and N-acetylhexosamines. *Biochem. J.* 235:707-713

* Rosenblatt, D.S., Hosack, H., Matiaszuk, N.V., Cooper, B.A. and Laframboise, R. (1985) Defect in vitamin $B_{12}$ release from lysosomes: newly described inborn error of vitamin $B_{12}$ metabolism. *Science* 228:1319-1321

* Rosenblatt, D.S., Laframboise, R., Pichette, J., Langevin, P., Cooper, B.A. and Costa, T. (1986) New disorder of vitamin $B_{12}$ metabolism (cobalamin F) presenting as methylmalonic aciduria. *Pediatrics* 78:51-54

* Rosenblatt, D.S. and Cooper, B.A. (1990) Inherited disorders of vitamin $B_{12}$ utilization. *BioEssays* 12:331-334

* Sakmann, B. and Neher, E. (1983) "Single channel recording", Plenum Press New York.

* Sambrook, J.F. (1991) Pumping peptides. How do small peptides travel across membranes that separate intracellular components. *Curr. Biol.* 1:57-58

* Scarborough, G.A. (1985) Binding energy, conformational change and the mechanism of transmembrane solute movement. *Microb.* Rev. 49:214-231

* Schneider, D.L., Burnside, J., Gorga, F.R. and Nettleton, C.J. (1978) Properties of the membrane proteins of rat liver lysosomes. The majority of lysosomal membrane proteins are exposed to the cytoplasm. *Biochem. J.* 176:75-82

* Schneider, D.L. (1979) The acidification of rat liver lysosomes in vitro: a role for the membrane ATPase as aproton pump. *Biochem. Biophys. Res. Commun.* 87:559-565

* Schneider, D.L. (1981) ATP-dependent acidification of intact and disrupted lysosomes. Evidence for an ATP-driven proton pump. *J. Biol. Chem.* 256:3858-3864

* Schneider, D.L. (1983) ATP-dependent acidification of membrane vesicles isolated from purified rat liver lysosomes. Acidification activity requires phosphate. *J. Biol. Chem.* 258:1833-1838

* Simell, O. (1989) Lysinuric protein intolerance and other cationic aminoacidurias. In "The metabolic basis of inherited disease" (Scriver, C.R., Baudet, A.L. and Valle, D. eds) VI ed. chapter 100. Mc Graw-Hill New York

* Simon, S.M. and Blobel, G. (1991) A protein-conducting channel in the endoplasmic reticulum. *Cell* 65:371-380
* Smith, M.L., Greene, A.A., Mendoza, S.A. and Schneider, J.A. (1987) Lysosomal cystine transport. Effect of intralysosomal pH and membrane potential. *J. Biol. Chem.* 262:1244-1253
* Stanfield, P.R. (1987) Nucleotides such as ATP may control the activity of ion channels. *Trends in Neurosci.* 10:335-339
* Stein, W.D. (1967) The movement of molecules across cell membranes. Chapter 3. Academic Press, New York.
* Stein, W.D. (1986) Transport and diffusion across cell membranes. pp.356-366. Academic press, Orlando.
* Stewart, B.H., Collarini, E.J., Pisoni, R.L. and Christensen, H.N. (1989) Separate and shared lysosomal transport of branched and aromatic dipolar amino acids. *Biochim. Biophys. Acta* 987:145-153
* Stone, D.K., Xie, X.S. and Racker, E. (1983) An ATP-driven proton pump in clathrin-coated vesicles. *J. Biol. Chem.* 258:4059-4062
* Südhof, T.C., Fried, V.A., Stone D.K., Johnston, P.A. and Xie, X.S. (1989) Human endomembrane $H^+$ pump strongly resembles the ATP-synthetase of archaebacteria. *Proc. Natl. Acad. Sci. USA* 86:6067-6071
* Swallow, C.J., Grinstein, S. and Rotstein, O.D. (1990) A vacuolar type $H^+$-ATPase regulates cytoplasmic pH in murine macrophages. *J. Biol. Chem.* 265:7645-7654
* Tietze, F., Kohn, L.D., Kohn, A.D., Bernardini, I., Andersson, H.C., Adamson, M.D., Harper, G.S. and Gahl, W.A. (1989) Carrier-mediated transport of monoiodotyrosine out of thyroid cell lysosomes. *J. Biol. Chem.* 264:4762-4765
* Tilly, B., Mancini, G.M.S., Bijman, J., van Gageldonk, P.G.M., Beerens, C.E.M.T., Bridges, R., de Jonge, H.R. and Verheijen, F.W. (1991) Nucleotide-activated chloride channels in rat liver lysosomal membranes. (manuscript submitted)
* Trinchera, M., Ghidoni, R., Greggia, L. and Tettamanti, G. (1990) The N-acetylgalactosamine residue of exogenous $GM_2$ ganglioside is recycled for glycoconjugate biosynthesis in rat liver. *Biochem. J.* 266:103-106
* Turner, R.J. (1983) Quantitative studies of cotransport systems: models and vesicles. *J. Membr. Biol.* 76:1-15
* Tycko, B. and Maxfield, F.R. (1982) Rapid acidification of endocytic vesicles containing $\alpha$-macroglobulin. *Cell* 28:643-651
* Uchida, E., Ohsumi, Y. and Anraku, Y. (1985) Purification and properties of $H^+$-translocating $Mg^{++}$-adenosinetriphosphatase from vacuolar membranes of Saccharomyces cerevisiae. *J. Biol. Chem.* 260:1090-1095
* Vadgama, J.V., Chang, K., Kopple, J.D., Idriss, J.M. and Jonas, A.J. (1991) Characteristics of taurine transport in rat liver lysosomes. *J. Cell. Physiol.* 147:447-454
* Van Dyke, R.W., Hornick, C.A., Belcher, J., Scharshmidt, B.F. and Havel, R.J. (1985) Identification and characterization of ATP-dependent proton transport by rat liver multivesicular bodies. *J. Biol. Chem.* 260:11021-11026
* Van Dyke, R.W. (1988) Proton pump-generated electrochemical gradients in rat liver multivesicular bodies. Quantitation and effects of chloride. *J. Biol. Chem.* 263:2603-2611
* West, I.C. (1983) The biochemistry of membrane transport. Chapmanand Hall, London and New York.
* Xie, X.S., Stone, D.K. and Racker, E. (1983) Determinants of clathrin-coated vesicle acidification. *J. Biol. Chem.* 258:14834-14838

* Xie, X,S. and Stone, D.K. (1988) Partial resolution and reconstitution of the subunits of the clathrin-coated vesicles proton ATPase responsible for $Ca^{++}$-activated ATP hydrolysis. *J. Biol. Chem.* 263:9859-9867

* Zhang, F. and Schneider, D.L. (1983) The bioenergetics of Golgi apparatus function: evidence for an ATP-dependent proton pump. *Biochem. Biophys. Res. Commun.* 114:620-625

Chapter 3

# GENETIC DISORDERS OF LYSOSOMAL TRANSPORT

Extensive reviews about the lysosomal transport defects are available (Gahl, 1989), therefore the clinical details will not be discussed here. Only essential features will be recalled in order to introduce some considerations which relate to the general problems addressed in our studies.

Four human inherited diseases are currently classified as lysosomal membrane transport defects; cystinosis, sialic acid storage diseases, Niemann-Pick disease type C and cobalamin F mutation. Among these only cystinosis and sialic acid storage diseases have been directly demonstrated to be monogenetic disorders caused by a primary defect of a lysosomal carrier. The experimental evidence for sialic acid storage disorders includes part of the work presented in this thesis and will be discussed in detail in the next chapters.

The evidence that Niemann-Pick disease type C (or II C) represents a lysosomal transport defect is still indirect. In Niemann-Pick disease type C, lysosomes accumulate large amounts of cholesterol and sphingomyelin. The suppressed sphingomyelinase activity and some aspects of the phenotype have warranted the classification of this disorder among Niemann-Pick diseases. According to more recent investigations, the storage of cholesterol appears to correlate with the primary defect, since the sphingomyelinase activity can be normalized in cells cultured under conditions which prevent cholesterol storage (Thomas et al., 1989). Several lines of evidence indicate that cholesterol derived from lysosomal degradation of low-density lipoproteins is not available for esterification in the endoplasmic reticulum, in the presence of a normal acyltransferase activity, therefore the metabolic defect

could reside in a mechanism of transport of cholesterol from the lysosome to the endoplasmic reticulum/Golgi apparatus (Liscum et al., 1989). Due to the chemical structure, it seems unlikely that cholesterol is blocked in the lysosome because of a deficient transmembrane simple carrier, but a more complex type of transport, perhaps vesicular, might be involved (van Meer, 1989). Although a certain clinical heterogeneity has been described, patients cannot be distinguished from those with a primary sphingomyelinase deficiency (Niemann-Pick diseases type IA and IB), presenting with a neurovisceral lipidosis (for a review see Spence, 1989).

The cobalamin F mutation (Cbl F) is the denomination of one of the complementation groups found for genetic defects of vitamin $B_{12}$ metabolism, clinically and biochemically related to methylmalonic acidurias (Cooper and Rosenblatt, 1987). Only two patients with this phenotype have been described to date (Rosenblatt et al., 1986 and Shih et al., 1989). In both cases, the small-for-date babies showed feeding difficulties, growth and developmental delay, persistent cutaneous rash and stomatitis, macrocytic anemia, homocysteinemia and methylmalonic aciduria. Both cases responded clinically and biochemically to hydroxycobalamin supplementation. However, one of these infants has been found dead in the crib (Shih et al., 1989). Metabolic loading of patient fibroblasts with [$^{57}$Co]cyanocobalamin preincubated with human serum (which contains transcobalamin II), followed by subcellular fractionation has shown that the increased intracellular content of cobalamins due to the intralysosomal accumulation of cyanocobalamin, after normal endocytosis and uncoupling of the transcobalamin II-cobalamin complex. This leads to cellular deficiency of the active coenzymes methylcobalamin and adenocobalamin (Rosenblatt et al., 1985). It was therefore deduced that the patient cells fail to transfer cobalamin from the lysosome to the cytosol, in the absence of a putative cobalamin membrane transporter. Microscopically, biopsy material and cultured cells do not show typical signs of lysosomal storage. A significant increase of lysosomal cobalamin has been shown only by sophisticated autoradiographic techniques, at the electron microscope level (Vassiliadis et al., 1991). The description of a lysosomal carrier for cobalamins (Jonas and Idriss, 1991) substantiates the hypothesis of a membrane transport defect and will allow the molecular definition of the primary defect.

Cystinosis has been the first genetic defect of a lysosomal membrane carrier that was discovered. The classic form of nephropathic cystinosis is essentially characterized by severe growth retardation and generalized renal tubular defect (Fanconi syndrome) leading to kidney failure within the first decade. Cystine crystals are deposited not only in the kidney, but also in the cornea, giving intense photophobia, in bone marrow and in other organs causing deterioration of several other functions. By the age of ten years, untreated patients require kidney transplantation which, until few years ago, was the only form of treatment possible for this disorder. The introduction of therapy with cysteamine will probably change the natural history of this disorder.

In 1976, it was observed that cystine storage in cystinotic lysosomes decreases upon

administration of thiols like cysteamine (Thoene et al., 1976). Only six years later the metabolic defect was identified in a specific transporter of the lysosome membrane (Gahl et al., 1982). Subsequent studies on the permeability of the lysosome membrane to different sorts of amino acids led to to the discovery of another transporter for cationic amino acids which can accomodate the mixed disulfide cysteine-cysteamine. The first follow-up studies in patients treated with cysteamine have shown depletion of cystine storage in peripheral leucocytes and significant growth improvement of the patients (Gahl et al., 1987). Renal damage, once established, seems to be irreversible but cysteamine therapy has been able to prevent development of renal Fanconi syndrome (Da Silva et al., 1985). Corneal crystals can be cleared upon treatment with cysteamine eyedrops, at high doses (Kaiser-Kupfer et al., 1987).

There are at least two other congenital disorders, a subtype of glycogenosis type II and Batten's disease (ceroidlipofuscinosis), which are lysosomal disorders with a possible involvement of membrane components. In the first, lysosomal glycogen degradation is impaired while the acid maltase activity is normal. It has been hypothesized, without experimental evidence, that impaired glucose efflux might cause maltase inhibition *in situ* (Ullrich et al., 1989). In the latter disease, accumulation of the 3.5-kDa intramembrane subunit of the mitochondrial ATP synthetase, highly homologous to the 17-kDa subunit of the lysosomal proton pump, accumulates in the lysosome (Hall et al., 1991).

REFERENCES


* Cooper, B.A. and Rosenblatt, D.S. (1987) Inherited defects of vitamin $B_{12}$ metabolism. *Annu. Rev. Nutr.* 7:291-320

* Da Silva, V.A., R.P. Zurbrugg, P. Lavanchy, A. Blumberg, H. Suter, S.R. Wyss, C.M. Luthy and O.H. Oetliker (1985). Long-term treatment of infantile nephropathic cystinosis with cysteamine. *N. Engl. J. Med.* 23:1460-1463

* Gahl, W.A., Bashan, N., Tietze, F., Bernardini, I. and Schulman, J.D. (1982) Cystine transport is defective in isolated leukocyte lysosomes from patients with cystinosis. *Science* 217:1263-1265

* Gahl, W.A., G.F. Reed, J.G. Thoene, J.D. Schulman, W.B. Rizzo, A.J. Jonas, D.W. Denman, J.J. Schlesselmann, B.J. Corden, and J.A. Schneider (1987). Cysteamine therapy for children with nephropathic cystinos. *N. Engl. J. Med.* 316:971-977.

* Gahl, W.A., Renlund, M. and Thoene, J.G. (1989) Lysosomal transport disorders: cystinosis and sialic acid storage disorders. In: "The metabolic basis of inherited disease" (Scriver, C.R., Baudet, A.L., Sly, W.S., Valle, D. eds.) chapter 107. McGraw-Hill Information Services Company.

* Hall, N.A., Lake, B.D., Dewji, N.N. and Patrick, A.D. (1991) Lysosomal storage of subunit *c* of mitochondrial ATP synthase in Batten's disease (ceroidlipofuscinosis). *Biochem. J.* 275:269-272

* Jonas, A.J. and Idriss, J.M. (1991) Vitamin $B_{12}$ transport by rat liver lysosomal membrane vesicles. *J. Biol. Chem.* 266:9438-9441

* Kaiser-Kupfer, M.I., Fujikawa, L., Kuwabara, T., Jain, S. and Gahl, W.A. (1987) Removal of corneal crystals by topical cysteamine in nephropathic cystinosis. *N. Engl. J. Med.* 316:775-779

* Liscum, L., Ruggiero, R.M. and Faust, J.R. (1989) The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick type C fibroblasts. *J. Cell Biol.* 108:1625-1636

* van Meer, G. (1989) Lipid traffic in animal cells. *Ann. Rev. Cell Biol.* 5:247-275

* Rosenblatt, D.S., Hosack, H., Matiaszuk, N.V., Cooper, B.A. and Laframboise, R. (1985) Defect in vitamin $B_{12}$ release from lysosomes: newly described inborn error of vitamin $B_{12}$ metabolism. *Science* 228:1319-1321

* Rosenblatt, D.S., Laframboise, R., Pichette, J., Langevin, P., Cooper, B.A. and Costa, T. (1986) New disorder of vitamin $B_{12}$ metabolism (cobalamin F) presenting as methylmalonic aciduria. *Pediatrics* 78:51-54

* Shih, V.E., Axel, S.M., Tewksbury, J.C., Watkins, D., Cooper, B.A. and Rosenblatt, D.S. (1989) Defective lysosomal release of vitamin $B_{12}$ (cbl F): a hereditary cobalamin metabolic disorder associated with sudden death. *Am. J. Med. Genet.* 33:555-563

* Spence, M.W. and Callahan, J.W. (1989) Sphingomyelin-cholesterol lipidoses: the Niemann-Pick group of diseases. In: "The metabolic basis of inherited disease" (Scriver, C.R., Baudet, A.L., Sly, W.S., Valle, D. eds.) chapter 66. pp.1655-1676. McGraw-Hill Information Services Company.

* Thoene, J.G., R.G. Oshima, J.C. Crawhall, D.L. Olson, and J.A. Schneider (1976). Cystinosis. Intracellular cystine depletion by amino thiols in vitro and in vivo. *J. Clin. Invest.* 58:180-187

* Thomas, G.H., Tuck-Muller, C.M., Miller, L.S. and Reynolds, L.W. (1989) Correction of sphingomyelinase deficiency in Niemann-Pick type C fibroblasts by removal of lipoprotein fraction from culture media. *J. Inher. Metab. Dis.* 12:139-151

* Ullrich, K., von Bassewitz, D., Shin, J., Korinthenberg, R., Sewell, S., von Figura, K. (1989) Lysosomal glycogen storage disease without deficiency of acid alpha-glucosidase. *Genetics of neuromuscular disorders.* pp. 163-171. Alan R. Liss Inc.

* Vassiliadis, A., Rosenblatt, D.S., Cooper, B.A. and Bergeron, J.J.M. (1991) Lysosomal cobalamin accumulation in fibroblasts from a patient with an inborn error of cobalamin metabolism (cbl F complementation group): visualization by electron microscope radioautography. *Exp. Cell Res.* 195:295-302

Chapter 4

# SIALIC ACID STORAGE DISORDERS

## Sialic acid metabolism

The major type of sialic acid in humans is N-acetylneuraminic acid (NeuAc). In other mammalians, a variable but often minor percentage of the sialic acids is represented by N-glycolylneuraminic acid (NeuGc). These monocarboxylic N-substituted monosaccharides are essential components of oligosaccharides (OGS) either present in glycoproteins or gangliosides. OGS-bound sialic acids are involved in main metabolic functions like immunological processes, hormonal responses, signal transmission in neurons, tumor progression, cell adhesion and protection from premature degradation (for a review: Schauer, 1982).

The essential steps of intracellular sialic acid metabolism (as NeuAc) are schematically depicted in fig. 3. The steps indicated as 1, 2, 3 are respectively catalyzed by UDP-GlcNAc-2-epimerase (Thomas et al., 1985), the key enzyme of NeuAc synthesis, $\alpha$-neuraminidase (Verheijen et al., 1985) and the lysosomal carrier for acidic monosaccharides (publication III). They are particularly mentioned here because they are respectively linked to three distinct human monogenetic disorders.

A defective feed-back inhibition of the epimerase at step 1 by CMP-NeuAc, leading to hyperproduction of free NeuAc, has been found in patients with a syndrome characterized by massive sialuria and cytosolic accumulation of free sialic acid (Thomas et al., 1985; Weiss et al., 1989; Seppala et al., 1991). Three unrelated patients with this disorder have been identified to date (Fontaine et al., 1968; Wilken et al., 1987). Another patient excreting

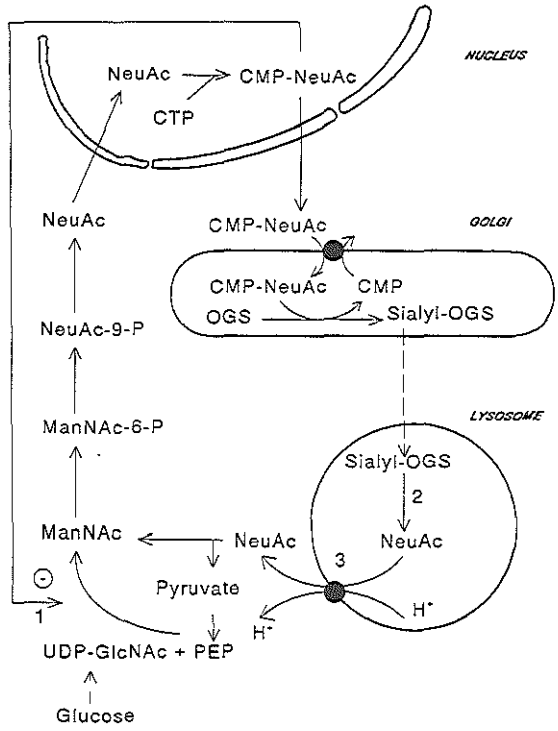


**Figure 3.** Metabolism of sialic acid. CMP: cytidine-monophosphate; CTP: cytidine-triphosphate; UDP-GlcNAc: uridine-diphosphate-N-acetylglucosamine; ManNAc: N-acetylmannosamine; PEP: phospho-enol-pyruvate.

large quantities of sialic acid, described by Palo et al. (1985), presented with epilepsy, neuromotor impairment, skeletal changes but no visceromegaly. No evidence of lysosomal storage was observed and also the possibility of sialic acid overproduction has been excluded (Dr. M. Renlund, personal communication). Her disease remains undiagnosed.

Defective intralysosomal degradation of glycoproteins (Cantz et al., 1977; Cantz and Messer, 1979) and gangliosides (Mancini et al., 1986; Lieser et al., 1989) by the enzyme $\alpha$-neuraminidase (step 2, fig.3) is the defect in patients with sialidosis, where sialyl-OGS and a little free sialic acid, accumulate in the lysosomes (Cantz et al., 1977; Durand and O'Brien, 1982). Some lysosomal storage diseases present with secondary neuraminidase deficiency and share some clinical aspects with sialidosis, namely I-Cell disease and galactosialidosis (Hoogeveen et al., 1980).

Following degradation of sialyl-OGS, the efflux of free NeuAc out of the lysosome precedes its cytoplasmic reutilization (step 3, fig. 3). The observation of patients with lysosomal storage of free sialic acid, in whom the other steps of sialic acid metabolism appeared normal, suggested a defective efflux of sialic acid from patient lysosomes, hence

the existence of a specific transport protein for sialic acid (Renlund et al., 1983). The clarification of the biochemical mechanism of lysosomal NeuAc transport and of the primary metabolic defect in these patients has been the subject of this thesis.

**Clinical aspects of sialic acid storage diseases**

Sialic acid storage diseases (SASD) are characterized by lysosomal accumulation of free (unbound) sialic acid and excessive sialuria. Two distinct phenotypes are known: Salla disease, the adult type (SD) and the infantile sialic acid storage disease (ISSD). Salla disease was originally described in a group of patients originating from a circumscribed area in northern Finland, which lend the eponym (Aula et al., 1979). The disease is usually evident in the first months of life with hypotonia and ataxia. Nystagmus, as a sign of central nervous system involvement, can precede the ataxia. Very often permanent exothropia persists. The facies is not specifically malformed, but often hypertelorism, long philtrum and a slightly coarse features can be observed. Developmental delay is invariably present, with major handicaps in mentation, walking and speeching abilities. Patients usually reach adult life and are severely mentally retarded with an I.Q. often below 20. Growth retardation is present in half of the patients. Neurological signs in adult life are ataxia and dysdiadochokynesis, athetosis, brisk tendon reflexes (often with an extensor plantar response), epilepsy in about one third of the cases, low voltage EEG, dysarthric and dyspraxic speech. CT-scans show enlarged ventricles, cortical and basal brain atrophy. Visceromegaly and skeletal abnormalities do not belong to the usual presentation of SD (Renlund et al., 1983; Renlund, 1984). To date 85 patients have been diagnosed in Finland, where the relevant gene appears to be particularly abundant, with a carrier frequency in the population in northern Finland of 1:40 (Aula et al., 1986), high enough to justify heterozygote screening (Prof. Dr. P.P. Aula, personal communication). Life span does not seem to be shortened and the oldest patient deceased at the age of 72 (Mancini et al., 1991).

ISSD is a severe neurovisceral disease, described so far in 15 patients. The major clinical findings are listed in table III. This form does not show ethnical prevalence and has not been diagnosed in Finland. Constant features are the visceromegaly, the growth retardation, the severe psychomotor delay, the facial dysmorphysms and the hypopigmentation. Skeletal changes, though often present as dysostosis multiplex, are not constant. On the other hand primary skeletal abnormalities are never seen in Salla disease.

Non-Finnish patients with a clinical presentation similar to Salla disease have been classified in a separate table (table IV). Among these, some have intermediate features which would make a classification in either the SD or ISSD group difficult. For example, the patients of Ylitalo et al. (1986) showed a striking difference between the almost normal level of mentation and the severe motor impairment. In Salla disease, the opposite situation is more often encountered (Renlund et al., 1983). The skeletal changes observed in the patients by Wolburg-Buchholz and Fois, though not radiologically representative for dysostosis

TABLE III

ISSD: Clinical Manifestations

| Author | Hepatosplenomegaly/ascites | Skeletal changes | Growth retardation | Neonatal Hypotonia | Hypopigmentation | Facies | Signs of Cardiorespiratory failure | Psychomotor development | Ocular findings | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|
| *Horowitz, 1981 | Yes | No | | | | | | | | †5 mo. |
| *Cooper, 1988 | | | | | | | | | | |
| -case 1 | Yes | No | | No | | Dysmorphic | Yes | Delayed | | |
| -case 2 | Yes | Yes | | | | Dysmorphic | Yes | Delayed | | |
| *Tondeur, 1982 | | | | | | | | | | |
| -case 1 | Yes | No | Yes | Yes | Yes | Gargoylic | Yes | Delayed | Normal | † 5 yr. |
| -case 2 | Yes | No | Yes | Yes | Yes | Coarse | Yes | Delayed | Normal | alive 2 yr. |
| *Stevenson, 1983 | | | | | | | | | | |
| -case 1 | Yes | Yes | Yes | No (hypertonus) | Yes | Coarse; prominent filtrum | Yes | Delayed | Squint + Epicanthus | † 3½ yr. |
| -case 2 | Yes | Yes | Yes | No (hypertonus) | Yes | Dysmorphic | Yes | Delayed | Retinal pallor; salt & pepper appearance | Alive, 15 mo. |
| *Paschke, 1986 (2 cases, one published | Yes | No | Yes | Yes (Truncal) | Yes | Long philtrum | Yes | Delayed | Normal | † 10 mo. |
| *Cameron, 1990 | Yes | No | Yes | Yes | Yes | Dysmorphic, coarse | Yes | Delayed | Strabismus pale retina | † 13 mo. |
| *Lake, 1989, case 1 (+ prenatal) | Yes | | Yes | Yes | | Mildly dysmorphic | | Severely delayed | | † 17 mo. |
| *Sperl, 1990 | | | | | | | | | | |
| -case 1 | Yes | No | Yes | Yes | Yes | Dysmorphic prominent filtrum | Yes | Severely delayed | Strabismus | † 1 yr. |
| -case 2 | Yes | | | Yes | Yes | Dysmorphic | Yes | | | † 3 mo. |
| *Clements, 1988 | | | | | | Coarse | | | | |
| -case 1 | Yes | | | | | Coarse | | | | † 18 mo. |
| -case 2 | Yes | | | | | | | | | † 1 mo. |

**TABLE IV**

**Non-Finnish (Sporadic) Salla Disease and Intermediate Cases**

| Author | Hepatospleno-megaly | Skeletal changes | Growth retardation | Muscular tone | Hypo-pigmentation | Facies | Cardiorespiratory failure | Psychomotor development | Ocular findings | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|
| *Wolburg-Buchholz, 1985<br>-case 1 (3 in total) | - | +/- | | Hypotonia + Tetraplegia | | Dysmorphic | | Severely delayed | | Alive 9 yr. |
| *Ylitalo, 1986<br>-case 1<br>-case 2 | | - | | Hypotonia | | | | Near to normal mentation<br>Delayed motor functions | Normal | Alive 6 yr. |
| | | | | Hypotonia | | | | -The same | Nystagmus | Alive 8 yr. |
| *Echenne, 1986 | - | - | | Normal | | Not dysmorphic<br>Hoarsness of the voice<br>Macrocephaly | | Delayed | Normal | Alive 5 yr. |
| *Fois, 1987 | - | +/- | + | Trunk hypotonia<br>limbs hypertonia | + | Dysmorphic | - | Severely delayed | Retinal pallor | Alive 13 yr. |
| *Mancini, (submitted) | - | + | -(?) | Limbs hypertonia | | Dysmorphic | | Mildly delayed | Retinal pallor<br>Corneal opacities<br>Strabismus | Alive 10 yr. |
| *Baumkötter, 1985 | + | + | + | Hypotonia | - | Coarse | Yes | Mildly delayed | Mortal | Alive 5 yr. |

multiplex, are rare in Salla disease. The disease of Baumkötter's patient resembles ISSD in terms of visceral and skeletal involvement, but not in the clinical course which is slowly progressive (Baumkötter et al., 1985). The first Dutch patient with SASD has recently been described (Mancini et al., submitted). He combines the neurological features of Salla disease with skeletal abnormalities observed in ISSD. The slowly progressive course of the neurological disease, instead, reminds Salla patients. The corneal opacities, among the ocular findings, are unique to this case. Particularly, clear corneas have been classified among the clinical criteria to exclude other disorders of sialic acid metabolism (Stevenson et al., 1983). The clinical course in this patient is mild enough to remind SD. These observations suggest a wide clinical heterogeneity, while at the moment the biochemical studies (see publication II and V) show a similar metabolic defect in patients with different clinical forms of the disease. Speculations about a possible genetic heterogeneity are at the moment premature.

All the patients with sialic acid storage disorders are distinguished from the other form of sialuria, which is caused by overproduction of NeuAc, on the basis of the lysosomal localization of the stored product (Virtanen et al., 1980; Mendla et al., 1988). In general, in SASD the levels of storage and excretion correlate with the clinical severity (Gahl et al., 1989a; Mancini et al., 1991), but the ethiopathogenetic correlation between the sialic acid storage and the clinical manifestations is still unclear. The only neuropathological study in SD has not identified the involvement of specific areas of the nervous system; the grey and the white matter as well as the central and the peripheral nervous system appeared aspecifically affected (Autio-Harmainen et al., 1988). In SD, storage material has been found also in other organs than the nervous system, without leading to clinical dysfunction (Renlund et al., 1983).

In ISSD instead, the visceral and neuronal storage cause malfunctions of the involved organs. In particular, the two patients described by Sperl et al. (1990) presented with a severe nephrotic syndrome dominating the clinical picture and determining the outcome. Extensive microscopical investigations have showed in these patients specific glomerular damage of the podocytes and the mesangial cells, without macroscopic damage of the basement membrane. However, detachment of the cells from the membrane was observed, which was temptatively ascribed to altered glycoprotein synthesis. Epithelia of the proximal tubule also showed marked vacuolation of the cell cytoplasm. Again, it is not clear whether these patients represent a distinct phenotype.

For many other ISSD patients, cardiorespiratory failure with cardiac and pulmonal infiltration has instead been the main cause of death. Lysosomal storage has been observed in post-mortem specimens from skin, cardiac muscle, liver, renal tubules and glumeruli, fat cells, alveolar macrophages, bone marrow, spleen, lymph nodes, neurons, axons and glial cells (Hancock et al., 1982; Stevenson et al., 1983).

The biochemical diagnosis of SD and ISSD is based on the microscopical finding of lysosomal accumulation in peripheral blood cells and skin biopsies and on the detection of high levels of free sialic acid in urine, leucocytes and cultured fibroblasts. The routine use

of leucocyte subpopulations as a source for sensitive analysis and a possible tool for heterozygote detection, has been proposed (Mancini et al., submitted). Heterozygote (intermediate ) values of sialic acid transport have been detected in parents of SD patients using lymphoblast lysosomal membranes (publication V). Easy screening of sialic acid in urine is done by thin layer chromatography of a properly concentrated and partially purified sample (Baumkötter et al., 1985; Mancini et al., submitted).

Prenatal diagnosis has been performed for SD and ISSD with prediction of affected foetuses, by measurements of sialic acid content of amniotic fluid, amniocytes (Renlund and Aula, 1987; Vamos et al., 1986) and chorionic villi (Lake et al., 1989), or by microscopical analysis of chorionic villi (Vamos et al., 1986; Lake et al., 1989). We have monitored a pregnancy at risk for SASD, by determination of sialic acid in chorionic villi and amniotic fluid, with prediction of an unaffected foetus (Mancini et al., submitted).

We have recently described a lysosomal membrane carrier, specific for the transport of acidic monosaccharides, among which sialic acid and glucuronic acid (see chapter 5). Defective carrier-mediated transport of both sialic acid and glucuronic acid was found in the lysosomal membrane of cells from patients with different clinical forms of SASD (publication V). These studies defined the primary metabolic defect in these patients, but also revealed, unexpectedly, that glucuronic acid is another monosaccharide which cannot be transported out of the lysosomes of SASD patients. Increased amounts of glucuronic acid have been recently found in cultured fibroblasts of ISSD patients, confirming our results (Blom et al., 1990). It is therefore interesting to find out whether this (and other?) metabolite(s) accumulates in the tissues of SASD patients, contributing to the pathological processes.

As stressed by Sperl et al. (1990), it is curious that ISSD, sialidoses and Hurler disease (a genetic disorder of uronic acids degradation) are the only lysosomal disorders which can present with a distinct nephrotic phenotype. It is temptating to speculate about the responsability of an abnormal metabolism of polyanions like glycosaminoglycans and sialoglycoproteins in the onset of nephrosis in these disorders.

Also, it is not clear whether in SASD the pathologic evolution is linked to impairment of cellular sialic acid turnover or to its storage in the lysosome with secondary alteration of the organelle function. The first hypothesis has been forwarded to explain the abnormal podocyte detachment from the basement membrane in cases of ISSD (Sperl et al., 1990). To support the second hypothesis, it has been suggested that the function of lysosomal neuraminidase is inhibited in SASD, by the accumulation *in situ* of its reaction product (Baumkötter et al., 1985). This however, should not produce cellular deficiency of sialic acid, because, independently of the lysosomal recycling, sialic acid can be synthesized *de novo* from glucose. The fact that sialuria patients, not affected by a lysosomal disease, share with sialic acid transport defects the hepatosplenomegaly, the developmental delay and the progressive course suggest that the type of substrate involved, and not the localization of the metabolic block, is the factor which more directly correlates with the clinical symptoms.

Therapy of SASD is at the moment only supportive.

REFERENCES


* Aula, P., Autio, S., Raivio, K.O., Rapola, J., Thoden, C.J., Koskela, S.L. and Yamashina, I. (1979). "Salla Disease". A new lysosomal storage disorder. *Arch. Neurol.* 36:88-94.

* Aula, P., Renlund, M., Raivio, K.O. and Koskela, S.L. (1986) Screening of inherited oligosaccharidurias among mentally retarded patients in northern Finland. *J. Ment. Def. Res.* 30:365-368

* Autio-Harmainen, H., Oldorfs, A., Sourander, P., Renlund, M., Dammert, K. and Similä, S. (1988) Neuropathology of Salla disease. *Acta Neuropathol.* 75:481-490.

* Baumkötter, J., Cantz, M., Mendla, K., Baumann, W., Friebolin, H., Gehler, J. and Spranger J. (1985) N-acetylneuraminic acid storage disease. *Hum. Genet.* 71:155-159.

* Blom, H.J., Andersson, H.C., Seppala, R., Tietze F. and Gahl, W.A. (1990) Defective glucuronic acid transport from lysosomes of infantile free sialic acid storage disease fibroblasts. *Biochem. J.* 268:621-625

* Cameron, P.D., Dubowitz, V., Besley, G.T.N. and Fensom, A.H. (1990) Sialic Acid Storage Disease. *Arch. Dis. Child.* 65:314-315.

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

* Cantz, M. and Messer, H. (1979) Oligosaccharide and ganglioside neuraminidase activities of mucolipidosis I (sialidosis) and mucolipidosis II (I cell disease) fibroblasts. *Eur. J. Biochem.* 97:113-118

* Clements, P.R., Taylor, J.A. and Hopwood, J.J. (1988) Biochemical characterization of patients and prenatal diagnosis of sialic acid storage disease for three families. *J. Inher. Metab. Dis.* 11:30-44.

* Cooper, A., Sardahrwalla, I.B., Thornley, M. and Ward,K.P. (1988) Infantile Sialic Acid Storage Disease in Two Siblings. *J. Inher. Metab. Dis.* 11, Suppl. 2:259-262.

* Durand, P. and O'Brien, J.S. (1982) Genetic errors of glycoprotein metabolism. Springer Verlag, Berlin.

* Echenne, B., Vidal, M., Maire, I., Michalski, J.C., Baldet, P. and Astruc, J. (1986) Salla Disease in one Non-Finnish patient. *Eur. J. Pediatr.* 145:320-322

* Fois, A., Balestri, P., Farnetani, M.A., Mancini, G.M.S., Borgogni, P., Margollicci, M.A., Molinelli, M., Alessandrini, C. and Gerli, R. (1987). Free Sialic Acid Storage Disease. A new Italian case. *Eur. J. Pediatr.* 146:195-198.

* Fontaine, G., Biserte, G., Montreuil, J., Dupont, A., Ferriaux, J.P., Strecker, G., Spik, G., Puvion, E., Puvion-Dutilleul, F., Sezille, G. and Pique, M. (1968) La sialurie: un trouble metabolique original. *Helv. Paediat. Acta* 23:1-32 (suppl. XVII).

* Gahl, W.A., Renlund, M. and Thoene, J.G. (1989) Lysosomal transport disorders: cystinosis and sialic acid storage disorders. In: "The metabolic basis of inherited disease" (Scriver, C.R., Baudet, A.L., Sly, W.S., Valle, D. eds.) chapter 107. McGraw-Hill Information Services Company.

* Hancock, L.W., Thaler, M.M., Horwitz, A.L. and Dawson, G. (1982) Generalized N-acetylneuraminic acid storage disease: quantitation and identification of the monosaccharide accumulating in brain and other tissues. *J. Neurochem.* 38:803-809.

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

* Horwitz, A.L., Hancock, L.W., Dawson, G. and Thaler, M.M. (1981) Generalized sialic acid storage disease. *Pediat. Res.* 15:363.

* Lake, B.D., Young, E.P. and Nicolaides, K. (1989) Prenatal diagnosis of sialic acid storage disease in a twin pregnancy. *J. Inher. Metab. Dis.* 12:152-156.
* Lieser, M., Harms, E., Kern, H., Bach, G. and Cantz, M. (1989) Ganglioside $GM_3$ sialidase activity in fibroblasts of normal individuals and of patients with sialidosis and mucolipidosis IV. Subcellular distribution and some properties. *Biochem. J.* 260:69-74
* Mancini, G.M.S., Hoogeveen, A,T., Galjaard, H., Månsson, J.E. and Svennerholm, L. (1986) Ganglioside $GM_1$ metabolism in living human fibroblasts with ß-galactosidase deficiency. *Hum. Genet.* 73:35-38
* Mancini, G.M.S., Verheijen, F.W., Beerens, C.E.M.T., Renlund, M. and Aula, P.P. (1991) Sialic acid storage disorders: observations on clinical and biochemical variation. *Dev. Neurosci.* in press
* Mancini, G.M.S., Hu, P., Verheijen, F.W., van Diggelen, O.P., Janse, H.C., Kleijer, W.J., Beemer, F.A. and Jennekens, F.G.I. Salla disease variant in a Dutch patient. Potential value of polymorphonuclear leucocytes for heterozygote detection. (*submitted*)
* Mendla, K., Baumkötter, J., Rosenau, C., Ullrich-Bott, B. and Cantz, M. (1988) Defective lysosomal release of glycoprotein-derived sialic acid in fibroblasts from patients with sialic acid storage disease. *Biochem. J.* 250:261-267
* Palo, J., Rauvula, H., Finne, J., Haltia, M. and Palmgren, K. (1985) Hyperexcretion of free N-acetylneuraminic acid. A novel typpe of sialuria. *Clin. Chim. Acta* 145:237-242
* Paschke, E., Trinkl, G., Erwa, W., Pavelka, M., Hutz,I. and Roscher A. (1986) Infantile type of sialic acid storage disease with sialuria. *Clin. Genet.* 29:417-424.
* Renlund, M., Aula, P.P., Raivio, K.D., Autio, S., Sainio, K., Rapola, J. and Koskela, S.L.(1983). Salla Disease: A new lysosomal storage disorder with disturbed sialic acid metabolism. *Neurology* 33:57-66.
* Renlund, M. (1984). Clinical and laboratory diagnosis of Salla disease in infancy and childhood. *J. Pediat.* 104:232-236.
* Renlund, M. and Aula, P. (1987) Prenatal detection of Salla disease based upon increased free sialic acid in amniocytes. *Am. J. Med. Genet.* 28:377-384.
* Schauer, R. (1982) Sialic acids. Chemistry, metabolism and function. Series: "Cell biology monographs". Vol. 10. Springer Verlag. Wien.
* Seppala, R., Tietze, F., Krasnewich, D., Weiss, P., Ashwell, G., Barsh, G., Thomas, G.H., Packman, S. and Gahl, W.A. (1991) Sialic acid metabolism in sialuria fibroblasts. *J. Biol. Chem.* 266:7456-7461
* Sperl, W., Gruber, W., Quatacker, J., Monnens, L., Thoenes, W., Fink, F.M. and Paschke E. (1990). Nephrosis in two siblings with infantile sialic acid storage disease. *Eur. J. Pediatr.* 149:477-482.
* Stevenson, R.E., Lubinsky, M., Taylor, H.A., Wenger, D.A., Schroer, R.J. and Olmstead, P.M. (1983) Sialic Acid Storage Disease with Sialuria: Clinical and Biochemical Features in the Severe Infantile Type. *Pediatrics* 72:441-449.
* Thomas, G.H., Reynolds, L.W. and Miller, C.S. (1985) Overproduction of N-acetylneuraminic acid (sialic acid) by sialuria fibroblasts. *Pediatr. Res.* 19:451-455
* Tondeur, M., Libert, J., Vamos, E., van Hoof, F., Thomas, G.H. and Strecker, G. (1982). Infantile form of Sialic Acid Storage Disorder: Clinical, Ultrastructural and Biochemical Studies in Two Siblings. *Eur. J. Pediat.* 139:142-147.
* Vamos, E., Libert, J., Elkhazen, N., Jauniaux, E., Hustin, J. and Wilkin, P. (1986) Prenatal diagnosis and confirmation of infantile sialic acid storage disease. *Prenat. Diagn.* 6:437-446.

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

* Virtanen, I., Ekblom, P., Laurila, P., Nordling, S., Raivio, K.O. and Aula, P. (1980) Characterization of storage material in cultured fibroblasts by specific lectin binding in lysosomal storage diseases. *Pediat. Res.* 14:1199-1203

* Weiss, P., Tietze, F., Gahl, W.A., Seppala, R. and Ashwell, G. (1989) Identification of the metabolic defect in Sialuria. *J.Biol.Chem.* 264:17635-17636.

* Wilken, B., Don, N., Greenway, R., Hammond, J. and Sosula, L. (1987) Sialuria: a second case. *J. Inher. Metab. Dis.* 10:97-102

* Wolburg-Buchholz, K., Schlote, W., Baumkötter, J., Cantz, M., Holder, H. and Harzer, K. (1985). Familial Lysosomal Storage Disease with Generalized Vacuolization and Sialic Aciduria. Sporadic Salla Disease. *Neuropediatrics* 16:67-75.

* Ylitalo, V., Hagberg, B., Rapola, J., Mansson, J-E, Svennerholm, L., Sanner, G. and Tonnby, B. (1986) Salla Disease Variants. Sialoylaciduric Encephalopathy with Increased Sialidase Activity in Two Non-Finnish Children. *Neuropediatrics* 17:44-47.

Chapter 5

# THE EXPERIMENTAL WORK

## Introduction

The project described in this thesis started when the only transport proteins known to exist in the lysosome membrane were the proton pump and the cystine carrier. However, Renlund et al. (1983) suggested the possibility of a defective sialic acid efflux from the lysosome in patients with Salla disease (SD). In this period, patients with infantile sialic acid storage disease (ISSD) were considered as a separate clinical entity, as well as the non-lysosomal sialurias (Tondeur et al., 1982; Stevenson et al., 1983; Renlund, 1984). In one patient of non-Finnish ancestry we found evidence for a lysosomal storage disease and increased free sialic acid in urine and fibroblasts, and clinical features resembling those of SD and ISSD. The patient described in publication I, in collaboration with prof. Dr. A. Fois (Siena, Italy) is of italian ancestry and from pedigree studies it can be assumed that his disease represents either a recessively inherited monogenetic disorder or, less likely, is the result of a spontaneous mutation. Clinically he resembles the most severe patients affected by Salla disease and does not present the visceral and skeletal abnormalities typical of ISSD, except for the presence of retinal pallor.

The phenotype of this patient seemed somehow intermediate between SD and ISSD, suggesting that the two syndromes could be related to a common metabolic defect, manifesting with wide clinical variability. Having cultured fibroblasts from patients with classical Salla disease, ISSD and the intermediate form available, we wanted to test the hypothesis of Renlund about a possible defective lysosomal efflux of sialic acid and to

investigate whether a common metabolic defect is underlying all clinical forms.

To study the efflux of sialic acid from lysosomes, we needed to preload the lysosomal compartment of cultured fibroblasts from patients and controls with similar levels of a radiolabeled, thus easily measurable, counterpart of the acidic monosaccharide. To achieve this, we have synthesized the methylester of N-acetylneuraminic acid, in collaboration with Dr. J. van Pelt and prof. Dr. J.P. Kamerling (Utrecht, the Netherlands). The methylester has lost the charge of the carboxylic group, therefore it should be able to diffuse across biological membranes. A similar approach had previously been used to study efflux of amino acids from the lysosomal compartment (Goldman and Kaplan, 1973). The experimental data in publication II indicate that a lysosomal sialic acid pool can be generated by exposure of cultured cells to the sialic acid methylester. The intralysosomal conversion of the methylester to free sialic acid was shown in lysosome-rich granular fractions from cultured fibroblasts. When the efflux of sialic acid from patient and control lysosomes was followed in time, cell lines from the three main different categories of patients showed an impaired release. Although these data suggested a defective transport mechanism, this method did not allow conclusions about the type of transport mechanism that is involved. Nonetheless, the advantage of this approach was the independence from other metabolic pathways in the investigation of a particular lysosomal transport event, in partially purified lysosomes.

Independent studies, undertaken with different techniques to load intact lysosomes with sialic acid, led to very similar conclusions (Jonas et al., 1986; Renlund et al., 1986; Paschke et al., 1986; Mendla et al., 1988; Tietze et al., 1989). Here too, kinetic determinations of the mechanism involved were not possible and the only report showing a relation between lysosomal loading and efflux rate supported the model of unassisted simple diffusion (Renlund et al., 1986). Although a sialic acid transport defect had been established, it was not certain that the defect represented the primary genetic mutation and that a carrier-mediated system was involved.

We therefore searched for a reliable and precise biochemical method enabling the investigation of transport kinetics. Uptake studies in vesiculated membranes had already been used to investigate transport in other organelles (Turner, 1983). Purified biological membranes, resealed to form empty vesicles, avoid the disturbancies usually introduced by the organelle content and metabolism, and easily allow study of transport in both directions. In 1985 Bame and Rome had reported the preparation of highly purified membrane vesicles from lysosomes, which allowed the demonstration of the kinetic function of the integral membrane enzyme acetyl-CoA: $GlcNH_2$ N-Acetyl transferase (Bame and Rome, 1985; Ohsumi et al., 1983). We investigated the utility of such rat liver membrane preparations to study lysosomal sialic acid transport. Using this approach, described in publication III, we could for the first time demonstrate that the transport of sialic acid in the lysosome is a carrier-mediated process. The mechanism is that of secondary active cotransport of sialic acid with protons. A proton gradient created with impermeable buffers, set at different pH at the two sides of the membrane, provided the driving force for sialic acid movements across the

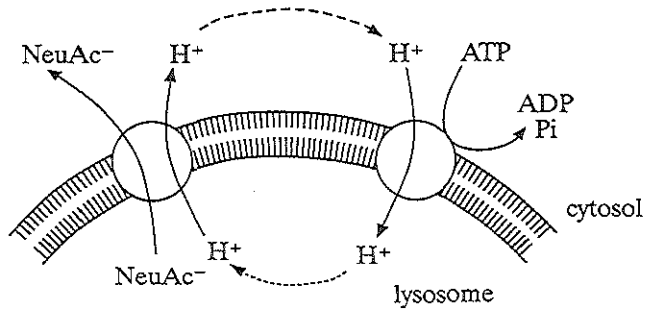


**Figure 4.** Model for lysosomal sialic acid transport. A transmembrane proton gradient is maintained by an ATP-dependent proton pump. Sialic acid efflux is driven by the transmembrane proton gradient.

membrane. The initial uptake process showed a transient overshoot, which proved the energetic activation of the carrier by protons, with actual transport of both sialic acid and protons (Turner, 1983).

The experimental data also showed for the first time the existence of proton cotransport mechanisms in the lysosomal membrane, providing a model to explain other lysosomal transport mechanisms, which might behave in a similar way, like that for vitamin $B_{12}$, sulfate and perhaps cystine (see table I and chapter 2).

A model for the physiological efflux of sialic acid out of the lysosome is depicted in figure 4. In traditional studies performed with intact lysosomes, pH manipulation can be only done outside the organelle and the extent of the internal acidification by the proton pump cannot easily be controlled. In that case, cotransport systems with protons are therefore much more difficult to detect.

The sialic acid carrier showed *trans*-stimulation properties and its function was also slightly stimulated by nucleotides, most likely via a regulatory mechanism independent of the proton pump activation. Studies on the substrate specificity revealed that other acidic sugars were a substrate for the sialic acid carrier as well. Among these, glycuronic acids (aldo-hexoses, monocarboxylated at $C_6$) were of special interest because they occur physiologically in the lysosome as degradation products of glycosaminoglycan metabolism. In fact, intravesicular glucuronic acid could *trans*-stimulate the uptake of sialic acid, demonstrating unequivocally that it is a substrate for the sialic acid carrier.

The lysosomal carrier for sialic acid thus recognized a category of acidic sugars,

while neutral sugars like glucose were not transported via this route. Since the possibility that glucose, produced by glycogen degradation, leaves the lysosome via a specific carrier (Maguire et al., 1983) had recently been debated (Bird et al., 1987; Bird et al., 1988; Iveson et al., 1989), it seemed opportune to compare the kinetics of glucose transport with those of sialic acid in our purified vesicle system.

The experimental data in publication IV led us to the discovery that the lysosome membrane contains a distinct carrier for neutral monosaccharides besides that for monocarboxylic sugars (see also table II, chapter 2). The biochemical properties of this carrier are quite different from those of the sialic acid carrier. The system does not work as a proton cotransporter although it showed an optimal function at acidic pH. Jadot et al. (1989) have shown that glucose transport in intact lysosomes can be inhibited by diethylstilbestrol (DES). In our system, DES does not inhibit glucose uptake and countertransport (G. Mancini, F. Verheijen, C. Beerens; unpublished results), but it is possible that the results of Jadot reflect the effect of a decreased lysosomal acidification (DES inhibits the proton pump; Cuppoletti et al., 1987), whereby glucose transport was also slowed down.

The $K_t$ for transport is quite high (about 75 mM), agreeing with data from previous reports on intact lysosomes (Hales et al., 1984). The substrate specifity included a number of neutral D-hexoses and the mammalian L-fucose, but apparently excluded pentoses which were formerly claimed to share the lysosomal glucose transport system (ibidem). The acidic pH optimum of this transporter discriminates it from the other six immunological distinct glucose carriers already known to exist (Gould and Bell, 1990). This opens new questions about the metabolic role and the structural feature of a new member of the glucose transporter family.

With respect to our observations on sialic acid transport in rat liver lysosomal membrane preparations, the next question to be answered was whether the $H^+$/sialic acid cotransporter exists in the human lysosome as well. We also wanted to know whether the human system has also substrate specificity for uronic acids or whether the latter are transported by an independent system. And the ultimate question in this stage of our work was to define the primary molecular defect in the different forms of SASD.

The possibility to study these questions came from the use of purified membrane vesicles from human fibroblast lysosomes. In publication V it is illustrated that lysosomal membranes from human sources show an identical transport system for both acid sugars. Then we tested cell lines from two ISSD patients, two Salla disease patients and both patients described in publication I and by Mancini et al. (submitted). As a result, the $H^+$-driven transport of both sialic acid and glucuronic acid was deficient in all patient cell lines tested.

The transport defect affects acidic sugars only, since neutral monosaccharides are normally transported in all the patients tested. Evidence that the transport defect represents the primary genetic mutation came from the observation of intermediate transport rates in obligate heterozygotes for this autosomal recessive disease. This result could be obtained by

application of the same technical assay on transformed lymphoblasts from patients and heterozygotes, in collaboration with prof. Dr. P. Aula (Turku, Finland).

Although the defect in SASD has now been clarified, still several problems have to be solved. For example, it is clear that SD and ISSD are two forms of the same metabolic disorder, but the clinical heterogeneity of these diseases still needs to be explained. Also, it will be interesting to investigate whether glucuronic acid, besides sialic acid, is accumulated in patient lysosomes *in vivo*. Storage of glucuronic acid has been recently reported in fibroblasts from ISSD patients (Blom et al., 1990), but has never been studied in tissues.

A logical follow-up of these studies was to attempt to isolate and characterize the lysosomal acidic sugar carrier protein. An important prerequisite for such studies is to have an assay system for the purified protein once extracted from its membraneous environment. One approach to this problem is to develop a functional reconstitution system by which the protein is first solubilized and then incorporated in an artificial liposomal membrane vesicle before its transport function is being assayed. Reconstitution can be only successful if the protein is stable under the extraction conditions employed, if it *likes* the artificial phospholipid membrane environment which is used, and it returns to its native (active) conformational state once incorporated into the artificial membrane (Maloney and Ambudkar, 1989; Maloney, 1990). Another useful approach for the purification of a transport protein would be specific labeling (for example at the active site) enabling the follow-up of the various purification steps. The development of a sialic acid derivative as a photoaffinity probe has recently been successful for the identification of two different sialidases (van der Horst et al., 1990a and 1990b).

A successful functional reconstitution of the lysosomal sialic acid carrier was initially achieved after solubilization of rat liver lysosomal membranes with a non-ionic detergent with high critical micelle concentration, Mega 10. The solubilized protein fraction was then inserted into artificial liposomes of egg yolk phospholipids (F. Verheijen, G. Mancini, C. Beerens, unpublished data). This system contained the active sialic acid transporter, without obvious changes in its biochemical properties. Though this technique showed us that the carrier retains its activity after solubilization and can be reintegrated in an artificial membrane in the absence of native phospholipids, it was not easy to vary the protein/lipid ratio during treatment without loosing transport activity. This would make reconstitution after a few purification steps, which are inevitably associated with the loss of protein, quite complicated.

Therefore we developed another reconstitution system, based on solubilization with Triton X-100 and detergent removal, after addition of phospholipids, on a small Amberlite column. This system allowed us to measure transport activity with as little as 1 $\mu$g of incorporated protein, independently of the amount of lipid (publication VI). The proteoliposomes used are of much smaller size (about 3 $\mu$l/mg protein compared with the 10 $\mu$l of the other reconstitution system) and are better sealed, showing low aspecific diffusion. Saturability of the glucuronic acid uptake process was observed practically in the absence of

a leakage component. Side-chain modification studies have contributed to identify a number of amino acid residues essential for the transport function of the carrier. Particularly, in the native and reconstituted carrier, cystine residues cannot be modified without loss of activity. Arginine and histidine residues, often present at the substrate-binding site of proteins interacting with anionic substrates (Riordan et al., 1977), were also essential for the transport function. However, the latter inactivation could be prevented by the concomitant incubation with the substrates sialic acid and glucuronic acid. This result demonstrates that arginine and/or histidine residues are likely present at or near the active site of the sialic acid carrier. These results also justify attempts to specifically label the transporter on the basis of a selection, induced by the substrate, in a protein mixture (Fox and Kennedy, 1965; Wright and Peerce, 1984; Peerce, 1989).

The availability of such a reconstitution system, means an important step in the purification of the acid sugar transporter and of the other carrier proteins in the lysosomal membrane.

REFERENCES


* Bame, K.J. and Rome, L.H. (1985) Acetyl coenzyme A:α-glucosaminide N-Acetyltransferase. Evidence for a transmembrane acetylation mechanism. *J. Biol. Chem.*260:11293-11299

* Bird, S.J., Forster, S. and Llouyd, J.B. (1987) Translocation of sugars into rat liver lysosomes. Evidence against a common carrier for D-glucose and D-ribose. *Biochem. J.* 245:929-931

* Bird, S.J., Forster, S. and Lloyd, J.B. (1988) Lysosomal sugar transport: facilitated or free diffusion? *Biochem. J.* 252:623

* Blom, H.J., Andersson, H.C., Seppala, R., Tietze F. and Gahl, W.A. (1990) Defective glucuronic acid transport from lysosomes of infantile free sialic acid storage disease fibroblasts. *Biochem. J.* 268:621-625

* Cuppoletti, J., Aures-Fisher, D. and Sachs, G. (1987) The lysosomal $H^+$ pump: 8-azido-ATP inhibition and the role of chloride in $H^+$ transport. *Biochim. Biophys. Acta* 899:276-284

* Fox, C.F. and Kennedy, E.P. (1965) Specific labeling and partial purification of the M protein, a component of the ß-galactoside transport system of Escherichia Coli. *Proc. Natl. Acad. Sci. USA* 54:891-898.

* Goldman, R. and Kaplan, A. (1973) Rupture of rat liver lysosomes mediated by L-amino acid esters. *Biochim. Biophys. Acta* 318:205-216

* Gould, G.W. and Bell, G.I. (1990) Facilitative glucose transporters: an expanding family. *Trends in Biochem. Sci.* 15:18-23.

* Hales, C.N., Docherty, K. and Maguire, G.A. (1984) Sugar transport in lysosomes. In "Lysosomes in biology and pathology" (J.T. Dingle, R.T. Dean, W. Sly eds.) pp.165-174. Elsevier Science Publisher.

* van der Horst, G.T.J., Mancini, G.M.S., Brossmer, R., Rose, U. and Verheijen, F.W. (1990a) Photoaffinity labeling of a bacterial sialidase with an aryl azide derivative of sialic acid. *J. Biol. Chem.* 265:10801-10804.

* van der Horst, G.T.J., Rose, U., Brossmer, R. and Verheijen, F.W. (1990b) Photoaffinity labeling of the lysosomal neuraminidase from bovine testis. *FEBS Lett.* 277:42-44.

* Iveson, G.P., Bird, S.J., Lloyd, J.B. (1989) Passive diffusion of non-electrolytes across the lysosome membrane. *Biochem. J.* 261:451-456

* Jadot, M., Wattiaux-De Coninck, S. and Wattiaux, R. (1989) The permeability of lysosomes to sugars. Effect of diethylstilbestrol on the osmotic activation of lysosomes induced by glucose. *Biochem. J.* 262:981-984.

* Jonas, A.J. (1986) Studies of lysosomal sialic acid metabolism: retention of sialic acid by Salla disease lysosomes. *Biochem.Biophys.Res.Commun.* 137: 175-181

* Lloyd, J.B. (1969) Studies on the permeability of rat liver lysosomes to carbohydrates. *Biochem. J.* 115:703-707

* Maguire, G.A., Docherty, K. and Hales, C.N. (1983) Sugar transport in rat liver lysosomes. Direct demonstration by using labeled sugars. *Biochem. J.* 212:211-218

* Maloney, P.C. and Ambudkar, S.V. (1989) Functional reconstitution of prokaryote and eukaryote membrane proteins. *Archiv. Biochem. Biophys.* 269:1-10

* Maloney, P.C. (1990) Microbes and membrane biology. *FEMS Microbiol. Rev.* 87:91-102

* Mancini, G.M.S., Hu, P., Verheijen, F.W., van Diggelen, O.P., Janse, H.C., Kleijer, W.J., Beemer, F.A. and Jennekens, F.G.I. Salla disease variant in a Dutch patient. Potential value of polymorphonuclear leucocytes for heterozygote detection. (*submitted*)

* Mendla, K., Baumkötter, J., Rosenau, C., Ullrich-Bott, B. and Cantz, M. (1988) Defective lysosomal release of glycoprotein-derived sialic acid in fibroblasts from patients with sialic acid storage disease. *Biochem. J.* 250:261-267

* Ohsumi, Y., Ishikawa, T., and Kato, K. (1983) A rapid and simplified method for the preparation of lysosomal membranes from rat liver. *J. Biochem.* 93:547-556

* Paschke, E., Hofler, G. and Roscher, A. (1986) Infantile sialic acid storage disease: the fate of biosynthetically labeled N-acetyl[$^3$H]neuraminic acid in cultured human fibroblasts. *Pediat. Res.* 20:773-777

* Peerce, B.E. (1989) Identification of the intestinal $Na^+$-phosphate cotransporter. *Am. J. Physiol.* 256:G645-652.

* Renlund, M., Chester, M.A., Lundblad, A., Parkkinen, J. and Krusius, T. (1983) Free N-acetylneuraminic acid in tissues in Salla disease and the enzymes involved in its metabolism. *Eur. J. Biochem.* 130:39-45.

* Renlund, M. (1984) Clinical and laboratory diagnosis of Salla disease in infancy and childhood. *J. Pediat.* 104:232-236.

* Renlund, M., Tietze, F. and Gahl, W.A. (1986). Defective sialic acid egress from isolated fibroblast lysosomes of patients with Salla disease. *Science* 232:759-762.

* Riordan, J.F., McElvany, K.D. and Borders, C.L. Jr. (1977) Arginyl residues: anion recognition sites in enzymes. *Science* 195:884-886.

* Stevenson, R.E., M. Lubinsky, H.A. Taylor, D.A. Wenger, R.J. Schroer, and P.M. Olmstead (1983) Sialic acid storage disease with sialuria: clinical and biochemical features in the severe infantile type. *Pediatrics* 72:441-449.

* Tietze, F., Seppala, R., Renlund, M., Hopwood, J.J., Harper, G.S., Thomas, G.H. and Gahl, W.A. (1989) Defective lysosomal egress of free sialic acid (N-acetylneuraminic acid) in fibroblasts of patients with infantile free sialic acid storage disease. *J. Biol. Chem.* 264:15316-15322.

* Tondeur, M., Libert, J., Vamos, E., van Hoof, F., Thomas, G.H. and Strecker, G. (1982). Infantile form of sialic acid storage disorder: clinical, ultrastructural and biochemical studies in two siblings. *Eur. J. Pediat.* 139:142-147.

* Turner, R.J. (1983) Quantitative studies of cotransport systems: models and vesicles. *J. Membr. Biol.* 76:1-15

* Wright, E.M. and Peerce, B.E. (1984) Identification and conformational changes of the intestinal proline carrier. *J. Biol. Chem.* 259:14993-14996

**Publication I**

Free sialic acid storage disease. A new Italian case
*Eur. J. Pediatr.* 146:195-198 (1987)

Eur J Pediatr (1987) 146:195–198


# Free sialic acid storage disease

## A new Italian case

**A. Fois[1], P. Balestri[1], M. A. Farnetani[1], G. M. S. Mancini[1], P. Borgogni[1], M. A. Margollicci[1], M. Molinelli[1], C. Alessandrini[2], and R. Gerli[2]**

[1]Clinica Pediatrica and [2]Istituto di Istologia e Embriologia Generale, Università di Siena, Siena, Italy

**Abstract.** Increased amounts of free sialic acid were found in cultured fibroblasts and urine of a 4-year-7-month-old Italian boy with mental retardation, hypotonia, failure to thrive, coarse facial features, convergent strabismus, pale skin and fair hair. Ultramicroscopic examination of conjunctival and skin tissues showed a number of membrane-bound vacuoles containing low-density granular material in the cytoplasm of the fibroblasts. The clinical, biochemical and ultrastructural findings are similar to those described in Salla disease. Neuraminidase activity is normal.

The molecular basis of the sialic acid storage disease is not known. Evidence for defective transport of sialic acid across the lysosomal membrane has been demonstrated in the patient's fibroblasts. It is possible that this might represent the metabolic abnormality.

**Key words:** Free sialic acid storage disease – Salla disease

*Offprint requests to:* A. Fois, Clinica Pediatrica, Università degli Studi, Via P. A. Mattioli, 10, I-53100 Siena, Italy

### Introduction

Two groups of hereditary disorders of sialic acid metabolism have been identified. In the first there is a storage of sialic acid bound to oligosaccharides and glycoproteins. These patients have a defect of the lysosomal enzyme neuraminidase (sialidase) and for this reason the name of "sialidoses" has been suggested [7]. In the second group there is a markedly increased excretion of free sialic acid (N-acetyl-neuraminic acid) in the urine. These subjects have different clinical symptoms. The most consistent number of patients has been observed in Finland [1, 11, 12, 14]. The disease is characterized by autosomal recessive transmission, early onset, severe psychomotor retardation, ataxia, athetosis, spasticity and impaired speech. Thick calvaria, exotropia and growth retardation are also present in about half of the patients. Ultramicroscopic examination of skin biopsies shows membrane-bound vacuoles containing fibrillo-granular material in different cell types. The daily excretion of free sialic acid amounts to about ten times the normal values. The activities of the lysosomal enzymes in serum, fibroblasts and lymphocytes are normal. This condition has been named "Salla disease" from the region where the first cases were born.

Other sporadic patients with sialuria but a clinical picture different from that of the Finnish patients have also been described [2–4, 10, 15, 19, 21]. We present a case of sialic acid storage disease with sialuria. The clinical, ultrastructural and biochemical findings are similar to those described in Salla disease.

### Clinical data

Patient P.E., a 4-year-7-month-old boy, was born from non-consanguineous parents in Terni (Central Italy). Pregnancy and delivery were uneventful. An 8-year-old brother and 5-year-old sister are in good health. Neuromotor retardation was noted in the patient from the first months of life. At 8 months head control was not yet acquired, he could not sit unsupported and was not able to reach for objects. At 26 months, with high fever, he developed a generalized convulsion which lasted about 20 min. For this reason he was hospitalized in the Pediatric Institute, University of Siena. Physical examination showed a boy whose weight was 9.800 kg (< 3rd centile) and height 75 cm (< 3rd centile). Head circumference was 47.5 cm (25th centile). He showed mild coarse facial features with flared nostrils, long philtrum, flat nasal bridge, large mouth, bilateral exo-

tropia, short neck. The skin was pale with fair hair. There was a moderate thoracic kyphosis with increased anteroposterior diameter. Liver and spleen were not enlarged. Neurological examination showed marked hypotonia: he could sit unsupported only for a few seconds. He could reach for objects and bring them to his mouth. He could not speak.

**Laboratory findings**

At the age of 26 months results of the following laboratory examinations were normal: blood glucose, urea nitrogen, bilirubin, $Na^+$, $K^+$ and $Cl^-$, serum proteins, triglycerides and lipoproteins, uric acid, plasma and urine amino acids, CSF, $T_3$, $T_4$, and TSH, oral xylose loading test, karyogram, chest and bone X-rays. Urinary glycosaminoglycan and oligosaccharide excretion was also normal.

Fundoscopic examination showed only a slight pallor of the optic disc. A marked diffuse slowing with 2–3/s asynchronous slow waves was present in the EEG.

CT scan indicated an increase of the CSF space over the cortex and enlarged lateral ventricles.

Three percent of mononuclear cells in the peripheral blood showed azurophilic cytoplasmic granulations; there was no evidence of vacuolated cells in peripheral blood and bone marrow.

*Special studies*

Biopsies from conjunctiva and skin were prepared for electron microscopy. Skin biopsy was utilized also for fibroblast culture.

*Electron microscopy*

Small tissue specimens of conjunctiva and skin were fixed in 2.5% glutaraldehyde in 0.1 *M* cacodilate buffer, pH 7.2 for 2 h at 4° C, washed in the same buffer, postfixed in 1% osmium-tetroxide for 2 h, dehydrated in ethanol and included in epon-araldite. Semi-thin and ultra-thin sections were cut with an LKB NOVA ultramicrotome. The ultra-thin sections were stained with uranyl acetate and lead citrate and examined under a Philips 400 electron microscope. Conjunctival fibroblasts (Fig. 1), endothelial and Schwann cells revealed numerous cytoplasmic vacuoles of different size containing granular, electron-dense material surrounded by membrane. Some fused vacuoles formed vacuoles of larger dimension. Mast cells and epithelial cells had an apparently normal ultrastructure. The cytoplasmic organelles were normal. Ultrastructural findings similar to those described in conjunctival biopsy were also observed in the skin (Fig. 2).

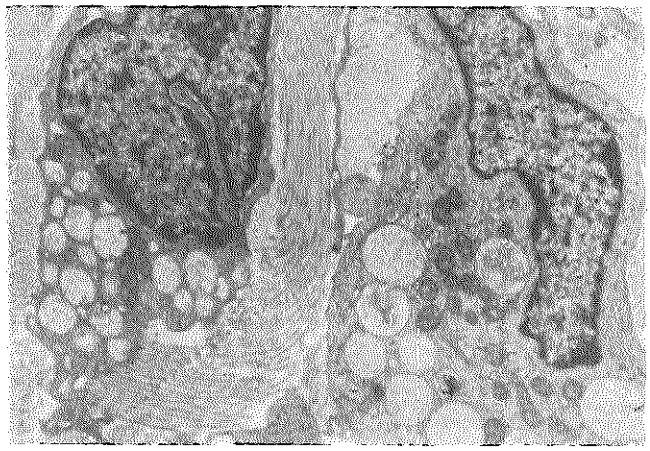

**Fig. 1.** Two conjunctival fibroblasts containing cytoplasmatic vacuoles of different dimensions

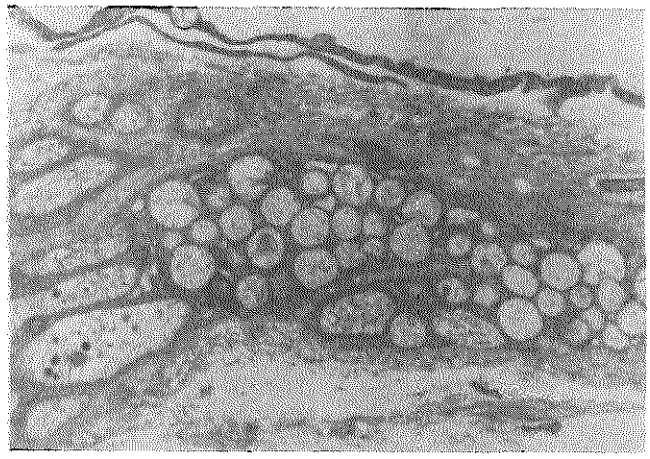

**Fig. 2.** Skin biopsy: the cytoplasm of a Schwann cell is filled with vacuoles containing granular electron-dense material

*Fibroblast culture*

Skin fibroblasts were grown in Ham's F10 medium supplemented with 10% fetal calf serum and antibiotics. Cells were harvested by trypsinization, extensively washed with phosphate buffered saline and the pellet kept frozen until use.

Table 1. Lysosomal hydrolase activities

| | Leucocytes (nmol/h per mg protein) | | Serum (nmol/h per ml) | | Fibroblasts (nmol/h/mg protein) | |
|---|---|---|---|---|---|---|
| | Patient | n.v.[a] | Patient | n.v.[a] | Patient | n.v.[a] |
| α-Neuraminidase | 1.04 | 1–5 | | | 89 | 40–100 |
| β-Exosaminidase | 653 | 650–3660 | 800 | 300–1700 | | |
| Isoenzyme A | 68.4% | 60–74% | | | | |
| Isoenzyme B | 31.4% | 25–40% | | | | |
| α-Galactosidase | 9.24 | 8–50 | 3 | 2–15 | | |
| β-Galactosidase | 38.7 | 25–190 | 23.5 | 3–60 | | |
| β-Glucuronidase | 187.5 | 57–350 | 100 | 15–385 | | |
| α-Mannosidase | 70.4 | 50–233 | 28.7 | 3–100 | | |
| α-Fucosidase | 21.8 | 20–110 | 346.7 | 30–1500 | | |
| Arylsulphatase A | | | | | | |
| pH 5 | 148.8 | 100–260 | | | | |
| pH 6 | 90.6 | 48–140 | | | | |
| pH 5/pH 6 | 1.64 | 1.5–2.4 | | | | |
| Arylsulphatase B | 83.4 | 70–160 | | | | |
| β-Glucosidase | 19.4 | 1–29 | | | | |

[a] n.v. = normal values

Table 2. Sialic acid content in cultured fibroblasts, urine and serum

| | | Patient P.E. | Normal controls | (n) |
|---|---|---|---|---|
| Cultured fibroblasts (nmol/mg protein) | Free | 22.19 | 1.54 ± 0.52 | (3) |
| | Total | 24.74 | 13 ± 0.83 | (3) |
| Urine (mg/24 h) | Free | 58.8 | 4.21 ± 1.9 | (19) |
| | Total | 59.9 | 5.59 ± 2.35 | (19) |
| Serum (nmol/ml) | Free | 0.066 | 0.027 ± 0.009 | (7) |
| | Total | 1.78 | 0.63 ± 0.11 | (7) |

*Lysosomal hydrolase activities*

Lysosomal hydrolase activities in serum, leucocytes and cultured fibroblasts are shown in Table 1.

Leucocytes and cultured fibroblasts were analysed for neuraminidase activity on a substrate of sodium 4-methylumbelliferyl-α-D-N-acetylneuraminate.

When re-examined at the age of 4 years 7 months, the patient still could not walk and could pronounce only a few syllables. The legs were hypertonic. Chest X-rays gave normal results. Funduscopic examination confirmed the previously reported pallor of the optic disc. EEG showed a diffuse 18–20 c/s activity during wakefulness and spontaneous sleep. There was no evidence of vacuolated cells in peripheral blood.

*Sialic acid determination*

Qualitative determination of sialic acid in urine was performed by thin layer chromatography according to the method of Michalski et al. [9] directly spotting 20 µl urine samples on a precoated silica gel 60 thin layer chromatographic plate. Quantitative assays of sialic acid in urine, serum and cultured fibroblasts were done essentially as described by Warren's procedure [20]. An increase of free sialic acid to about 15 times the normal control values was present in the patient's fibroblasts (Table 2). The 24 h excretion of total sialic acid varied between 50.2 and 69.7 mg (mean value = 59.9 mg). The amount of free sialic acid varied between 48.9 and 67.5 mg (mean value = 58.8 mg) or about 14 times the normal control values (Table 2). The free sialic acid in the serum was only 2.4 times the normal (Table 2).

## Discussion

The clinical, ultrastructural and biochemical picture of Salla disease, as described above, has been now reported in 49 Finnish subjects [1, 11–14]. Besides this consistent group, other patients with similar biochemical findings but a different clinical picture have been observed. Fontaine et al. in 1968 [3] described a boy with massive sialuria, low weight and height, hepatomegaly, convulsions and retarded psychomotor development. Hancock et al. [4] published data on a 5-month-old boy of German-Swedish ancestry who died after rapid progression with massive ascites, hepatomegaly, probable mental retardation and no signs of skeletal involvement. At autopsy the brain was enlarged and firm. Electron microscopy showed granular and flocculär material in liver, brain and cultured skin fibroblasts. The group of Strecker [19] has published two more cases born from unrelated Yugoslavian parents. They showed coarse facies, hepatosplenomegaly, marked neuromotor retardation, pale complexion, fair hair and vacuolated peripheral lymphocytes. Ultrastructural studies of conjunctiva, skin, bone marrow and liver demonstrated a generalized lysosomal storage of polysaccharide-like material. Urine and cultured fibroblasts contained increased (18–25 fold) amounts of free sialic acid. Biochemical analysis of crude sonicates of the cultured fibroblasts revealed increased levels of sialic acid [16]. Two more unrelated infants with sparse white hair, coarse facies, hepatosplenomegaly, neuromotor retardation, mild signs of dysostosis multiplex and clear vacuoles in fibroblasts, visceral, central nervous system cells and lymphocytes have been described [15]. Urine, liver, leucocytes and sonicated cultured skin fibroblasts contained increased amounts of free sialic acid. Lysosomal hydrolases were normal. A 9-year-old patient without visceromegaly, with severe neuromotor retardation, epileptic fits and biochemical and ultrastructural findings similar to Salla disease, has recently been reported by Wolburg-Buchholtz et al. [21]. Two sisters exhibited similar symptoms and died at the ages of 8 and 17 years. Recently another Italian case, born of consanguineous Sicilian parents, has been reported by Baumkötter et al. [2]. This patient however also had hepatosplenomegaly and mild signs of dysostosis multiplex. The clinical picture better resembled a generalized storage disorder. Free sialic acid was increased 13, 28 and 46 times more than normal control values in urine, cultured fibroblasts and liver tissues, respectively. Variants of Salla disease have also been described in two patients [22]. The neurological picture in the early period

of life was considered compatible with a non-progressive cerebral palsy. There was no overt intellectual deterioration but severe hypotonia and ataxia or dyskinesia. Later however the picture of a progressive encephalopathy became evident. These patients also had five to ten times increased amounts of free sialic acid in the urine with normal values of neuraminidase and several other lysosomal enzymes in cultured skin fibroblasts. An increase of neuraminidase activity in lymphocytes was present. Another patient with sialuria and epileptic fits [10] had, at the age of 26, no other clinical findings. Microscopic or ultrastructural findings of storage disease were also absent.

Thus it seems apparent that two main forms of sialic acid storage disease exist: Salla disease or "Salla variants" characterized by a slow progression of symptoms and the severe infantile form, a rapidly progressive neurodegenerative disorder with visceral and/or skeletal involvement, leading to a vegetative state or death in the first years of life.

In our case the severe neuromotor retardation, the moderately dysmorphic facial features and the ultramicroscopic findings are similar to those described in Salla disease. The increased excretion of free sialic acid in the urine and the increase of the same substance in the cultured skin fibroblasts are in agreement with this diagnosis.

The basic metabolic defect of all these cases, characterized by free sialic acid in the urine, is not known. Hancock et al. [5] and Renlund et al. [13] have hypothesized the possibility of a transport defect of free sialic acid across the lysosomal membrane. An over-production of free N-acetyl-neuraminic acid in fibroblasts from the original sialuria patient reported by Fontaine et al. [3], has also been considered to be at least in part responsible for the increased sialuria. Indirect evidence that this may result from either hyperactivity or increased levels of the enzyme uridine diphosphate N-acetylglucosamine-2-epimerase has been presented [17].

In our patient a defective efflux of N-acetylneuraminic acid has been demonstrated from lysosome-enriched fractions prepared from fibroblasts treated with labelled methylester of N-acetylneuraminic acid [8]. In the same study, a similar defect was also found in cultured fibroblasts obtained from one of the patients studied by Tondeur et al. [19] and in another cultured fibroblast cell line belonging to a patient with classic Salla disease [12].

This observation, together with the recent studies of Tietze et al. [18] and Jonas and Huls [6], may indicate the existence of a specific transport system for free N-acetyl-neuraminic acid across the lysosomal membrane. An abnormality of this transport mechanism could explain the basis and perhaps the different clinical expression of the sialic acid storage disease. A better knowledge of this mechanism could clarify whether the different forms of free sialic acid storage disorders are due to the same genetic mutation with clinical heterogeneity or conversely represent the end point of different genetic abnormalities causing storage and increased excretion of free sialic acid.

## References


1. Aula P, Autio S, Raivio KO, Rapola J, Thoden C-J, Koskela S-L, Yamashina I (1979) "Salla disease". A new lysosomal storage disorder. Arch Neurol 36:88–94
2. Baumkötter J, Cantz M, Mendla K, Baumann W, Friebolin H, Gehler J, Spranger J (1985) N-acetylneuraminic acid storage disease. Hum Genet 71:155–159
3. Fontaine G, Biserte G, Montreuil J, Dupont A, Farriaux J-P, Strecker G, Spik G, Puvion E, Puvion-Dutilleul F, Sezille G, Picqué MT (1968) La sialurie: un trouble métabolique original. Helv Paediatr Acta 23 [Suppl] XVII:1–32
4. Hancock LW, Thaler MM, Horwitz AL, Dawson G (1982) Generalized N-acetylneuraminic acid storage disease: quantitation and identification of the monosaccharide accumulating in brain and other tissues. J Neurochem 38:803–809
5. Hancock LW, Horwitz AL, Dawson G (1983) N-acetylneuraminic acid and sialoglycoconjugate metabolism in fibroblasts from a patient with generalized N-acetylneuraminic acid storage disease. Biochim Biophys Acta 760:42–52
6. Jonas AJ, Huls MH (1985) Lysosomal efflux of free sialic acid: implications for Salla disease. Pediatr Res 19, 4 (part 2):313A
7. Lowden JA, O'Brien JS (1979) Sialidosis: a review of human neuraminidase deficiency. Am J Hum Genet 31:1–18
8. Mancini GMS, Verheijen FW, Galjaard H (1986) Free N-acetylneuraminic acid (NANA) storage disorders: evidence for defective transport of NANA across the lysosomal membrane. Hum Genet (in press)
9. Michalski JC, Montreuil J, Strecker G (1983) A thin layer chromatographic technique for screening for sialuria. Clin Chim Acta 129:99–101
10. Palo J, Rauvala H, Finne J, Haltia M, Palmgren K (1985) Hyperexcretion of free N-acetylneuraminic acid. A novel type of sialuria. Clin Chim Acta 145:237–242
11. Renlund M, Chester MA, Lundblad A, Aula P, Raivio KO, Autio S, Koskela S-L (1979) Increased urinary excretion of free N-acetylneuraminic acid in thirteen patients with Salla disease. Eur J Biochem 101:245–250
12. Renlund M, Aula P, Raivio KO, Autio S, Sainio K, Rapola J, Koskela S-L (1983) Salla disease: a new lysosomal storage disorder with disturbed sialic acid metabolism. Neurology (NY) 33:57–66
13. Renlund M, Chester A, Lundblad A, Parkkinen J, Krusius T (1983) Free N-acetylneuraminic acid in tissues in Salla disease and the enzymes involved in its metabolism. Eur J Biochem 130:39–45
14. Renlund M (1984) Clinical and laboratory diagnosis of Salla disease in infancy and childhood. J Pediatr 104:232–236
15. Stevenson RE, Lubinsky M, Taylor HA, Wenger DA, Schroer RJ, Olmstead PM (1983) Sialic acid storage disease with sialuria: clinical and biochemical features in the severe infantile type. Pediatrics 72:441–449
16. Thomas GH, Scocca J, Libert J, Vamos E, Miller CS, Reynolds LW (1983) Alterations in cultured fibroblasts of sibs with an infantile form of free (unbound) sialic acid storage disorder. Pediatr Res 17:307–312
17. Thomas GH, Reynolds LW, Miller CS (1985) Overproduction of N-acetylneuraminic acid (sialic acid) by sialuria fibroblasts. Pediatr Res 19:451–455
18. Tietze F, Thomas G, Gahl W (1985) Decreased sialic acid clearance in granular fractions of fibroblasts from a patient with free sialic acid storage disease. Am J Hum Genet 37, 4 [Supplement July '85]:A19
19. Tondeur M, Libert J, Vamos E, Van Hoof F, Thomas GH, Strecker G (1982) Infantile form of sialic acid storage disorder: clinical, ultrastructural and biochemical studies in two siblings. Eur J Pediatr 139:142–147
20. Warren L (1959) The thiobarbituric assay of sialic acid. J Biol Chem 234:1971–1975
21. Wolburg-Buchholz K, Schlote W, Baumkötter J, Cantz M, Holder H, Harzer K (1985) Familial lysosomal storage disease with generalized vacuolization and sialic aciduria. Sporadic Salla disease. Neuropediatrics 16:67–75
22. Ylitalo V, Hagberg B, Rapola J, Månsson J-E, Svennerholm L, Sanner G, Tonnby B (1986) Salla disease variants. Sialoylaciduric encephalopathy with increased sialidase activity in two non-Finnish children. Neuropediatrics 17, 1:44–47


Received March 10, 1986 / Accepted July 10, 1986

**Publication II**

Free N-acetylneuraminic acid (NANA) storage disorders:
evidence for defective NANA transport across the lysosomal membrane.
*Hum. Genet.* 73:214-217 (1986)

Hum Genet (1986) 73:214–217

Human Genetics



# Free N-acetylneuraminic acid (NANA) storage disorders: evidence for defective NANA transport across the lysosomal membrane

G.M.S. Mancini*, F.W. Verheijen, and H. Galjaard

Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, NL-3000 DR Rotterdam, The Netherlands

**Summary.** To study the biochemical defect underlying N-acetylneuraminic acid (NANA) storage disorders (NSD), a tritium-labeled NANA-methylester was prepared and its metabolism was studied in normal and mutant human fibroblasts. The uptake of methylester, its conversion into free NANA, and the release of free NANA was studied in lysosome-enriched fractions. In three clinically different types of NSD accumulation of free NANA was observed and the half-life of this compound was significantly increased. Our observations indicate the existence of a transport system for NANA across the lysosomal membrane, which is deficient in all variants of NSD.

## Introduction

N-Acetylneuraminic acid (NANA) storage disorders (NSD) represent a heterogeneous group of genetically inherited metabolic diseases. All variants of NSD are characterized by severe psychomotor retardation and an early onset of the symptoms. However, striking differences are found in the progression of the symptoms and in the lifespan. Patients with so called "Salla" disease present severe symptoms and early onset, but a slow progression of the disease (Aula et al. 1979; Renlund et al. 1983a; Baumkötter et al. 1985). In contrast, patients with the severe infantile form, die in early childhood (Horwitz et al. 1981; Tondeur et al. 1982; Stevenson et al. 1983).

Biochemically, all types of the disease are characterized by accumulation of free NANA in lysosomes in several organs and cultured fibroblasts (Hancock et al. 1983; Virtanen et al. 1980), and by excessive urinary excretion of free NANA. The biochemical defect underlying these diseases is so far unknown. All the enzymes known to be involved in NANA metabolism were proven to be normally active in liver and cultured fibroblasts from patients with "Salla" disease (Renlund et al. 1983b). It has been hypothesized that NSD results from a defective transfer of NANA across the lysosomal membrane towards the cytosol (Hancock et al. 1983; Renlund et al. 1983b).

In order to obtain experimental evidence for such a defective NANA transport system, we have loaded living fibroblasts with tritium-labeled NANA ($^3$H)-NANA) using its methylester, which as an uncharged molecule diffuses into the lysosome. Here it is converted into free NANA and methanol, similarly to what is described for other methylesters (Reeves 1979). The release of radiolabeled-free NANA could then be followed after isolation of lysosome-enriched fractions from the fibroblasts. Under these conditions, we have studied cultured fibroblasts derived from a patient with "Salla" disease (Renlund et al. 1983a), an infant affected by a severe form of NSD (Tondeur et al. 1982), and from a 5-year-old child of Italian ancestry with a milder infantile form (A. Fois, personal communication).

## Materials and methods

### *NANA-methylester synthesis*

Tritium labeling of NANA was performed at our request by Amersham (England) giving a specific activity of 50 mCi/mmole. The NANA-methylester was prepared as described earlier (Yu and Ledeen 1969), by incubating 100 mg of NANA with 500 mg of Dowex 50 × 8 (H+ form) in the presence of 15 ml of methanol for two hours at room temperature. After incubation, the methanolic solution of the methylester was dried under vacuum rotation, resuspended in the appropriate volume of water, and kept frozen at −20° C until used. The purity of the preparation was checked by thin layer chromatography (TLC).

### *Cell lines*

Skin fibroblast cultures from the patient with "Salla" disease were kindly provided by Dr. P. Aula (Children's Hospital, University of Helsinki, Finland). Fibroblasts from the severe infantile form of NANA storage disease were a gift from Dr. E. Vamos (Laboratory of Cytogenetics and Cell Culture, Hospital St.-Pierre, Brussels, Belgium) and from the Italian patient from Professor A. Fois (Department of Pediatrics, University of Siena, Italy). All cell lines were grown in Ham's F10 medium supplemented with 10% fetal calf serum and antibiotics.

### *Loading of living fibroblasts with ($^3$H)-NANA-methylester*

In experiments performed in cell suspension, fibroblasts from two 75 cm$^2$ Falcon flasks were harvested under controlled trypsinization, washed twice with RPMI 1640 medium, and in-

* Working as a fellow from the Department of Pediatrics, University of Siena, Italy

*Offprint requests to:* F.W. Verheijen, Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, NL-3000 DR Rotterdam, The Netherlands

cubated 1 hour at 37°C in 1 ml of RPMI medium containing 10 m*M* unlabeled NANA-methylester and 50 µCi of ($^3$H)-NANA-methylester. At the end of the incubation, the cells were washed extensively with RPMI, and in case of "chase" experiments, cell pellets were resuspended in fresh RPMI and divided into fractions which were incubated at 37°C for different times (0, 15, 30, and 60 min). The content of unlabeled sialic acid reached after loading was measured in the cell homogenate by the thiobarbituric acid assay (Warren 1959).

For long-term accumulation experiments fibroblast monolayers were used; 75 cm$^2$ flasks were incubated at 37°C for 20 h in Ham's F10 medium containing 1 m*M* NANA-methylester and 50 µCi of ($^3$H)-NANA-methylester. After incubation lysosome-enriched fractions were prepared as follows. The cells were homogenized with 17 strokes of a Teflon-glass homogenizer at 0°C in 0.25 M sucrose containing 10 m*M* HEPES buffer and 1 m*M* EDTA, pH 7. After centrifugation at 100x*g* for 10 min, to remove nuclei and unbroken cells, the supernatant was spun down at 2000x*g* for 10 min and the new supernatant was centrifuged at 12 000x*g* for 15 min. The final pellet, representing the lysosome-enriched preparation, was resuspended in water. The specific activity of β-hexosaminidase in this fraction was six-fold higher then that in the total cell homogenate. The radiolabeled compounds in the lysosome-enriched fraction were separated by TLC on a 0.2 mm silica gel covered glass plate (Merck) in butanol-acetic acid-water (100:50:50, v/v/v). Silica fractions of 0.5 cm were then scraped off, the samples were eluted with 500 µl of 1 N NaOH, and the radioactivity was counted with 10 ml of Instagel (Packard) in a liquid scintillation counter.

The radioactivity of the fractions identified by the corresponding marker as free NANA was expressed as a percentage of the initial ($^3$H)-NANA dpm per unit of β-hexosaminidase activity. The logarithm of this percentage was plotted against time of clearance. Half-lives of NANA release were calculated by linear regression.

*Lysosomal latency*

The integrity of the lysosomal preparations at each time point was determined by measuring the β-hexosaminidase activity as described previously (Galjaard 1980), in 0.3 *M* sucrose in the presence or absence of 0.1% Triton X-100. Latency was defined as a percentage of the difference between the activity in the presence or absence of Triton X-100 and the activity in the presence of Triton X-100. In all our experiments 80 to 90% of β-hexosaminidase was latent in the lysosomal preparations.

## Results

The methylester of NANA was synthesized by incubation with DOWEX 50 × 8 (H+ form) as described previously (Yu and Ledeen 1969), and the purity of the preparation was examined by TLC (Fig. 1). This compound proved to be stable in aqueous solutions over a pH range from 4 to 7, and no spontaneous conversion into free NANA was observed in the culture medium under our experimental conditions (data not shown).

Pilot experiments with unlabeled NANA-methylester showed that living fibroblasts in suspension, exposed to this compound for two hours, accumulated intracellular levels of thiobarbiturate assay positive material of up to 22 nmol/mg protein; without loading this amount is 0.5-1 nmol/mg protein.

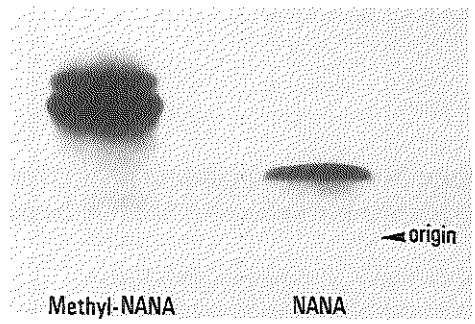


Fig. 1. Thin layer chromatography of ($^3$H)-NANA and its methylester obtained after incubation with Dowex 50 × 8, in the presence of methanol

To investigate whether NANA-methylester could reach the lysosomal compartment, and whether it was converted to free NANA, normal fibroblasts in suspension were incubated in RPMI medium containing 10 m*M* of cold NANA-methylester and 50 µCi of the tritium-labeled NANA-methylester. After periods of 15, 30, 60, and 120 min lysosome-enriched fractions were prepared by differential centrifugation and applied to a TLC plate, in order to separate the labeled compounds. The areas identified by the corresponding markers as free NANA and NANA-methylester were scraped off and the radioactivity was counted. As shown in Fig. 2, lysosome-enriched fractions accumulate an increasing content of NANA-methylester, and meanwhile part of it is converted into free NANA, presumably by a lysosomal esterase. Between one and two hours of incubation the uptake of methylester and conversion into free NANA seems to reach a plateau. Therefore, and to prevent cell damage in the following studies on the release of NANA a limited period (1 h) of loading was chosen as a starting point for the "chase" experiments.

To compare the long-term accumulation of free NANA in normal and mutant cells, the following experiment was carried out. Fibroblast cultures in monolayer were exposed to the tritium-labeled NANA-methylester for 20 h. After preparation of the respective lysosome-enriched fractions, the radiolabeled compounds were separated by TLC, and the radioactivity was determined. In the three different patients a peak at the position corresponding to free NANA is present, which is not the case in controls (Fig. 3). The radiolabeled products close to the origin in control cells probably reflect incorporated NANA in other products because of the long incubation period.

To study the background of the accumulation in the mutant cells "chase" experiments were performed. Cell suspensions were incubated for one hour with ($^3$H)-NANA-methylester, followed by a "chase" for up to one hour. The release of NANA from lysosome-enriched fractions was measured by counting the radioactivity of NANA after separation from other radiolabeled compounds on TLC. This radioactivity was expressed as a percentage of the initial dpm per unit of β-hexosaminidase activity in each lysosomal preparation. Within the short period of experimentation the latter activity var-

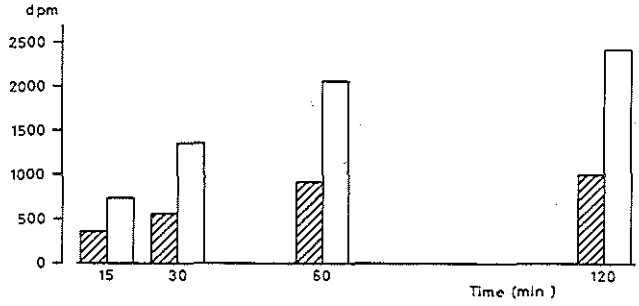


Fig. 2. Loading of control fibroblasts in suspension. Uptake of ($^3$H)-NANA-methylester and conversion into ($^3$H)-NANA in lysosome-enriched fractions obtained by differential centrifugation at different time points. NANA and NANA-methylester were separated by TLC. The corresponding fractions were scraped off and counted. ▨, NANA, □, methyl-NANA

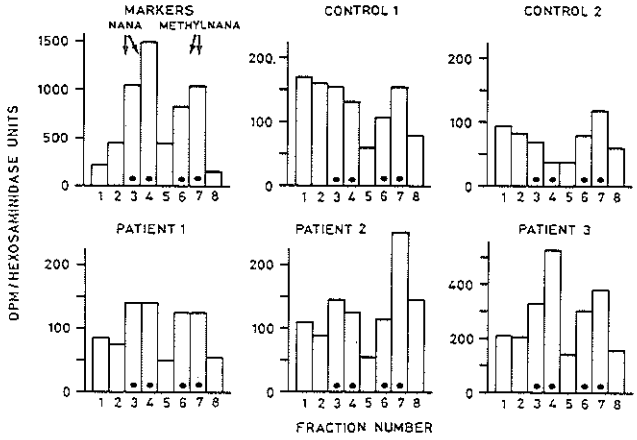


Fig. 3. Accumulation of free NANA in lysosome-enriched fractions from normal and mutant fibroblasts. The TLC pattern of radiolabeled compounds from each cell line is shown in every graph. *Patient 1:* Italian 5-year-old child with milder late infantile form. *Patient 2:* "Salla" disease. *Patient 3:* infantile form of NSD. The *black dots* indicate the position of the corresponding markers NANA and methyl-NANA

**Table 1.** Clearance of ($^3$H)-NANA after loading of fibroblasts in suspension. Different lysosome-enriched fractions prepared after "chase" periods of 0, 15, 30, and 60 min, were applied on TLC plates. Radioactivity in the area corresponding to NANA was determined and expressed as percentage of the initial ($^3$H)-NANA dpm per unit β-hexosaminidase activity. The logarithm of this percentage was plotted against time of clearance. Half lives were calculated by linear regression

| Cell strain | T 1/2 (min) |
|---|---|
| Control 1 | 67 |
| Control 2 | 37 |
| Control 3 | 62 |
| Mean | 55 |
| Infantile NANA storage disease | 107 |
| Late infantile NANA storage disease | 146 |
| "Salla" disease | ∞ |

ied not more than 20%. The logarithm of this percentage was then plotted against time and, in order to quantify the release of NANA, the half-life of intralysosomal free NANA was calculated by linear regression (Table 1). These results of three independent experiments indicate that the half-life of NANA in cells from all the different variants was significantly higher than in normal cells. These data provide experimental evidence for a defective release of free NANA from the lysosome in all these types of NSD.

## Discussion

Previous studies (Hancock et al. 1983) showed that NANA is not taken up by cultured fibroblasts most likely because of its charge. Therefore we prepared the methylester of NANA, which is uncharged and which was expected to reach the lysosomes as demonstrated for other monosaccharides (Hales et al. 1984).

The results of our experiments showed that the NANA-methylester is taken up by human fibroblasts and is subsequently converted into free NANA in the lysosome-enriched fractions. Since the methylester is chemically stable over a pH range between 4 and 7, this conversion is probably the result of an enzymatic reaction. A similar mechanism has been described for amino acid methylesters (Reeves 1979). These methylesters have been successfully used to study amino acid transport across the lysosomal membrane, which clarified the

basic defect in cystinosis; a lysosomal storage disease due to a defective transport of cystine across the lysosomal membrane (Gahl et al. 1982).

Since the conversion of NANA-methylester to free NANA was found to occur rather rapidly, it was possible to study the transport of NANA across the lysosomal membrane in normal and mutant human fibroblasts.

During long-term incubation with NANA-methylester all three types of mutant cells showed an accumulation of free NANA in the lysosome-enriched fractions. The results of these loading experiments, where the conversion of methylester into free NANA is independent of neuraminidase activity, exclude the possibility that increased neuraminidase activity or other mechanisms of NANA hyperproduction are responsible for NANA storage and sialuria, as has been described for one case with massive sialuria (Thomas et al. 1985).

The data from our "chase" experiments show a markedly increased half-life of free NANA in lysosome-enriched fractions of cells from all three types of patients tested. This implies a defective transport of free NANA across the lysosomal membrane. This would be the third example of a genetically determined transport defect across the lysosomal membrane, after cystinosis and vitamin B12 storage disease (Gahl et al. 1982; Rosenblatt et al. 1985). Our studies indicate a similar biochemical defect in different clinical variants of NSD. In order to relate the severity of the clinical features and the biochemical defect, somatic cell hybridization studies and characterization of the responsible transport protein(s) are required to obtain definite answers.

*Acknowledgements.* We acknowledge the help of Dr. J. P. Kamerling and Dr. H. van Pelt (Organic Chemistry Laboratory, University of Utrecht) with the preparation of the NANA-methylester. We thank Dr. P. Aula (Helsinki), Dr. E. Vamos (Brussels), and Professor A. Fois (Siena) for providing the cell material from patients with NSD.

## References

Aula P, Autio S, Raivio KO, Rapola J, Thoden CJ, Koskela SL, Yamashina I (1979) Salla disease. A new lysosomal storage disorder. Arch Neurol 36:88–94

Baumkotter J, Cantz M, Mendla K, Baumann W, Friebolin H, Gehler J, Spranger J (1985) N-Acetylneuraminic acid storage disease. Hum Genet 71:155–159

Gahl WA, Bashan N, Tietze F, Bernardini I, Schulman JD (1982) Cystine transport is defective in isolated leucocyte lysosomes from patients with cystinosis. Science 217:1263–1265

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

Hales CN, Docherty K, Maguire GA (1984) Sugar transport in lysosomes. In: Dingle JT, Dean RT, Sly W (eds) Lysosomes in biology and pathology. Elsevier, Amsterdam, pp 165–174

Hancock LW, Horwitz AL, Dawson G (1983) N-Acetylneuraminic acid and sialoglycoconjugate metabolism in fibroblasts from a patient with generalized N-acetylneuraminic acid storage disease. Biochim Biophys Acta 760:32–52

Horwitz AL, Hancock L, Dawson G, Thaler MH (1981) Generalized sialic acid storage disease. Pediatr Res 15:563

Reeves JP (1979) Accumulation of amino acids by lysosomes incubated with amino acid methylesters. J Biol Chem 254:8914–8921

Renlund M, Aula P, Raivo KO, Autio S, Sainio K, Rapola J, Koskela SL (1983a) Salla disease: a new lysosomal storage disorder with disturbed sialic acid metabolism. Neurology (NY) 33:57–66

Renlund M, Chester MA, Lundblad A, Parkkinen J, Krusius T (1983b) Free N-acetylneuraminic acid in tissues in Salla disease and the enzymes involved in its metabolism. Eur J Biochem 130:39–45

Rosenblatt DS, Hosack A, Matiaszuk NV, Cooper BA, LaFramboise R (1985) Defect in vitamin B12 release from lysosomes: newly described inborn error of vitamin B12 metabolism. Science 228:1319–1321

Stevenson RE, Lubinsky M, Taylor HA, Wenger DA, Schroer RJ, Olmstead PM (1983) Sialic acid storage disease with sialuria: clinical and biochemical features in the severe infantile type. Pediatrics 72:441–449

Thomas GH, Reynolds LW, Miller CS (1985) Overproduction of N-acetylneuraminic acid (sialic acid) by sialuria fibroblasts. Pediatr Res 19:451–455

Tondeur M, Libert J, Vamos E, van Hoof F, Thomas GH, Strecker G (1982) Infantile form of sialic acid storage disorder: clinical, ultrastructural and biochemical studies in two siblings. Eur J Pediatr 139:142–147

Virtanen I, Ekblom P, Laurila P, Nordling S, Raivio KO, Aula P (1980) Characterization of storage material in cultured fibroblasts by specific lectin binding in lysosomal storage diseases. Pediatr Res 14:1199–1203

Warren L (1959) The thiobarbituric acid assay of sialic acids. J Biol Chem 234:1971–1975

Yu RK, Ledeen R (1969) Configuration of the ketosidic bond of sialic acids. J Biol Chem 244:1306–1313

Received February 28, 1986 / Revised March 20, 1986

**Note added in proof.** Other evidence in support of defective NANA transport in Salla disease was recently published by Renlund et al. (1986). J Clin Invest 77:568–574.

**Publication III**

Characterization of a proton-driven carrier for sialic acid in the lysosomal membrane. Evidence for a group specific transport system for acidic monosaccharides.
*J. Biol. Chem.* 264:15247-15254 (1989)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1989 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 264, No. 26, Issue of September 15, pp. 15247-16254, 1989
Printed in U.S.A.

# Characterization of a Proton-driven Carrier for Sialic Acid in the Lysosomal Membrane

## EVIDENCE FOR A GROUP-SPECIFIC TRANSPORT SYSTEM FOR ACIDIC MONOSACCHARIDES*

(Received for publication, January 17, 1989)

Grazia M. S. Mancini‡§, Hugo R. de Jonge¶, Hans Galjaard‡, and Frans W. Verheijen‡

*From the ‡Department of Cell Biology and Genetics and the ¶Department of Biochemistry I, Erasmus University, 3000 DR Rotterdam, The Netherlands*

**Highly purified lysosomal membrane vesicles, obtained from rat liver lysosomes, were used to study characteristics of NeuAc transport across the lysosomal membrane. Uptake of [$^{14}$C]NeuAc was found to be strongly influenced by a pH gradient across the membrane. When a proton gradient (pHin > pHout) was generated by impermeable buffers, NeuAc uptake above equilibrium level (overshoot) was observed. The influence of membrane diffusion potentials was ruled out by experiments where $K^+$ and valinomycin were present. The overshoot appeared to be specifically produced by protons, since gradients of other cations ($Na^+$ and $K^+$) did not give stimulation. Proton-driven uptake was saturable ($K_t$ = 0.24 mM) and mediated by a single system, as shown by linearity of the Scatchard plot. Stimulation of transport was also obtained by preincubation of vesicles with MgATP and the effect was blocked by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, but not by the protonophore carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone. Monocarboxylic sugars like glycuronic acids were competitive inhibitors of sialic acid transport. Transstimulation of [$^{14}$C] NeuAc uptake was observed when vesicles were preloaded either with unlabeled NeuAc or with glucuronic acid. The data demonstrate that lysosomal membrane vesicles from rat liver are a suitable system for kinetic studies of solute transport events. The presence of a proton-driven carrier in the lysosomal membrane specific for sialic acid and other acidic sugars, including glucuronic acid, is shown. The possible physiological significance of these findings for the human lysosomal carrier and the patients with a sialic acid transport defect is discussed.**

In eukaryotic cells digestion of products derived from intra- and extracellular metabolism essentially occurs in lysosomes, acidic organelles rich in hydrolytic enzymes. The intralysosomal acidic pH is maintained by a membrane ATPase acting as an electrogenic proton pump (1–5). The acidic environment provides optimal conditions for enzymatic catalysis. The lysosomal membrane is impermeable to macromolecules and intermediate oligomeric metabolites, which are therefore retained until their complete digestion. The fact that only low molecular weight monomeric metabolites can leave the lysosomes led in the past to the assumption that the lysosomal membrane works as an aspecific sieve (6).

The selective permeability of the membrane and its role in the regulation of catabolite flux became evident when a fatal human disorder, nephropathic cystinosis, was ascribed to a genetic defect in a specific lysosomal carrier for the disulfide amino acid cystine (7). Since then, the presence and function of other lysosomal transport systems for amino acids have been investigated, and differences between lysosomal and other cellular amino acid transport mechanisms have been found (8–14).

Recently, Salla disease and other forms of NeuAc storage disorders have been imputed to a defective lysosomal efflux of the anionic sugar NeuAc through the lysosomal membrane (15–17). In search for the existence of a carrier, we have investigated the kinetic properties of the sialic acid transport mechanism, by studying the flux of radiolabeled sialic acid into highly purified lysosomal membrane vesicles prepared by hypotonic shock of rat liver lysosomes (18, 19). This approach offers the advantage that transport events can be followed independently of the intralysosomal content. As previously demonstrated, carrier-mediated solute movements across a biomembrane can be studied in both directions, under the proper thermodynamic conditions (10, 11). Thus, although the physiological net flux of sialic acid is directed most likely from the lysosome toward the cytosol, the characterization of the [$^{14}$C]NeuAc uptake process into lysosomal vesicles does not require maintenance of the *in situ* membrane orientation upon resealing. By this approach, we succeeded in demonstrating and characterizing a proton-driven lysosomal transport system for sialic acid. Moreover, the technique of measuring transport in vesiculated lysosomal membranes, developed in this study, may find a more general application in the exploration of other solute transport mechanisms across the lysosomal membrane.

## EXPERIMENTAL PROCEDURES

*Lysosomal Membrane Vesicles*—For the preparation of lysosomal membranes, the procedure of Ohsumi *et al.* (18) was followed, with the modifications introduced by Bame and Rome (19). Shortly, lysosomes were isolated from livers of adult Wistar rats after homogenization in isotonic sucrose (0.25 M), followed by several differential centrifugation steps. Lysosomal membranes, obtained after selective hypotonic shock, were centrifuged at 50000 × *g* for 30 min and washed with 0.5 M NaCl or KCl. The final pellet was resuspended in 20 mM

* A preliminary report of this work has appeared in abstract form (Mancini, G. M. S., and Verheijen, F. W. (1988) *J. Cell Biol.* 107, 571 (abstr.)). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. Cell Biology and Genetics, Postbox 1738, 3000 DR, Rotterdam, The Netherlands.

Na- or K-Hepes,[1] pH 7.4, containing 0.1 mM EDTA to give a protein concentration of 8–10 mg/ml. Small aliquots were then frozen in liquid nitrogen and stored at −70 °C. Full transport activity was preserved for at least 1 month. The yield of purified membrane protein was about 150 μg/g liver (wet weight). Activities of β-glucocerebrosidase and tartrate-inhibitable acid phosphatase were measured as lysosomal membrane markers during the isolation procedure (18–20). Both enzyme-specific activities were 80–100-fold enriched in the final membrane preparation compared with the total homogenate, which is in agreement with previous results (18). In the lysosomal vesicles described by Ohsumi *et al.*, the presence of plasma membranes as contaminant was originally reported. Therefore, the activities of alkaline phosphodiesterase (21) and ouabain-sensitive $Na^+/K^+$ ATPase (22) were assayed as plasma membrane markers during the purification procedure. The relative enrichment in the final preparation compared with the total homogenate was, respectively, 9–10-fold, with an average recovery of 0.3% of these enzymes, compared with the 8% of the lysosomal membrane-associated markers. During purification, 75% of the original β-glucocerebrosidase was present in the pellet of the lysosomal/mitochondrial fraction whereas only 5% of the total alkaline phosphodiesterase was recovered in the same pellet. This confirmed to us that plasma membrane is eventually present as a minor contaminant.

In order to rule out the possibility that the observed transport activity is due to the presence of contaminating membranes, lysosomal membranes were also isolated by a different procedure, according to the method of Wattiaux *et al.* (23). The relative enrichment of acid phosphatase and β-glucocerebrosidase in the vesicles prepared by the latter procedure was respectively 25- and 30-fold, whereas alkaline phosphodiesterase and $Na^+/K^+$ ATPase were both 3-fold enriched. Also these lysosomal membrane vesicles showed proton-dependent sialic acid transport, but the method of Ohsumi *et al.* (18) was preferred because it was more suitable for large scale isolations.

The ultrastructure of the membrane preparation was examined by transmission electronmicroscopy, using negative staining as described (19).

*Sialic Acid Uptake*—For a typical uptake experiment, aliquots of [$^{14}$C]NeuAc were freeze-dried. When uptake was measured in the absence of a $H^+$ gradient, the material was redissolved in 5 μl of 20 mM Na- or K-Hepes, pH 7.4. When uptake was assayed in the presence of a $H^+$ gradient, the substrate was redissolved in 5 μl of 240 mM MES-free acid, which titrated the extravesicular pH during incubation to 5.5. Membrane vesicles were rapidly thawed at 37 °C and preequilibrated briefly at 20 °C. Uptake was initiated by adding 25 μl of membrane suspension to the substrate tube at 20 °C. After the required incubation period, uptake was stopped by adding 70 μl of ice-cold incubation buffer, as indicated in the legends of the figures. The sample (100 μl) was immediately applied to a 2-ml Sephadex G-50 (Fine) column (0.5 × 5 cm), equilibrated with incubation buffer and run at 0.3 ml/min at 4 °C. Subsequently, the vesicles were eluted quantitatively from the column by addition of 1 ml of ice-cold incubation buffer. Radioactivity of [$^{14}$C]NeuAc trapped in the vesicles was counted by liquid scintillation in 10 ml of Insta-Gel (Packard Instruments). In the experiments where the extravesicular pH was manipulated by addition of impermeable buffers, a discrepancy between internal and external osmolarity was present. However, these differences did not significantly affect initial uptake rates, but only equilibrium levels. This technique enabled us to perform experiments in small incubation volumes and to avoid the effect of viscosity of osmolyte sugars on the elution rate during Sephadex filtration. As blanks, 25 μl of membrane suspension was added to a tube containing [$^{14}$C]NeuAc in 75 μl of ice-cold buffer and directly run on a column as described. The radioactivity recovered in the eluate did not differ from that obtained running [$^{14}$C]NeuAc only, suggesting that at 4 °C vesicular binding/uptake of the substrate was negligible. The blank was subtracted from all determinations. Typically, $4.0 \times 10^5$ dpm applied to the column gave an average blank of 70 dpm. Details for different assay conditions are given in the legends of the figures. The gel filtration procedure was compared with other separation techniques, like filtration through nitrocellulose (MF-GS type, 0.22-μm pore size, Millipore) or glass fiber filters (GF/C, Whatman). In the first case aspecific binding of labeled sialic acid to the filter (approximately 1% of the applied counts) was observed, and the second method led to an uncontrolled loss of vesicles through the filter. Therefore, the gel filtration technique was preferred. Experiments were performed in duplicate and the intra-assay variation was always less than 10% of the mean value. The variation in maximum uptake rate observed between different vesicle preparations was at most 30%. The figures show representative experiments obtained with two to six separate batches of vesicles. Quantitative results are indicated as mean value ± standard errors for different determinations performed with the same vesicle preparation. The Student's *t* test was used to estimate the significance of differences between mean values.

*Miscellanea*—*N*-Acetyl-[4,5,6,7,8,9-$^{14}$C]neuraminic acid (specific activity 280 mCi/mmol), [$^{14}$C]glucosamine HCl (270 mCi/mmol) and [U-$^{14}$C]sucrose (540 mCi/mmol) were purchased as aqueous solutions containing 2% ethanol (Amersham Corp.). NEM was from Merck (Darmstadt). Valinomycin was provided by Boehringer (Mannheim). Sephadex G-50 (Fine) was from Pharmacia LKB Biotechnology Inc. MgATP, NaGTP, DIDS, monensin, FCCP and all the compounds used for inhibition studies were obtained from Sigma, except for NeuAc1Me which was synthesized from NeuAc as previously described (16). Purity of sialic acid analogs, used for inhibition studies, was checked by thin layer chromatography on precoated silica gel plates (Merck, Darmstadt), developed in butanol/acetic acid/water (2:1:1) and stained with orcinol (24). Osmolarity was measured with an Advanced Instruments freezing point depression osmometer. β-Glucocerebrosidase activity in the presence of 0.3% Triton X-100 and 0.6% sodium taurocholate was measured as described (20). Acid phosphatase activity was assayed in the presence and absence of 80 mM Na-K-tartrate, using *p*-nitrophenolphosphate as substrate, and the lysosomal activity was calculated subtracting the activity in presence by that in the absence of tartrate (25).

## RESULTS

*General Properties of Lysosomal Membrane Vesicles*—Previous studies have shown that lysosomal membranes prepared under the reported conditions reseal spontaneously to form vesicles (18, 19). Studies with transmission electron microscopy revealed a vesicle diameter between 50–200 nm, confirming previous observations. The intravesicular volume was estimated by comparing the uptake of [$^{14}$C]NeuAc at equilibrium (60 min of incubation) with uptake of the impermeable [$^{14}$C]sucrose. When we studied uptake of 0.03 mM [$^{14}$C]NeuAc at pH 7.4, as calculated from the radioactivity of [$^{14}$C]NeuAc associated with the vesicles at equilibrium, the intravesicular volume was 1.12 ± 0.05 μl/mg protein, whereas the uptake of [$^{14}$C]sucrose in parallel experiments accounted for 0.2 ± 0.005 μl/mg protein. In order to know whether uptake of NeuAc is taking place into lysosomal membranes or other contaminating compartments, we compared the vesicle-associated volume after uptake of [$^{14}$C]NeuAc with that observed after uptake of [$^{14}$C]glucosamine, at equilibrium. This last compound is known to freely cross the lysosomal membrane (19). Since the two volumes coincided, we deduced that the two sugars entered the same vesicle population. This excludes that sialic acid uptake predominantly takes place in a compartment possibly present as a contaminant and distinct from the lysosomal vesicles. Moreover, these results show that the sucrose-impermeable volume is at least 0.9 μl/mg protein and that this space does not change significantly following one freeze-thawing step. Finally, to prove that the sucrose impermeable space represents the internal compartment of the vesicles, we studied the uptake of 0.03 mM [$^{14}$C]NeuAc at equilibrium at increasing medium osmolarity, promoting vesicle shrinkage by addition of sucrose or Hepes buffer. Indeed, equilibrium uptake of [$^{14}$C]NeuAc decreased progressively with increasing medium osmolarity and in both cases the relationship between the uptake and the reciprocal of osmo-

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ManNAc, *N*-acetylmannosamine; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; 4MU-NeuAc, 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid; NEM, *N*-ethylmaleimide; NeuAc2en, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; NeuAc1Me, *N*-acetylneuraminic acid methylester; NeuGc, *N*-glycolylneuraminic acid.

larity was linear (data not shown). The extrapolated value of sialic acid uptake at infinite osmolarity was close to zero, implying that the amount of [$^{14}$C]NeuAc bound to the vesicles but not internalized is negligible.

*Effect of a $H^+$ Gradient on Sialic Acid Uptake*—The influence of extravesicular pH on [$^{14}$C]NeuAc uptake was investigated by adjusting the external pH with MES and MOPS impermeable buffers. The data presented in Fig. 1 show a progressive increase of initial sialic acid uptake with decreasing pH of the external medium.

In order to find out whether this increase reflected the titration of specific groups on a transport protein at acidic pH, or was dependent on the presence of a proton gradient across the membrane, we assayed uptake rates in the presence and absence of monensin. This ionophore is known to exchange $H^+$ and $Na^+$, thus equilibrating the pH difference across the membrane, in the presence of excess $Na^+$. In our case, the titration of vesicles prepared in 20 mM Na-Hepes, pH 7.4, with 40 mM MES-free acid, in the presence of monensin, brought the pH inside and outside the vesicles to 5.5. The experiment in Fig. 2 shows that the presence of an inwardly directed proton gradient ($pH_{in} = 7.4 > pH_{out} = 5.5$), in the absence of monensin, drives uphill transport of sialic acid, transiently above equilibrium level. Initial uptake rates were about 9-fold stimulated in the presence of a proton gradient. The "overshoot" was totally abolished in the presence of monensin, at the same extravesicular pH (5.5). The uptake rates at pH 5.5 (in = out) are close to those observed at pH 7.4 (in = out), indicating that, in the absence of a proton gradient, the transporter does not display a sharp pH optimum.

The effect of a diffusion potential, possibly generated by proton movements across the membrane, was evaluated studying sialic acid transport in vesicles voltage-clamped with $K^+$ and valinomycin. When vesicles were prepared in 20 mM K-Hepes and preequilibrated with 0.01 mM valinomycin for 10 min at 20 °C, the initial rate of uptake for 0.15 mM [$^{14}$C] NeuAc, measured afterwards in the presence of a proton gradient, was 95.3 ± 2.4 pmol/mg protein/30 s, compared with 96.1 ± 4.3 in the absence of valinomycin. The difference was not statistically significant ($n = 5$). Similar average values were found when vesicles were prepared in Na-Hepes (mean 97.9 ± 5.6; $n = 5$). These experiments virtually rule out a role for a proton diffusion potential or requirement of $Na^+$ in the overshoot of sialic acid uptake observed in Fig. 2.

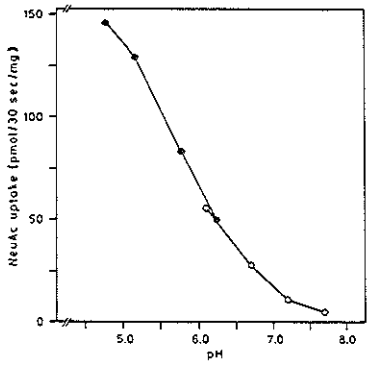


FIG. 1. **Effect of extravesicular pH on NeuAc uptake in lysosomal membrane vesicles.** Vesicles (190 μg of protein), prepared in 20 mM Na-Hepes, pH 7.0, were incubated for 30 s at 20 °C in medium containing 0.1 mM [$^{14}$C]NeuAc and 80 mM MES (●——●) or MOPS (○——○), titrated to the indicated pH with NaOH.

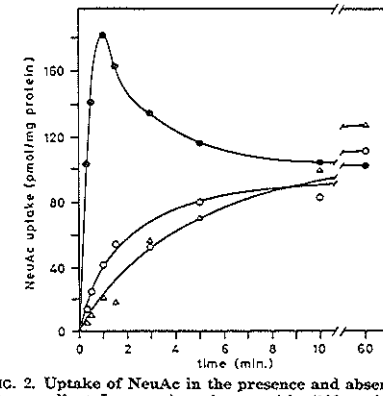


FIG. 2. **Uptake of NeuAc in the presence and absence of a proton gradient.** Lysosomal membrane vesicles (190 μg of protein), prepared in 20 mM Na-Hepes, pH 7.4, were preincubated for 10 min at 20 °C, in 20 mM Na-Hepes, pH 7.4 (●——●; Δ——Δ), or 20 mM Na-Hepes, 40 mM MES, pH 5.5 plus 0.01 mM monensin (○——○). Subsequently, transport of 0.1 mM [$^{14}$C]NeuAc was measured at 20 °C in 20 mM Na-Hepes, pH 7.4 (Δ——Δ; $pH_{in} = pH_{out} = 7.4$), or in 20 mM Na-Hepes, 40 mM MES, pH 5.5, plus 0.01 mM monensin (○——○; $pH_{in} = pH_{out} = 5.5$), or in 20 mM Na-Hepes, 40 mM MES, pH 5.5 (●——●; $pH_{in} = 7.4 > pH_{out} = 5.5$).

Further, the influence of inside positive diffusion potentials, generated by other cations than protons, was examined by studying the effect of a 20 mM $Na^+$ or $K^+$ gradient (inside, 20 mM Na- or K-Hepes; outside, 40 mM Na- or K-Hepes), the latter in the presence and absence of 0.01 mM valinomycin, on the uptake of 0.1 mM [$^{14}$C]NeuAc at pH 7.4 (in = out). In all cases, no significant increase of initial uptake rates of [$^{14}$C] NeuAc was observed above the values already reported in Fig. 2 at pH 7.4 (in = out). From these last experiments it can be also concluded that sialic acid transport is not coupled to $Na^+$ or $K^+$. Instead, these data altogether indicate that an inwardly directed proton gradient provides the driving force for sialic acid transport in lysosomal membrane vesicles.

In the experiments shown in Table IA, preincubation of the vesicles at pH 7.4 (in = out) with 2 mM MgATP was able to produce 2-fold increase of [$^{14}$C]NeuAc uptake. This effect might be explained by generation of a proton gradient by the lysosomal proton pump in inside-out vesicles. However, this would require membrane permeability for ATP, since the ATP-binding site is present on the external surface of the lysosomes. To investigate this hypothesis, we have studied the effect of ATP in the presence of inhibitors of the lysosomal proton pump and of the protonophore FCCP, which should prevent the formation of a proton gradient. As shown in Table IA, both DIDS and NEM prevent ATP stimulation, however, the latter only at concentrations higher (1 mM) than those known to inhibit the proton pump (50 μM). The experiments in Table IB indicate that the effects of DIDS cannot be ascribed to a direct inhibition of the carrier, in contrast to the effect of NEM, but seems to be specific for the ATP-stimulated transport. In another set of experiments, it was found that the presence of the protonophore FCCP did not affect the ATP stimulation (Table IA), excluding an effect of proton pumping in the inside-out vesicle population. Control experiments in the presence of $MgSO_4$ excluded that Mg was

TABLE I

*Effect of nucleotides and lysosomal $H^+$ pump inhibitors on sialic acid transport*

Part A. Vesicles in 20 mM Na-Hepes, pH 7.4 were preincubated for 10 min in the presence of 2 mM MgATP, or MgATP plus NEM, or plus DIDS, or plus FCCP, or $MgSO_4$ only, or NaGTP only. Uptake of 0.1 mM [$^{14}$C]NeuAc was studied for 30 s at 20 °C in the same buffer at pH 7.4. Part B. Vesicles in 20 mM Na-Hepes, pH 7.4, were preincubated for 10 min with NEM or DIDS at the indicated final concentration. In all these experiments, uptake was studied in the presence of a $H^+$ gradient generated by the addition of 40 mM MES free acid. Values are expressed as means ± S.E.

| | n* | Uptake |
|---|---|---|
| | | *pmol/mg protein* |
| A | | |
| No addition | 10 | 12.1 ± 1.7 |
| MgATP | 12 | 24.0 ± 2.0[b] |
| MgATP + 0.5 mM DIDS | 3 | 14.3 ± 0.9 |
| MgATP + 50 μM NEM | 4 | 18.2 ± 1.5[b] |
| MgATP + 1 mM NEM | 2 | 12.8 ± 0.1 |
| MgATP + 10 μM FCCP | 6 | 24.3 ± 2.1[b] |
| $MgSO_4$ | 2 | 12.9 ± 1.9 |
| NaGTP | 4 | 29.3 ± 1.2[b] |
| B | | |
| No addition | 3 | 73.1 ± 5.0 |
| 0.5 mM DIDS | 3 | 64.7 ± 0.6 |
| 50 μM NEM | 2 | 84.3 ± 4.0 |
| 1 mM NEM | 3 | 11.4 ± 1.3[b] |

* *n*, number of determinations.
[b] $p < 0.05$

responsible for the increased uptake. Moreover, stimulation was also observed after addition of NaGTP. From all these experiments we conclude that the stimulation of sialic acid uptake by ATP is not due to proton pumping in the inside-out vesicle population, but is probably ascribed to a yet unknown regulation mechanism by nucleotides.

*Concentration Dependence of Sialic Acid Uptake*—Incubations for only 30 s were considered to approximate zero trans conditions and therefore were chosen to determine initial uptake rates of NeuAc as a function of substrate concentration. The results of these experiments, performed in the presence and absence of a proton gradient (Fig. 3*A*), show the characteristic features of the Michaelis Menten kinetics typical of carrier-mediated transport. In the presence of a proton gradient, the apparent affinity constant $K_t$, as calculated by linear regression analysis from Lineweaver-Burk plots, showed an average value of 0.24 ± 0.07 mM ($n = 3$). At pH 5.5 (in = out, monensin added) the apparent $K_t$ increased to 0.7 mM ($n = 2$). As shown in the figure, the $V_{max}$ also differed in the presence and absence of a proton gradient (respectively, 450 and 250 pmol/30 s/mg protein). Scatchard plots of the data (Fig. 3, *B* and *C*) reveal a linear relationship in both cases (respectively, $r = 0.97$ and $r = 0.98$), indicating the operation of a single carrier and excluding the contribution of different transport mechanisms within the concentration range studied.

*Cooperativity of Protons in Activating Sialic Acid Transport*—The previous experiments demonstrate that the carrier does not display a clear pH optimum in the absence of a proton gradient, since the uptake rates at pH 5.5 (in = out) and pH 7.4 (in = out) are similar. Assuming that this is also the case in the presence of a proton gradient, an evaluation of the degree of cooperativity of the protons in activating sialic acid transport should be possible. The experimental data of the pH-dependent uptake of [$^{14}$C]NeuAc shown in Fig. 1 were fitted to the Hill equation. Uptake was expressed as a function of the external proton concentration, after subtrac-

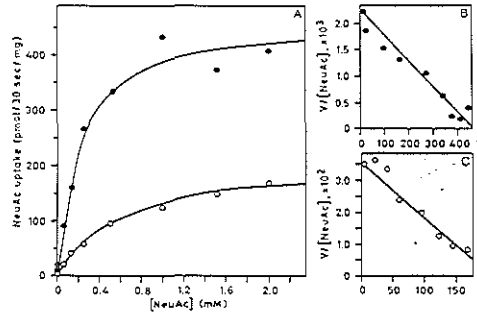


FIG. 3. **Concentration-dependent uptake of NeuAc into lysosomal membrane vesicles in the presence and absence of a proton gradient.** *A*, uptake in the presence of a proton gradient ($pH_{in} = 7.4 > pH_{out} = 5.5$; ●——●) and in the absence of proton gradient (monensin added; $pH_{in} = pH_{out} = 5.5$; ○——○) was performed in 20 mM Na-Hepes, 40 mM MES, pH 5.5, as indicated in Fig. 2, during 30 s at 20 °C, using different NeuAc concentrations. *B*, Scatchard plot of the uptake curve in the presence of a proton gradient ($pH_{in} > pH_{out}$), and *C*, the same in the presence of monensin ($pH_{in} = pH_{out}$).

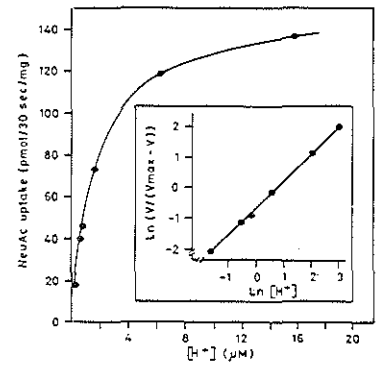


FIG. 4. **Proton gradient-dependent NeuAc uptake in lysosomal membrane vesicles, as a function of the extravesicular $H^+$ concentration.** The experimental data from Fig. 1 were fitted to the Hill equation after subtraction of a proton gradient-independent component of 15 pmol/30 s/mg protein. *Inset*, plot expressing the log form of the Hill equation. The slope of this curve was used to calculate the Hill coefficient *n*.

tion of the uptake component observed in the absence of a proton gradient at 0.1 mM NeuAc concentration (average 15 pmol/30 s/mg protein) (Fig. 4). The Hill coefficient *n*, indicating the degree of cooperativity, was determined using the log form of the Hill equation (*inset*, Fig. 4) and was found to be 0.8. This value suggests that probably there is no positive or negative cooperativity and that the sialic acid carrier interacts with one proton (26).

*Transstimulation Effect by Unlabeled Sialic Acid*—In order to demonstrate the symmetrical function of the carrier, the following experiment was performed. Vesicles were preloaded with 1 mM unlabeled sialic acid, and after 4-fold dilution of the extravesicular medium, uptake of [$^{14}$C]NeuAc was measured. As shown in Fig. 5, in the absence of a proton gradient (monensin added; $pH_{in} = pH_{out} = 5.5$), the presence of an outwardly directed gradient of unlabeled NeuAc strongly

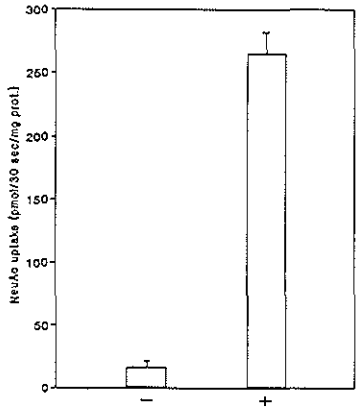


FIG. 5. **Transstimulation of [$^{14}$C]NeuAc uptake in lysosomal membrane vesicles preloaded with unlabeled NeuAc.** Vesicles (160 $\mu$g of protein) in 10 mM $Na^+$, 10 mM $K^+$, 20 mM Hepes, 40 mM MES, pH 5.5, plus 0.01 mM monensin, and 0.01 mM valinomycin were preincubated for 60 min at 20 °C in the presence (+) or absence (−) of 1 mM unlabeled NeuAc (final volume 25 $\mu$l). Transport was initiated by 4-fold dilution of the samples in the same prewarmed incubation buffer at pH 5.5 containing 0.027 mM [$^{14}$C]NeuAc and followed for 30 s at 20 °C. In the samples that were preincubated in the absence of unlabeled NeuAc, unlabeled NeuAc was added to the dilution buffer to give the same final concentration of 0.25 mM unlabeled sialic acid and 0.027 mM [$^{14}$C]NeuAc outside the vesicles, under both conditions. Results are indicated as average values ± S.E.

stimulated uptake of extravesicular [$^{14}$C]NeuAc. Since $K^+$ and valinomycin were present throughout the experiment, it can be ruled out that the stimulation of uptake is due to the generation of membrane potentials. This phenomenon of countertransport proves that, under appropriate biochemical conditions, sialic acid can be transported in both directions by the same carrier.

*Substrate Specificity Studies*—In order to investigate the substrate specificity of the transporter, we studied the *cis*-inhibitory effect of selected analogs on the proton-driven uptake of [$^{14}$C]NeuAc. As shown in Table II, analogs which were modified in the carboxyl group at carbon 1 (like NeuAc1Me) or at carbon 2 (4MU-NeuAc and NeuAc2en) of the native *N*-acetylneuraminic acid backbone led only to a weak inhibition. On the contrary, an analog like NeuGc, which is modified at the side chain on carbon 5, is a strong NeuAc transport inhibitor. This suggests that both the carboxyl group at carbon 1 and the alcoholic group at carbon 2 are important recognition sites for the transport protein.

Besides structural analogs, also other neutral and carboxylic monosaccharides and acidic aminoacids were tested for their *cis*-inhibition effect (Table II). ManNAc, a sialic acid precursor, GlcNAc, glucose, and anionic aminoacids, like aspartic and glutamic acid, did not affect transport at the concentration studied. On the other hand, glycuronic and aldonic acids strongly inhibited sialic acid transport. Glucose derivatives were apparently more effective than galactose ones. The strongest inhibitors were monocarboxylic monosaccharides with a negatively charged terminus, as shown by the fact that uronic and aldonic acid lactones were completely ineffective. But the position of the carboxyl in the molecule (uronic acids at carbon 6 and aldonic acids at carbon 1) and the formation of an hemiacetale ring (aldonic acids do not spontaneously form rings) are not crucial for recognition.

TABLE II

*Cis-inhibition of NeuAc uptake into lysosomal membrane vesicles by sialic acid analogs, other monosaccharides, and acidic amino acids*

Uptake of 0.15 mM [$^{14}$C]NeuAc was studied in the presence of an inwardly directed proton gradient under conditions similar to those of Fig. 2, except that vesicles were preequilibrated with 4 mM $K^+$ and 0.01 mM valinomycin. Inhibitors (7 mM final concentration) were titrated at pH 5.5 with NaOH, if necessary. Control uptake was 92.4 ± 5.7 pmol/30 s/mg protein. Uptake in presence of inhibitor is expressed as percentage of uninhibited rate for duplicate determinations.

| | % of uninhibited rate |
|---|---|
| Sialic acid analogs | |
| No addition | 100 |
| NeuAc | 10 |
| NeuGc | 12 |
| NeuAc1Me | 49 |
| 4MU-NeuAc | 58 |
| NeuAc2en | 50 |
| Other monosaccharides, acidic amino acids | |
| Glucuronate | 10 |
| Gluconate | 14 |
| Galacturonate | 34 |
| Galactonate | 36 |
| Pyruvate | 50 |
| Glucose | 80 |
| *N*-Acetylglucosamine | 100 |
| *N*-Acetylmannosamine | 100 |
| Gulonolactone | 88 |
| Mannuronolactone | 86 |
| Aspartate | 89 |
| Glutamate | 86 |

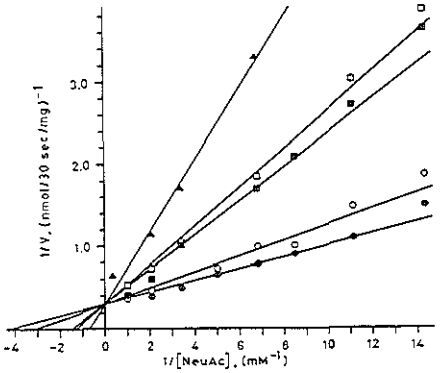


FIG. 6. **Double reciprocal plot of NeuAc uptake in the presence of different inhibitors.** Lysosomal membrane vesicles (200 $\mu$g of protein) in 20 mM Na-Hepes, pH 7.4, were preincubated 10 min at 20 °C in 16 mM Na-Hepes, 4 mM K-Hepes, and 0.01 mM valinomycin. Transport of increasing amounts of [$^{14}$C]NeuAc was measured for 30 s at 20 °C, in the presence of a proton gradient, as described in the legend of Fig. 2. The uptake medium contained 0.5 mM of the required inhibitor, titrated, to pH 5.5 with NaOH. ●——●, no inhibitor added; ○——○, NeuAc2en; ▣——▣, gluconic acid; □——□, NeuGc; ▲——▲, glucuronic acid.

*Inhibition Kinetics*—The inhibition kinetics of representative compounds of each class of inhibitors were studied in detail. Since some of these inhibitors might be potential substrates for the sialic acid carrier, we tested their effect at a concentration rather close to the $K_i$ for sialic acid. Proton-dependent transport was measured in voltage-clamped mem-

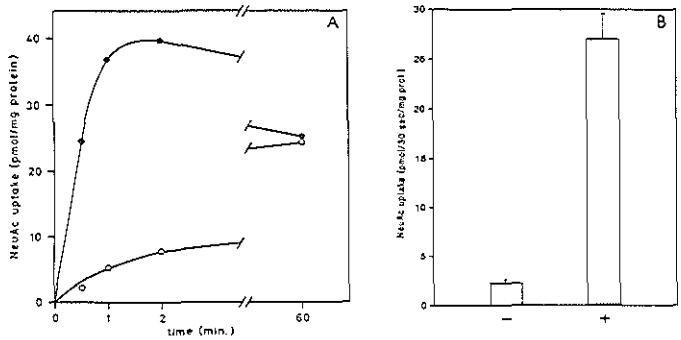


FIG. 7. **Transstimulation of NeuAc uptake in lysosomal membrane vesicles preloaded with glucuronic acid.** *A*, vesicles (160 μg of protein) in 20 mM Na-Hepes, 40 mM MES, pH 5.5, plus 0.01 mM monensin were preincubated for 60 min at 20 °C in the presence (●——●) or absence (○——○) of 1 mM glucuronic acid. Transport was initiated by 4-fold dilution of the sample in the same prewarmed incubation buffer at pH 5.5 containing [$^{14}$C] NeuAc at a final concentration of 0.027 mM. In the case where no preloading was performed (○——○), 0.25 mM glucuronic acid was added at the start of the transport assay. *B*, assay conditions were similar to those presented in the experiment of Fig. 5 except that vesicles were preincubated with 1 mM glucuronic acid (+). When no preloading was performed (−), 0.25 mM glucuronic acid was added at the start of the transport assay.

branes with $K^+$/valinomycin. The results were fitted to a double reciprocal plot and the approximate $K_i$ for the different inhibitors were calculated from the following relationship $K_i = K_t\,[I]/\{(1/x) - K_t\}$, where [I] is the inhibitor concentration, $x$ is the intercept of the line on the *abscissa* found by linear regression analysis, and $K_t$ is the apparent affinity constant for sialic acid. The latter value did not change in the presence of $K^+$/valinomycin. As illustrated in Fig. 6, all compounds displayed a competitive mode of inhibition of sialic acid transport. The calculated $K_i$ for glucuronic, gluconic acid, and NeuGc were, respectively, 0.11, 0.26, and 0.22 mM. For NeuAc2en we found a $K_i$ of 1.6 mM, indicating that this last compound has a much lower affinity for the carrier.

*Transstimulation of Sialic Acid Uptake by Glucuronic Acid*—Among the competitive inhibitors, glucuronic acid seemed of particular interest, since this compound naturally occurs in the lysosome, together with its stereoisomer iduronic acid, as breakdown product of glycosaminoglycans. Although the experiments mentioned above showed that glucuronic acid is competing at the binding site for sialic acid transport with high affinity, they do not prove that this compound is actually transported by the sialic acid carrier. In this latter case, glucoronic acid should be able to activate the carrier function and provoke transstimulation of [$^{14}$C]NeuAc uptake, similarly to unlabeled NeuAc. To test this assumption, vesicles were preloaded with unlabeled glucuronic acid and the uptake of trace amounts of [$^{14}$C]NeuAc, in the absence of a proton gradient (pH 5.5; in = out), was followed in time. Vesicles were incubated with monensin and in some instances with $K^+$/valinomycin to prevent undesired changes of the pH and the potential across the membrane as a consequence of glucuronic acid movements. The results are shown in Fig. 7, *A* and *B*. A transient accumulation (overshoot) of sialic acid, against a concentration gradient, was observed in glucuronic acid preloaded vesicles, receding to equilibrium levels after 1 h (Fig. 7*A*). Apparently, in this case, the magnitude of the outward directed glucuronic acid gradient drives transport of NeuAc in the opposite direction, thus moving it against its own concentration gradient (overshoot). The stimulation of NeuAc transport is not due to the generation of membrane potentials, since it is also observed in voltage-clamped membranes (Fig. 7*B*). This demonstrates that the lysosomal sialic acid carrier not only recognizes but also transports glucuronic acid.

## DISCUSSION

Investigations on lysosomal sugar transport mechanisms have been limited so far to uptake studies of neutral monosaccharides into rat liver lysosomes (27–30).

The existence of a lysosomal carrier for the anionic sugar sialic acid has been first supposed when initial biochemical studies on the metabolic defect in patients with free sialic acid storage disease showed no deficiency of lysosomal nor extralysosomal enzymes involved in sialic acid metabolism (32, 33). Lysosome-enriched fractions from cultured fibroblasts were used to provide evidence for defective sialic acid efflux in patients with NeuAc storage disorders (15, 16, 31) but appeared unsuitable for detailed kinetic studies of sialic acid transport. Jonas (31) reported that sialic acid uptake and efflux in rat liver and cultured human fibroblast lysosomes appear to be similar.

As presented in this paper, lysosomal membrane vesicles from rat liver exhibit appreciable rates of uptake of radiolabeled sialic acid, providing a convenient system to study transport kinetics in a system where both the extra- and the intravesicular medium can be easily adapted to the assay conditions.

The contribution of contaminating membranes, and particularly plasma membranes, in the observed sialic acid transport activity is excluded by the following considerations. First, the intravesicular volume was found to be identical either after uptake of [$^{14}$C]NeuAc or of [$^{14}$C]glucosamine, which is known to freely cross the lysosomal membrane (19). This suggests that the two compounds enter the same vesicle population and essentially that sialic acid is not only entering a compartment distinct from the lysosomal one. Second, the kinetics of sialic acid transport, represented in the Scatchard plots, demonstrate that, under our assay conditions, only one mechanism of transport is present, which therefore should be identical either in lysosomal or in contaminating compart-

ments. Third, lysosomal membrane vesicles obtained from highly purified lysosomes prepared by a different procedure (23) did show similar proton gradient-mediated sialic acid uptake. Also, partially purified lysosomal membrane preparations (lysosomal/mitochondrial fraction, during the procedure of Ohsumi *et al.*), containing the same amount of plasma membrane markers, but lower specific activities of lysosomal membrane markers, compared with the final preparation, did not show any proton driven sialic acid uptake (data not shown).

All these data indicate that sialic acid is taken up into a lysosomal compartment by a carrier-mediated mechanism and the possible contribution of contaminating (plasma-) membranes is undetectable.

Our experiments demonstrate that lysosomal sialic acid transport is a saturable process, mediated by a single carrier displaying transstimulation properties. Moreover, an "overshooting" uptake of NeuAc is observed when a pH gradient ($pH_{out} < pH_{in}$) is generated with impermeable buffers. This overshoot persisted following the short-circuiting of the membrane potential with $K^+$/valinomycin and therefore cannot be explained merely by the generation of a proton diffusion potential. Accordingly, no stimulation was observed when other inwardly directed cation gradients were generated ($Na^+$ and $K^+$), nor when the proton gradient was shunted by monensin, showing the specificity of the proton effect. The presence of a proton gradient not only increased the maximum rate of uptake ($V_{max}$) but also increased the affinity of sialic acid for the carrier ($K_t$). Since *in vivo* an outwardly directed proton gradient is present, due to the action of the lysosomal proton pump, it is likely that proton-driven sialic acid efflux represents the physiological mechanism of sialic acid export out of the lysosome.

Proton-driven solute transport has been described not only for several bacterial systems, but also for some plasma membrane carriers of eukaryotic cells, transporting either anionic or cationic compounds (34–39). Studies performed with intact lysosomes, showed that the lysosomal carrier for cystine and that for cationic amino acids (system c) are influenced by changes in the extralysosomal pH, but the underlying mechanism has not been elucidated yet (9, 10, 40, 41).

Our observations on proton gradient-dependent sialic acid transport suggest no cooperativity of the protons (or $OH^-$ ions) at the binding site of the carrier but did not allow us to discriminate between the operation of a $H^+$/sialic acid symporter or of a $OH^-$/sialic acid antiporter. In the latter case, however, the type of carrier must be different from the DIDS-sensitive anion exchangers (42).

The role of ATP in stimulating sialic acid transport is intriguing. Even if it is reasonable to think that, in our experiments, ATP stimulates the sialic acid carrier, in the inside-out vesicles, via generation of a proton gradient by the lysosomal proton pump, which is inhibited by DIDS, the experiments with the ionophore FCCP and with NEM do not support the hypothesis of proton pumping. The fact that the same stimulation is found both with ATP and GTP also excludes, in this case, the involvement of the lysosomal proton pump and rather suggests a kind of regulating mechanism by nucleotides. Little is known about regulating mechanisms of lysosomal carriers, and we believe that these observations deserve more detailed investigations.

In the rat liver lysosomal membrane, the sialic acid carrier displayed a preference for sialic acid analogs in which the carboxyl group at $C_1$ and the native hydroxyl group at $C_2$ are unaltered. In contrast, the viral, bacterial, and mammalian neuraminidase is only capable of recognizing the $\alpha$-2-glycosidic linkage of sialic acid residues (43), whereas the $\beta$-2-glycosidic bond of CMP-sialic acid is specifically recognized by the Golgi membrane carrier and sialyltransferases (44, 45). Indeed, free sialic acid is not a substrate for the Golgi carrier (45), precluding the access of the free sugar to this compartment. Our data indicate that the structural requirements for recognition by the lysosomal carrier are different from those of other proteins involved in sialic acid metabolism, with the possible exception of the enzyme N-acetylneuraminate-pyruvate lyase, which is localized in the cytosol.

Among other monocarboxylic sugars, uronic acids were found to be strong competitive inhibitors of lysosomal sialic acid transport. Glucuronic acid and its isomer iduronic acid occur in most mammalian tissues as acidic sugars of glycosaminoglycans. Because of their low p$K$, probably these acids are, like sialic acid (p$K$ < 2), present as charged anions at intralysosomal pH and, similarly might require a carrier to exit the lysosome. Our evidence that glucuronic acid is a substrate for the sialic acid carrier is not only based on *cis*-inhibition studies (sharing a $K_i$ in the same range as the $K_t$ for sialic acid), but also on transstimulation of [$^{14}$C]NeuAc uptake by intravesicular glucuronic acid, and this phenomenon is not generated by changes in membrane potentials. Therefore, the substrate specificity of the sialic acid carrier in rat lysosomal membranes apparently also includes other carboxylic sugars. Group specificity has already been demonstrated for several lysosomal aminoacid carriers (9, 11–13).

It is now interesting to study the relevance of our studies to the human lysosomal system, which might help in understanding the biochemical defect in patients with NeuAc storage disorders. Reinvestigation of storage products in biological material of these patients, which led in the past to the identification of sialic acid as the main accumulating compound, may reveal the presence of other carboxylic monosaccharides. Research in this direction is in progress in our laboratory. Positive results would imply that the human lysosomal sialic acid transport system has not only a substrate specificity similar to the rat liver system described here, but also that such a carrier displays an essential role in lysosomal disposal of both sialic acid and uronic acids. However, the full characterization of this and perhaps other transport mechanisms for uronic acids in the lysosomal membrane must await the availability of radiolabeled glycuronic acids of sufficiently high specific radioactivity.

*Acknowledgments*—We express our gratitude to C. Beerens for her skillful technical assistance and to Prof. R. Brossmer of the University of Heidelberg and Prof. H. Kamerling of the University of Utrecht for helpful discussions. We are indebted to R. Willemsen for performing electronmicroscopical studies and to P. Visser and M. Kuit for the preparation of the figures.

REFERENCES

1. Harikumar, P., and Reeves, J. P. (1983) *J. Biol. Chem.* **258**, 10403–10410
2. Moriyama, Y., Takano, T., and Ohkuma, S. (1984) *J. Biochem.* **95**, 995–1007
3. Schneider, D. L. (1983) *J. Biol. Chem.* **258**, 1833–1838
4. D'Souza, M. P., Ambudkar, S. V., August, J. T., and Maloney, P. C. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6980–6984
5. Harikumar, P., and Reeves, J. P. (1986) in *New Insights into Cell and Membrane Transport Processes* (Poste, G., and Crooke, S. T., eds) pp. 61–75, Plenum Publishing Co., New York
6. Goldman, R. (1976) in *Lysosomes in Biology and Pathology* (Dingle, J. T., and Dean, R. T., eds) pp. 309–333, Elsevier Scientific Publishing Company, Amsterdam.
7. Gahl, W. A., Bashan, N., Tietze, F., Bernardini, I., and Schulman, J. D. (1982) *Science* **217**, 1263–1265
8. Christensen, H. N. (1984) *Biochim. Biophys. Acta* **779**, 255–269

9. Pisoni, R. L., Thoene, J. G., and Christensen, H. N. (1985) *J. Biol. Chem.* **260**, 4791–4798
10. Pisoni, R. L., Thoene, J. G., Lemons, R. M., and Christensen, H. N. (1987) *J. Biol. Chem.* **262**, 15011–15018
11. Pisoni, R. L., Flickinger, K. S., Thoene, J. G., and Christensen, H. N. (1987) *J. Biol. Chem.* **262**, 6010–6017
12. Bernar, J., Tietze, F., Kohn, L. D., Bernardini, I., Harper, G. S., Grollman, E. F., and Gahl, W. A. (1986) *J. Biol. Chem.* **261**, 17107–17112
13. Collarini, E. J., Pisoni, R. L., and Christensen, H. N. (1987) *Faseb J.* **2**, A322
14. Stewart, B. H., Collarini, E. J., Pisoni, R. L., and Christensen, H. N. (1987) *Faseb J.* **2**, A322
15. Renlund, M., Tietze, F., and Gahl, W. A. (1986) *Science* **232**, 759–762
16. Mancini, G. M. S., Verheijen, F. W., and Galjaard, H. (1986) *Hum. Genet.* **73**, 214–217
17. Mendla, K., Baumkötter, J., Rosenau, C., Ulrich-Bott, B., and Cantz, M. (1988) *Biochem. J.* **250**, 261–267
18. Ohsumi, Y., Ishikawa, T., and Kato, K. (1983) *J. Biochem. (Tokyo)* **93**, 547–556
19. Bame, K. J., and Rome, L. H. (1985) *J. Biol. Chem.* **260**, 11293–11299
20. Galjaard, H. (1980) in *Genetic Metabolic Diseases: Early Diagnosis and Prenatal Analysis* (Galjaard, H., ed) pp. 821–822, Elsevier Scientific Publishing Co., Amsterdam
21. Hubbard, A. L., Wall, D. A., and Ma, A. (1983) *J. Cell Biol.* **96**, 217–229
22. Adams, R. J., Cohen, D. W., Gupte, S., Johnson, J. D., Wallik, E. T., Wang, T., and Schwartz, A. (1979) *J. Biol. Chem.* **254**, 12404–12410
23. Wattiaux, R., Wattiaux-de Conink, S., Ronveaux-Dupal, M. F., and Dubois, F. (1978) *J. Cell Biol.* **78**, 349–368
24. Michalski, J. C., Montreuil, J., and Strecker, G. (1983) *Clin. Chim. Acta* **129**, 99–101
25. Gieselmann, V., Hasilik, A., and Von Figura, K. (1984) *Hoppe Seyler's Z. Physiol. Chem.* **365**, 651–660
26. Segel, I. H. (1975) *Enzyme Kinetics*, John Wiley & Sons, New York
27. Maguire, G. A., Docherty, K., and Hales, C. N. (1983) *Biochem. J.* **212**, 211–218
28. Bird, S. J., Forster, S., and Lloyd, J. B. (1987) *Biochem. J.* **245**, 929–931
29. Maguire, G. A., Kay, J. D. S., and Hales, C. N. (1988) *Biochem. J.* **252**, 621–623
30. Bird, S. J., Forster, S., and Lloyd, J. B. (1988) *Biochem. J.* **252**, 623
31. Jonas, A. J. (1986) *Biochem. Biophys. Res. Commun.* **137**, 175–181
32. Renlund, M., Chester, M. A., Lundblad, A., Parkkinen, J., and Krusius, T. (1983) *Eur. J. Biochem.* **130**, 39–45
33. Hancock, L. W., Horwitz, A. L., and Dawson, G. (1983) *Biochim. Biophys. Acta* **760**, 42–52
34. Røigaard-Petersen, H., Jacobsen, C., and Sheikh, M. I. (1987) *Am. J. Physiol.* **253**, F15–20
35. Sokol, P. P., Holohan, P. D., and Ross, C. R. (1985) *J. Pharmacol. Exp. Ther.* **233**, 694–699
36. Weinberg, S. L., Burckhardt, G., and Wilson, F. A. (1986) *J. Clin. Invest.* **78**, 44–50
37. Ganapathy, V., Ganapathy, M. E., Nair, C. N., Mahesh, V. B., and Leibach, F. H. (1988) *J. Biol. Chem.* **263**, 4561–4568
38. Balkovetz, D. F., Leibach, F. H., Mahesh, V. B., and Ganapathy, V. (1988) *J. Biol. Chem.* **263**, 13823–13830
39. Blomstedt, J. W., and Aronson, P. S. (1980) *J. Clin. Invest.* **65**, 931–934
40. Smith, M. L., Greene, A. A., Potashnik, R., Mendoza, S. A., and Schneider, J. A. (1987) *J. Biol. Chem.* **262**, 1244–1253
41. Jonas, A. J. (1986) *Biochem. J.* **236**, 671–677
42. Motais, R. (1980) *Ann. N. Y. Acad. Sci.* **341**, 197–220
43. Corfield, A. P., and Schauer, R. (1982) in *Sialic Acids, Chemistry, Metabolism and Function* (Schauer, R., ed) pp. 225–234, Springer-Verlag, Wien.
44. Corfield, A. P., and Schauer, R. (1982) in *Sialic Acids, Chemistry, Metabolism and Function* (Schauer, R., ed) pp. 205–207, Springer-Verlag, Wien
45. Carey, D. J., Sommers, L. W., and Hirschberg, C. B. (1980) *Cell* **19**, 597–605

**Publication IV**

Glucose transport in lysosomal membrane vesicles.
Kinetic demonstration of a carrier for neutral hexoses.
*J. Biol. Chem.* 265:12380-12387 (1990)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1990 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 265, No. 21, Issue of July 25, pp. 12380–12387, 1990
Printed in U.S.A.

# Glucose Transport in Lysosomal Membrane Vesicles

## KINETIC DEMONSTRATION OF A CARRIER FOR NEUTRAL HEXOSES*

(Received for publication, December 29, 1989)

Grazia M. S. Mancini‡, Cecile E. M. T. Boerens, and Frans W. Verheijen

*From the Department of Cell Biology and Genetics, Erasmus University, 3000 DR, Rotterdam, The Netherlands*

**Lysosomal membrane vesicles isolated from rat liver were exploited to analyze the mechanism of glucose transport across the lysosomal membrane. Uptake kinetics of [$^{14}$C]D-glucose showed a concentration-dependent saturable process, typical of carrier-mediated facilitated transport, with a $K_t$ of about 75 mM. Uptake was unaffected by $Na^+$ and $K^+$ ions, membrane potentials, and proton gradients but showed an acidic pH optimum. Lowering the pH from 7.4 to 5.5 had no effect on the affinity of the carrier for the substrate but increased the maximum rate of transport about 3-fold. As inferred from the linearity of Scatchard plots, a single transport mechanism could account for the uptake of glucose under all conditions tested. As indicated by the transstimulation properties of the carrier, other neutral monohexoses, including D-galactose, D-mannose, D- and L-fucose were transported by this carrier. The transport rates and affinities of these sugars, measured by the use of their radiolabeled counterparts, were in the same range as those for D-glucose.**

**Pentoses, sialic acid, and other acidic monosaccharides including their lactones, aminosugars, *N*-acetylhexosamines, and most L-stereoisomers, particularly those not present in mammalian tissues, were not transported by this carrier. Glucose uptake and transstimulation were inhibited by cytochalasin B and phloretin. The biochemical properties of this transporter differentiate it from other well-characterized lysosomal sugar carriers, including those for sialic acid and *N*-acetylhexosamines. The acidic pH optimum of this glucose transporter is a unique feature not shared with any other known glucose carrier and is consistent with its lysosomal origin.**

Since the discovery of the lysosome, much insight has been obtained into the role of this organelle in the catabolism of a wide variety of macromolecules. Instead, less is known about the fate of the products of catabolism and the role of the lysosome membrane in the regulation of their efflux.

Specific carrier-mediated transport systems in the lysosomal membrane have been detected and characterized during the last few years (for review see Ref. 1). The occurrence of human diseases and, consequently, the use of human mutant cells have been helpful in elucidating the normal mechanisms of lysosomal membrane transport for metabolites like cystine (2), vitamin $B_{12}$ (3), and sialic acid (4–6).

The biochemistry of lysosomal transport systems for sugars like sialic acid, uronic, and aldonic acids and for *N*-acetylhexosamines has been recently investigated (7, 8). Although previous investigations suggested the existence of a specific carrier for glucose transport in the lysosomal membrane (9, 10), this evidence has recently been the object of debate in the literature, and experimental data have been provided in favor of a simple diffusion rather than carrier-mediated transport model (11–13). Also, it has been questioned whether hexoses and pentoses share the same lysosomal transport system (11, 12, 14). In this respect, most studies have been performed using the osmotic protection method (9, 15), or with tritosomal preparations (10), and some technical difficulties inherent to both these methodologies may have caused the questionable results. (13, 14, 16).

Recently, we have developed a new system for studying transport mechanisms in highly purified lysosomal membrane vesicles. These vesicles are prepared by selective hypotonic shock of purified lysosomes and are 50% inside-out oriented upon resealing of the membrane (20, 21). We have advantageously used uptake experiments, in the presence of an inward-directed pH gradient, for the kinetic characterization of a proton-driven lysosomal transport system for sialic acid and other acidic monosaccharides (7). This has been possible because carrier-mediated transport mechanisms are theoretically bidirectional and both uptake and efflux studies have proved to be equivalent for the demonstration of lysosomal carriers, although these systems are physiologically needed mainly for the efflux of catabolites (17–19). In the present study, we have followed the same approach to unequivocally establish the mechanism by which glucose and other neutral monosaccharides are transported across the lysosomal membrane. The data provide firm evidence for the existence of a lysosomal carrier-mediated transport system, distinct from the sialic acid and the *N*-acetylhexosamine transport systems, which specifically mediates the facilitated diffusion of neutral hexoses like D-glucose, D-galactose, D-mannose, and D- and L-fucose, with the exclusion of other L-stereoisomers, pentoses, aminohexoses, acidic monosaccharides, and their lactones.

### MATERIALS AND METHODS

Rat liver lysosomes were isolated by differential centrifugation, and highly purified membrane vesicles were prepared according to the procedure described by Ohsumi *et al.* (20) and Barne and Rome (21). The membrane preparation was suspended at a protein concentration of 8–10 mg/ml in 20 mM NaHepes[1], pH 7.4, containing 0.1 mM EDTA, unless otherwise specified in the text, and were kept at −70 °C before use. The purity of the membranes was assessed as described (7). The lysosomal membrane marker enzymes β-glucocerebrosidase and acid phosphatase were about 100-fold enriched in

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

‡ To whom correspondence should be addressed: Dept. Cell Biology and Genetics, P. O. 1738, 3000 DR, Rotterdam, The Netherlands.

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

the final preparation, with a final recovery of 8% of the marker enzymes present in the total homogenate.

The radiolabeled sugars of the highest specific activity available D[U-$^{14}$C]glucose, D[U-$^{14}$C]glucosamine, D[U-$^{14}$C]mannose, D[U-$^{14}$C] galactose, L[6-$^{3}$H]fucose were purchased from Amersham. All the sugars used in the transstimulation experiments, cytochalasin B, phloretin, valinomycin, and monensin were purchased from Sigma.

For a typical "zero-trans" uptake assay, 10 μl of vesicles were incubated at 20 °C with 20 μl of substrate solution usually containing 2 μCi of radiolabeled sugar, 20 mM NaHepes, pH 7.4 (or MES and MOPS buffers, for experiments at different pH) and the required amount of unlabeled monosaccharide to reach the appropriate final concentration. In order to compensate for the osmotic effect of the extravesicular sugars at the higher concentrations, the vesicles were permeabilized by previous freezing in liquid nitrogen and slow thawing at 0 °C, in the presence of an appropriate amount of buffer (NaHepes or NaMES), or KCl. This treatment allowed equilibration of the buffer across the membrane and apparently did not alter the transport capacity nor the vesicular structure. After incubation, the reaction was stopped by adding 70 μl of isoosmotic ice-cold incubation buffer and the sample (100 μl) was poured over a small Sephadex G-50 (Fine) column, run at 0.3 ml/min at 4 °C, and elution of the vesicles was further accomplished as described (7). As blanks, 10 μl of vesicles were added to 90 μl of ice-cold incubation buffer containing 2 μCi of radiolabeled sugar and directly eluted on the column. This blank averaged between 200 and 300 dpm.

For transstimulation experiments, the same amount of membranes (10 μl) was pre-equilibrated for 45 min at 20 °C with 5 μl of 20 mM NaHepes or Mes buffer containing 300 mM unlabeled sugar (100 mM final concentration), and the assay was started by adding 85 μl of NaHepes or NaMES buffer at 20 °C, containing 2 μCi of radiolabeled substrate, and 50 mM KCl to compensate for osmolarity. Control experiments were performed by pre-equilibration of the membranes with 50 mM KCl instead of the sugar and, at the start of the assay, 15 mM unlabeled sugar was added together with 2 μCi of label and 35 mM KCl (final concentrations), in order to maintain in both cases the same final extravesicular concentration of unlabeled sugar and the same osmolarity. Measurements of the sensitivity of [$^{14}$C]D-glucose uptake to medium osmolarity were performed as described (7). All the experiments were repeated with two to four different vesicle preparations and were routinely performed as duplicate or triplicate determinations. The intra-assay variation never exceeded 20% of the mean value. Statistical determinations were performed using the Student's *t* test. Calculation of the kinetic parameters was carried out using the computer program Enzfitter from Elsevier-BIOSOFT, Cambridge United Kingdom. No corrections were necessary to compensate for nonsaturable components of sugar uptake.

TABLE I

*Effect of inward directed $Na^+$ and $K^+$ gradients or membrane potentials on D-glucose uptake in lysosomal membrane vesicles*

10 μl of vesicles in 20 mM NaHepes, pH 7.4, were incubated with 0.12 mM [$^{14}$C]D-glucose for 30 s at 20 °C in 20 μl of the same buffer (no addition) or of buffer containing at final concentrations 40 mM NaHepes ($Na^+$ gradient), or 40 mM KHepes ($K^+$ gradient) or 40 mM KHepes plus 10 μM valinomycin, or without addition. Reactions were stopped with 70 μl of the corresponding ice-cold buffer and vesicles were collected as described under "Materials and Methods." Results are from a representative experiment and show average values of triplicate determinations plus standard deviations.

| | [$^{14}$C]D-Glucose uptake |
|---|---|
| | *nmol/mg protein* |
| No addition | 0.052 ± 0.002 |
| 20 mM $Na^+$ gradient | 0.053 ± 0.003 |
| 20 mM $K^+$ gradient | 0.051 ± 0.002 |
| 20 mM $K^+$ gradient plus valinomycin | 0.046 ± 0.003 |

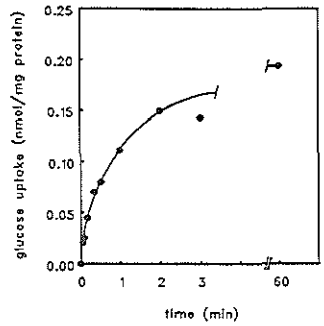


FIG. 1. **Time course of D-glucose uptake into rat liver lysosomal membrane vesicles.** Lysosomal membrane vesicles (78 μg of protein) in 20 mM NaHepes, pH 7.4, were incubated with 0.18 mM [$^{14}$C]D-glucose at 20 °C. At the end of incubation, the reaction was stopped by addition of ice-cold NaHepes buffer. The sample (100 μl) was poured over a Sephadex G-50 column, and the vesicles were eluted by addition of 1 ml of buffer. The radioactivity associated with the membranes was determined by liquid scintillation counting.

## RESULTS

Appreciable uptake of [$^{14}$C]D-glucose was observed in rat liver lysosomal membrane vesicles. In order to verify whether the vesicular uptake of glucose reflects transport into an osmotically active intravesicular space, rather than binding to the lysosomal membrane, we studied the sensitivity of the uptake at equilibrium (60 min of incubation) to increasing medium osmolarity, achieved by increasing the extravesicular concentration of an impermeant buffer (Hepes). As a consequence, glucose equilibrium uptake progressively decreased, indicating that the vesicle membrane is resealed and osmotically active. The relationship between the uptake and the reciprocal of osmolarity was found to be linear, and no significant uptake remained following extrapolation of the data to infinite osmolarity (results not shown).

Initial experiments demonstrated no influence of $Na^+$ or $K^+$ gradients on the rates of 0.12 mM [$^{14}$C]D-glucose uptake ($[Na^+$ or $K^+]_{ext}$ = 40 mM, $[Na^+$ or $K^+]_{in}$ = 20 mM) and also variations of the membrane potential in the presence of a 20 mM $K^+$ gradient plus valinomycin did not affect transport (Table I). The use of larger gradients did not change the results (not shown).

Initial uptake rates of [$^{14}$C]D-glucose were found to be linear up to 30 s of incubation, ensuring reliable zero-trans conditions for kinetic determinations (Fig. 1). It is noteworthy to mention that the intravesicular volume, as calculated from the equilibrium uptake levels, is about 1 μl/mg protein, similarly to what we previously observed for sialic acid uptake (7), suggesting that the same lysosomal compartment is permeable to both sugars.

Subsequently, we studied the concentration-dependent uptake of [$^{14}$C]D-glucose at the extravesicular pH of 7.4. The results are shown in Fig. 2. Kinetics of initial uptake show a saturable process typical of carrier mediated transport. The $K_t$ of this process averaged about 75 mM ($n$ = 4) and the $V_{max}$ about 11 nmol/15 s/mg protein. The Lineweaver-Burk transformation of the data (Fig. 2*B*), and the Scatchard plot (Fig. 2*C*), demonstrate linearization of the process, suggesting that, within the concentration range studied (90 μM to 200 mM), only one type of transport system is responsible for glucose uptake.

The sensitivity of the uptake to changes in the extravesicular pH was investigated within the pH range 5.5–7.9, using the impermeant buffers MOPS and MES. When we manipulated the extravesicular pH, keeping the internal pH at 7.4, we observed that uptake rates were significantly higher at acidic pH, with an optimum between pH 5.5 and 6.0 (Fig. 3). We had previously observed that the lysosomal carrier for sialic acid is also extremely sensitive to extravesicular acidi-

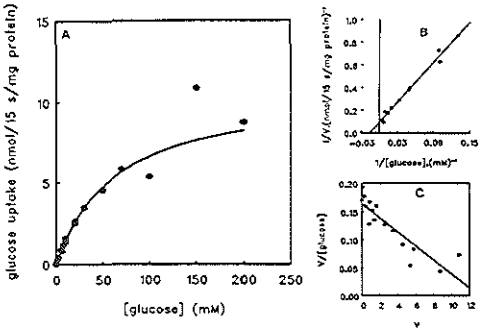


FIG. 2. **Kinetics of initial D-glucose uptake into lysosomal membrane vesicles at pH 7.4.** 10 $\mu$l of vesicles (90 $\mu$g of protein) in 170 mM NaHepes, pH 7.4, were incubated for 15 s at 20 °C with 20 $\mu$l of Hepes buffer containing 2 $\mu$Ci of [$^{14}$C]D-glucose and increasing amounts of unlabeled D-glucose. In order to compensate for the increasing medium osmolarity, the concentration of NaHepes was proportionally decreased. At the highest glucose concentration (200 mM), 10 mM NaHepes was present (isoosmotic with 170 mM NaHepes inside the vesicles). At the end of each incubation, the reaction was stopped with 70 $\mu$l of 170 mM NaHepes, and the membranes were eluted as described under "Materials and Methods." *A*, Michaelis-Menten analysis. *B*, Lineweaver-Burk transformation. *C*, Scatchard analysis plot.

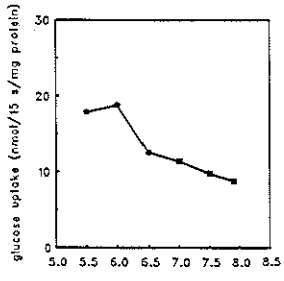


FIG. 3. **Effect of medium pH on the initial uptake rates of D-glucose in lysosomal membrane vesicles.** Lysosomal membrane vesicles, prepared in 20 mM NaHepes, pH 7.4, were 5-fold diluted in medium containing 25 mM MES or MOPS (final concentrations), titrated to the desired pH with NaOH, and 100 mM D-glucose plus 2 $\mu$Ci of [$^{14}$C]D-glucose. Although no compensation was introduced for the higher external osmolarity, this was maintained at comparable levels in all the experiments. Incubations were performed for 15 s at 20 °C and afterward, the vesicles were filtered and collected as described. ●——● = MES; ■——■ = MOPS.

fication, but in that case the effect of pH is essentially coupled to the presence of a proton gradient across the membrane (7). In order to find out whether this holds for the glucose carrier, we compared glucose uptake rates at acidic pH in the presence or absence of an inward-directed proton gradient ($pH_{in}$ 7.4 > $pH_{out}$ 5.5, or $pH_{in}$ = $pH_{out}$ = 5.5). The equilibration of the acidic pH across the membrane was achieved by adding MES-free acid to the vesicles and then shunting the pH gradient with the ionophore monensin, in the presence of sodium. As shown in Fig. 4, glucose initial uptake rates are about 2-fold stimulated at acidic pH, both in the absence and in the presence of an inward-directed proton gradient across the membrane, compared with conditions where $pH_{in}$ = $pH_{out}$ =

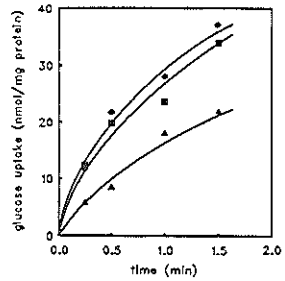


FIG. 4. **Time course of lysosomal vesicular uptake of 100 mM glucose at different pH values, either in the presence or absence of a proton gradient across the membrane.** Uptake at pH 7.4 in = out (▲——▲): vesicles were pre-equilibrated 30 min at 20 °C in 20 mM NaHepes, pH 7.4, and 50 mM NaCl. The assay was started by addition of 10 $\mu$l of membranes to 20 $\mu$l of 20 mM NaHepes pH 7.4, 150 mM D-glucose, and 2 $\mu$Ci of [$^{14}$C]D-glucose. Uptake at $pH_{in}$ 7.4 > $pH_{out}$ 5.5 (●——●): vesicles were equilibrated for 30 min at 20 °C like above. Transport was measured by adding 10 $\mu$l of membranes to 20 $\mu$l of 30 mM MES-free acid, 150 mM D-glucose, and 2 $\mu$Ci of [$^{14}$C]D-glucose. This addition titrated the extravesicular pH to 5.5. Uptake at pH 5.5 in = out (■——■): vesicles were pre-equilibrated in 10 mM NaHepes, 20 mM MES-free acid, pH 5.5, plus 0.01 mM monensin and 50 mM NaCl. At the start of the assay, 10 $\mu$l of membranes were added to 20 $\mu$l of 10 mM NaHepes, 20 mM MES-free acid, pH 5.5, plus 0.01 mM monensin, 150 mM D-glucose, and 2 $\mu$Ci of [$^{14}$C]D-glucose. Monensin was diluted from a concentrated stock in ethanol. In the absence of monensin, an equal amount of ethanol was added.

TABLE II

*Effect of inward- or outward-directed pH gradients across the lysosomal membrane on the uptake of 100 mM D-glucose*

Lysosomal membrane vesicles were washed and centrifuged at 50,000 × *g* with 8 mM NaMES, pH 5.5 (A and B) or NaHepes, pH 7.4 (C and D) plus 90 mM NaCl. Uptake was started by adding 10 $\mu$l of membranes to 40 $\mu$l of 75 mM NaHepes, pH 7.4 (A and D) or 75 mM NaMES, pH 5.5 (B and C) plus 125 mM D-glucose and 3 $\mu$Ci of [$^{14}$C]D-glucose. Incubations were performed for 30 s at 20 °C. Results are from quadruplicate determinations, plus standard deviations.

| | D-Glucose uptake |
|---|---|
| | *nmol/mg protein* |
| A. $pH_{in}$ 5.5 < $pH_{out}$ 7.4 | 18.8 ± 0.2 |
| B. $pH_{in}$ 5.5 = $pH_{out}$ 5.5 | 24.0 ± 0.2 |
| C. $pH_{in}$ 7.4 > $pH_{out}$ 5.5 | 24.1 ± 0.1 |
| D. $pH_{in}$ 7.4 = $pH_{out}$ 7.4 | 15.1 ± 0.05 |

7.4. Next we investigated whether the direction of the proton gradient (inward or outward directed) would differently influence glucose uptake. As shown in Table II, again highest uptake rates are observed at extravesicular acidic pH, independently of the presence of an inward-directed pH gradient. The effect of an acidic pH on the transport protein is not as efficient when the acidic pH is present at the opposite side of the membrane, compared with the substrate (outward-directed pH gradient). All these results exclude a cotransport mechanism linked to protons. Since the hepatocyte plasma membrane glucose carrier is apparently not sensitive to pH changes (22), our data also exclude that the observed uptake takes place into a contaminating plasma membrane compartment. Conversely, an acidic pH optimum is a typical hallmark for lysosomal proteins.

In order to investigate the impact of an acidic pH on the transport kinetics, the concentration-dependent uptake of glucose was followed at pH 5.5. The results of the experiments shown in Fig. 5 indicate that the uptake process at acidic pH

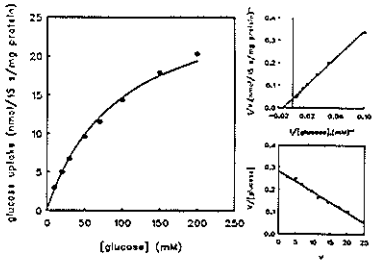


FIG. 5. **Kinetics of initial glucose transport at acidic pH.** Lysosomal membrane vesicles were pre-equilibrated in 20 mM Na-Hepes, pH 7.4, and 200 mM KCl by freeze-thawing, as described under "Materials and Methods." The assay was started by diluting 10 μl of membranes with 20 μl of 75 mM NaMES, pH 5.5 (final concentration 50 mM), 2 μCi of [$^{14}$C]D-glucose, and increasing concentrations of unlabeled D-glucose up to 200 mM. The increase of extravesicular osmolarity was compensated by decreasing the external KCl concentration. Incubations were performed for 15 s at 20 °C. *Right panels* respectively show Lineweaver-Burk (*above*) and Scatchard (*below*) transformations.

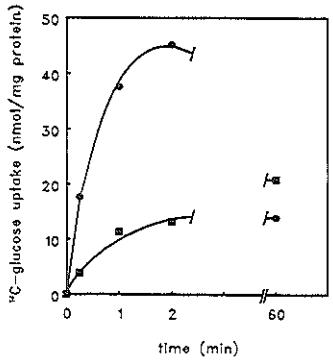


FIG. 6. **Transstimulation of [$^{14}$C]D-glucose uptake after preloading lysosomal membrane vesicles with unlabeled D-glucose.** 10 μl of vesicles in 20 mM NaHepes, pH 7.4, were preloaded with 100 mM D-glucose and the uptake of 2 μCi of [$^{14}$C]D-glucose was followed after diluting the sample 6.7 times exactly as described under "Materials and Methods" (●——●). Control experiments without preloading were performed in the presence of 15 mM unlabeled D-glucose and 2 μCi of [$^{14}$C]D-glucose, as similarly described (■——■). Uptake is expressed as [$^{14}$C]D-glucose radioactivity associated with the vesicles minus the blank.

is also saturable with an affinity constant of about 90 mM, very close to that observed at pH 7.4. Instead, the $V_{max}$ raises to circa 35 nmol/15 s/mg protein, which is significantly higher than that observed at pH 7.4. Separate experiments performed in the presence of monensin (at $pH_{in} = pH_{out} = 5.5$) produced very similar results, including the increase of $V_{max}$ (data not shown). These results reveal that the carrier increases the transport capacity at the optimal pH, without changes in the affinity for the substrate. Linearity of the Scatchard and Lineweaver-Burk plots suggest the operation of a single system, also under these assay conditions.

One of the properties of a carrier is the ability to transport the substrate in both directions and, consequently, to show under the proper conditions the phenomenon of transstimulation. When lysosomal vesicles were preloaded at glucose concentrations close to half saturation levels (100 mM), uptake of trace amounts of radiolabeled glucose (73 μM) was transstimulated by the outward-directed glucose gradient (total external glucose concentration 15 mM, Fig. 6). In these experiments we performed incubations at pH 7.4 to ensure linear uptake for longer incubation times. However, comparable levels of transstimulation were obtained when the experiments were performed at pH 5.5, with the only difference that the peak of overshoot was exhausted earlier, at 30 s of incubation instead of 2 min (data not shown).

Once we established these properties, we investigated the sensitivity of glucose uptake and transstimulation to known inhibitors of plasma membrane glucose carriers. As presented in Table III, cytochalasin B is a good *cis*-inhibitor and reduces glucose transstimulation, but this effect is only evident at concentrations much higher than those known to inhibit the hepatocyte plasma membrane carrier (1.3 μM) (22). Phloretin was found to be a stronger inhibitor when used at the same concentrations as cytochalasin B, in either *cis*-inhibition or transstimulation experiments. In the latter case the addition of 100 μM phloretin resulted in total abolition of the transstimulation effect (Table III). This inhibition effect seems to be more pronounced than that reported for the hepatocyte plasma membrane glucose carrier (22).

The transstimulation properties of the lysosomal glucose carrier were then used to investigate the substrate specificity of this transport system, assuming that all the solutes recognized and transported by the carrier with a similar affinity should be able to produce an equivalent stimulation. In Fig. 7

TABLE III

*Effect of cytochalasin B and phloretin on lysosomal glucose uptake and transstimulation*

A, uptake studies. 10 μl of vesicles in 20 mM NaHepes, pH 7.4, were preincubated 5 min at 20 °C with cytochalasin B or phloretin. Uptake of 0.1 mM D-glucose (2 μCi of [$^{14}$C]D-glucose) was later followed in the presence of the same concentration of inhibitor for 15 s at 20°C. Phloretin and cytochalasin B were added from concentrated ethanol solutions. The same amount of ethanol was added in the control experiments. Results are average of triplicate determinations. Control uptake (100%): 0.049 ± 0.001 nmol/mg protein. B, transstimulation. Lysosomal membrane vesicles (10 μl) were preloaded with 100 mM unlabeled D-glucose similarly to the experiment of Fig. 6 except that in the last 5 min cytochalasin B or phloretin were added. Uptake was studied after diluting the sample 6.7 times in buffer containing 20 mM NaHepes, pH 7.4, plus 2 μCi of [$^{14}$C]D-glucose and cytochalasin B or phloretin, and was followed for 2 min at 20 °C. Results of triplicate or quadruplicate experiments performed with two different batches of vesicles are reported. Control uptake without preloading (100%): 10.8 ± 0.3 nmol/mg protein.

| | % of uninhibited rate |
|---|---|
| A. *Cis*-Inhibition | |
| No addition | 100 |
| Cytochalasin B | |
| 30 μM | 62 |
| 200 μM | 37 |
| Phloretin | |
| 30 μM | 79 |
| 200 μM | 21 |
| | **% of glucose uptake** |
| B. Transstimulation | |
| Without preloading | 100 |
| After preloading | 280 |
| Preloading + cytochalasin B | |
| 10 μM | 254 |
| 100 μM | 154 |
| Preloading + phloretin | |
| 10 μM | 230 |
| 100 μM | 94 |

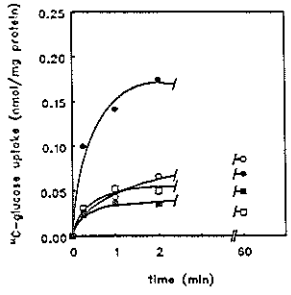


FIG. 7. **Transstimulation of [$^{14}$C]D-glucose uptake in lysosomal membrane vesicles after preloading with 100 mM of the unlabeled monosaccharides D-galactose and sialic acid.** Experimental conditions were similar to those indicated in Fig. 6. *Filled symbols*, experiments after preloading with 100 mM unlabeled sialic acid (■——■) or D-galactose (●——●). *Open symbols*, experiments without preloading but where 15 mM unlabeled sialic acid (□——□) or D-galactose (○——○) was added outside the vesicles to reach equal concentrations of external substrate.

it is shown that, for example, D-galactose produces a pattern of transstimulation similar to that observed when vesicles are preloaded with D-glucose (see Fig. 6). On the contrary, sugars which are not recognized by the carrier should not produce significant increase of [$^{14}$C]D-glucose uptake when they are accumulated at the *trans*-side of the membrane. In agreement with this assumption, we observed no transstimulation of [$^{14}$C]D-glucose transport when we preloaded the lysosomal membrane vesicles with high concentrations of *N*-acetylneuraminic acid (sialic acid) (Fig. 7). This demonstrates that sialic acid, a strongly acidic monosaccharide, is not transported by the glucose carrier and also that this latter carrier is distinct from the one specific for sialic acid and other acidic monosaccharides. Reciprocally, previous experiments showed that glucose is not a substrate for the sialic acid carrier (7).

The experiments illustrated in Figs. 6 and 7 show a peak of transstimulation at 2 min of incubation, under the conditions used. For screening of a wider group of sugars, we therefore studied the ability to produce transstimulation at this time point. As presented in Table IV, other neutral hexoses, namely D-mannose, D-galactose, and D- and L-fucose produced levels of transstimulation very close to those found for D-glucose uptake, and are therefore likely to be transported by the carrier with a similar affinity. Comparable levels of countertransport were achieved when, reciprocally, the uptake of 72 μM labeled D-galactose, D-mannose, or L-fucose was followed after preloading with 100 mM unlabeled D-glucose (data not shown). Interestingly, all these sugars (including D-fucose) are produced in the lysosome by the activity of specific acid hydrolases and are supposed to leave the lysosome without further catabolism (23). A number of other sugars were not recognized by the carrier under these conditions (Table IV). Among these, it is important to note that *N*-acetylglucosamine, which possesses a specific lysosomal carrier, is not recognized by the glucose transporter, even though it is a neutral monosaccharide. Other sugars not recognized by the glucose carrier include the L- stereoisomers L-glucose, L-galactose, and L-rhamnose (not represented in animal tissues), the acidic monosaccharides sialic acid, glucuronic acid, and gluconic acid and lactones of acidic monosaccharides (D-mannuronolactone, D-galactono-γ-lactone and L-ascorbic acid). Among the aminosugars, 6-amino-6-deoxy-D-glucose also gave a negative result, but a modest increase of uptake was observed with D-glucosamine.

TABLE IV

*Transstimulation of [$^{14}$C] D-glucose uptake by different monosaccharides*

Lysosomal membrane vesicles (10 μl) were preloaded with 100 mM of the indicated sugar and afterward the sample was 6.7 times diluted and incubated for 2 min at 20 °C in the presence of 2 μCi of [$^{14}$C]D-glucose. In the cases where no preloading was performed, 15 mM unlabeled sugar was added at the start of the transport assay, as already described. Solutions of the acidic sugars and the aminosugars were titrated to pH 7.4 by addition of an appropriate amount of NaOH or HCl. Uptake rates are represented as percentage of control [$^{14}$C]D-glucose uptake obtained without preloading with unlabeled sugar (average value 12 ± 2.6 nmol/mg protein, $n$ = 7). Results are from duplicate experiments performed with at least two different vesicle preparations.

| | [$^{14}$C]D-glucose uptake | |
|---|---|---|
| | After preloading | Without preloading |
| | % | |
| D-Glucose | 290 | 100 |
| D-Galactose | 302 | 114 |
| D-Mannose | 255 | 84 |
| D-Fucose | 231 | 101 |
| L-Fucose | 190 | 80 |
| D-Fructose | 173 | 100 |
| L-Glucose | 118 | 90 |
| L-Galactose | 114 | 79 |
| L-Rhamnose | 116 | 98 |
| D-Ribose | 132 | 95 |
| D-Arabinose | 100 | 84 |
| D-Glucuronic acid | 75 | 109 |
| D-Gluconic acid | 81 | 107 |
| N-Acetylneuraminic acid | 61 | 86 |
| N-Acetylglucosamine | 70 | 101 |
| D-Glucosamine | 141 | 96 |
| 6-Amino-6-deoxyglucose | 109 | 102 |
| D-Mannuronolactone | 105 | 100 |
| D-Galactonolactone | 101 | 101 |
| L-Ascorbic acid | 73 | 100 |

We further investigated this aspect by studying under identical conditions the uptake of [$^{14}$C]D-glucosamine after preloading the vesicles with either D-glucose or D-glucosamine. The latter compound is known to be taken up by lysosomal membrane vesicles (7, 21). While preloading with 100 mM, D-glucosamine did not produce any countertransport, a 50% increase over the basal [$^{14}$C]D-glucosamine uptake was seen after preloading with 100 mM D-glucose. This suggests that, although the glucose carrier is able to recognize glucosamine, its affinity for this compound must be much lower than that for D-glucose, since 100 mM unlabeled glucosamine could not transstimulate [$^{14}$C]D-glucosamine transport. A similar explanation may apply for D-ribose which showed a low level of transstimulation, although this effect does not seem to be shared by other pentoses (compare D-arabinose). Evidently, the affinity of the glucose carrier for D-ribose must be even lower than for D-glucosamine, which makes a possible physiological significance of such recognition rather questionable.

As reported in Table IV, the keto-hexose D-fructose showed intermediate levels of transstimulation, which were significantly higher than those obtained without preloading, but always lower than obtained by loading of aldo-hexoses.

We compared transport kinetics of the major substrates by studying the concentration-dependent uptake of [$^{14}$C]D-galactose, [$^{14}$C]D-mannose, and [$^{3}$H]L-fucose. In all cases the processes were saturable and the $K_t$ averaged about 50–75 mM, *i.e.* very close to the values observed for [$^{14}$C]D-glucose (Fig. 8). The processes linearized when plotted according to the Lineweaver-Burk and the Scatchard transformations. From our results, we conclude that all these hexoses share a common

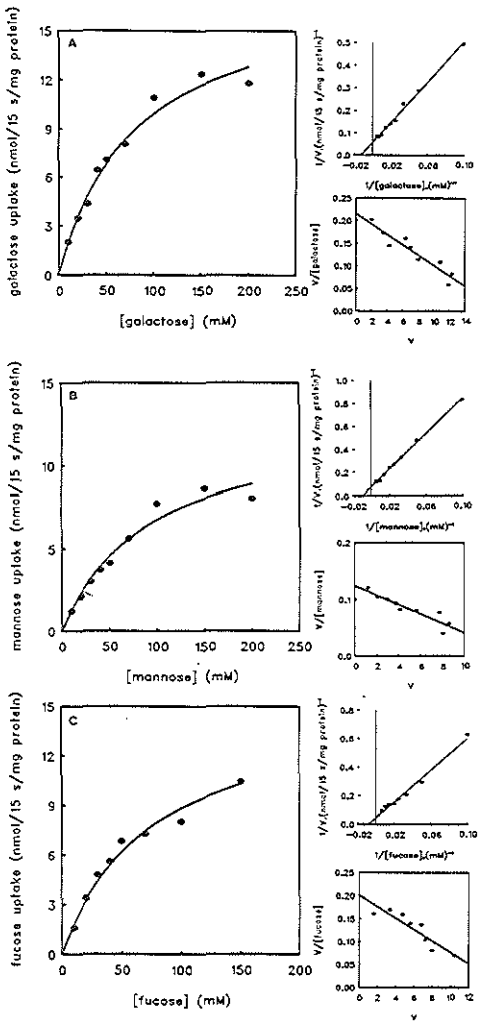


FIG. 8. **Kinetics of lysosomal vesicle transport of D-galactose, D-mannose, and L-fucose.** Experimental conditions were similar to those of the experiments in Fig. 5 at pH 5.5. *A*, uptake of [$^{14}$C]D-galactose in the presence of increasing amounts of unlabeled D-galactose. *B*, uptake of [$^{14}$C]D-mannose plus increasing amounts of unlabeled D-mannose. *C*, uptake of [$^{3}$H]L-fucose plus increasing amounts of unlabeled L-fucose. *Right panels* respectively show Lineweaver-Burk transformations (*above*) and Scatchard plots (*below*).

carrier with D-glucose in the lysosomal membrane and that in our system there are no other major mechanisms responsible for their transfer across the membrane.

## DISCUSSION

Early studies performed by Lloyd sustained that the majority of neutral monosaccharides, including glucose, could cross the lysosomal membrane by simple diffusion through the lipid bilayer (15). In contrast, using the osmotic protection method, but also studying uptake of radiolabeled glucose and ribose into rat liver tritosomes, Docherty *et al.* (9) and Maguire *et al.* (10) suggested the existence of a common lysosomal carrier for hexoses and pentoses. But in both cases, due to technical problems inherent to the methodologies, the contribution of diffusion components to the measured uptake could not be excluded (16).

Recently, by use of the same techniques, evidence has been provided against the existence of a common carrier for D-glucose and D-ribose (11), and again the possibility of simple diffusion, predicted on the basis of hydrogen bonding capacity, has been proposed as the main mechanism of glucose transfer across the lysosomal membrane (13).

Purified membrane vesicles have been increasingly used in the last decade as the most appropriate method for accurate and direct investigations of solute transport kinetics in biological membranes. We have recently developed an assay to study the transport properties of resealed lysosomal membrane vesicles (7).

In the study presented here, we have demonstrated the existence of a carrier in the lysosomal membrane, which facilitates the diffusion of neutral monosaccharides, in agreement with the previous suggestions by Maguire *et al.* (10) and by Jadot *et al.* (24). We found that transport is inhibited by cytochalasin B, similarly to what was observed by Maguire *et al.* (10), but phloretin is found to be a more powerful inhibitor.

This transport system possesses characteristics which differentiate it clearly from glucose carriers in mammalian plasma membranes, particularly that of rat liver hepatocytes (22, 25, 26).

The lysosomal glucose transporter showed an acidic pH optimum which accounted for an increase of the maximum transport capacity, without changes in the affinity for the substrate. Our data argue against the possibility that the carrier is involved in a mechanism of cotransport with protons. Presumably, the effect of the pH is played at the level of ionization of the carrier protein.

This pH optimum seems to be unique to the lysosomal system, since it has never been observed for other types of mammalian glucose carriers. Particularly, this carrier is distinct from the more widely represented $Na^+$-independent glucose carriers in the erythrocyte, hepatocyte, adipocyte, fibroblast, and skeletal muscle plasma and microsomal membranes which mediate facilitated diffusion of monosaccharides at neutral pH (27, 28).

The pH sensitivity of the lysosomal glucose transport system also differs from that observed for other well-characterized lysosomal sugar carriers (7, 8). In the case of the carrier for sialic acid, an extravesicular acidic pH provides the driving force for $H^+$/sialic acid cotransport, implying that the pH effect is strictly coupled to the presence of a proton gradient across the membrane (7). Here we show that such a model is not applicable to the lysosomal glucose carrier. Furthermore, the lysosomal carrier for *N*-Acetylhexosamines, recently characterized by Jonas *et al.* (8), was apparently insensitive to changes of the extralysosomal pH, and in this respect differs clearly from the glucose carrier.

The transport kinetics appeared to adapt well to the Michaelis-Menten equation and to the Lineweaver-Burk and Scatchard analysis and did not demonstrate any contribution of a nonsaturable component. This supports the idea that, in lysosomal membrane vesicles from rat liver, the rate of nonfacilitated diffusion of glucose and other monohexoses must

be very low in comparison with the transport rate supported by the glucose carrier.

This system also demonstrates transstimulation properties, which have provided a direct and sensitive method to investigate substrate specificity. Using this approach, the main substrates were found to be D-glucose, D-galactose, D-mannose, D- and L-fucose. All these sugars are products of intralysosomal catabolism, including D-fucose which, though xenobiotic, can be recognized by a lysosomal hydrolase biochemically similar to $\beta$-galactosidase (23). These sugars produce reciprocal transstimulation and display a similar affinity for the carrier, as judged from their $K_t$.

This carrier exhibits clear stereospecificity and in general L-hexoses are not transported. L-Fucose represents an exception, but this sugar is a component of oligosaccharide chains of mammalian glycoproteins, and it is normally present in the lysosome, due to the hydrolytic activity of acid $\alpha$-L-fucosidase. Its structural similarity to D-galactose probably makes recognition possible (23).

The substrate specificity studies also permitted us to gain additional information about other structural properties that play a role in substrate recognition by the carrier. For example, the presence of charges in the hexose molecule (aminosugars, carboxylic monosaccharides, and their lactones) decrease substrate recognition, whereas the presence of an hemiacetal bond (absent in fructose, gluconic acid, ascorbic acid, and other lactones of acidic monosaccharides) facilitates recognition. Similarly, the glucose carrier in placental brush border membrane shows some preference for hemiacetal-forming sugars and has a lower affinity for the ketohexose D-fructose (29).

Since our data exclude that ribose and pentoses in general are recognized by the glucose transporter, they agree with the conclusions of Bird *et al.* (11, 12), who argue against the existence of a lysosomal carrier common to hexoses and pentoses. Moreover, we have to consider that extralysosomal degradation of nucleosides by nucleosidases, the major intracellular source of pentoses, produces pentose-phosphates. In the lysosome, nucleosides are not further catabolized and escape via a specific carrier (30). This might imply that free pentoses do not require a lysosomal transport system. On the other hand, it has been suggested that, on the basis of their size and hydrogen bonding capacity, pentoses might diffuse across the lysosomal membrane unassisted by any carrier (1, 12). Clearly, studies with radiolabeled pentoses should be carried out to demonstrate the presence or absence of a separate pentose carrier.

The lysosomal glucose carrier exhibits a low substrate affinity, compared with the other known lysosomal carriers (1, 7, 8, 19, 30) and with the physiological intra- and extracellular glucose concentration, which is about 4–10 mM. These properties apparently do not support a model in which the lysosomal transport of monosaccharides is a rate-limiting step in the metabolism of lysosome-derived sugars, preventing under physiological conditions glucose accumulation in the lysosome. Nevertheless, a hypothetical genetic defect of this transport system might produce accumulation of neutral monosaccharides in the lysosome. It has been recently hypothesized that in some human forms of lysosomal glycogen storage disease without acid maltase deficiency (31–33), glucose release from the lysosome might be abnormal (33). In these cases, therefore, intralysosomal glycogen accumulation could be due to secondary inhibition of acid maltase by hexoses, accumulated because of a lysosomal transport defect.

Recently, a great deal of work has been carried out to demonstrate the central role of glucose transporters in the glycemic homeostasis, as a final target of insulin action on sensitive tissues like the adipose and muscular tissue (28, 34–38). One of the major insulin effects is to recruit glucose carriers to the plasma membrane from an intracellular microsomal pool (34, 35). Studies on the subcellular localization of the transporters have recently shown that immunologically distinct glucose carriers (either insulin-sensitive or not) can coexist in different intracellular compartments of insulin-responsive cell types (39). The characterization of a biochemically distinct lysosomal type of glucose transporter stimulates further investigations aimed at understanding whether a relationship exists with the other members of the family of glucose carriers, and whether the lysosomal membrane plays a role in the intracellular routing of these transport molecules.

*Acknowledgments*—We are thankful to Prof. H. Galjaard and Dr. Hugo R. de Jonge for advice and support in the course of our work and for critically reading the manuscript, and to Prof. Robert Wattiaux for fruitful discussions. We are indebted to Joop Fengler for printing the figures and to Jeannette Lokker for secretarial assistance.

REFERENCES

1. Forster, S., and Lloyd, J. B. (1988) *Biochim. Biophys. Acta* **947**, 465–491
2. Gahl, W. A., Bashan, N., Tietze, F., Bernardini, I., and Schulman, J. D. (1982) *Science* **217**, 1263–1265
3. Rosenblatt, D. S., Hosack, A., Matiaszuk, N. V., Cooper, B. A., and Laframboise, R. (1985) *Science* **228**, 1319–1321
4. Mancini, G. M. S., Verheijen, F. W., and Galjaard, H. (1986) *Hum. Genet.* **73**, 24–217
5. Renlund, M., Tietze, F., and Gahl, W. A. (1986) *Science* **232**, 759–762
6. Gahl, W. A., Renlund, M., and Thoene, J. G. (1989) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., and Valle, D. eds) VI ed, McGraw-Hill New York
7. Mancini, G. M. S., de Jonge, H. R., Galjaard, H., and Verheijen, F. W. (1989) *J. Biol. Chem.* **264**, 15247–15254
8. Jonas, A. J., Speller, R. J., Conrad, P. B., and Dubinsky, W. P. (1989) *J. Biol. Chem.* **264**, 4953–4956
9. Docherty, K., Brenchley, G. V., and Hales, C. N. (1979) *Biochem. J.* **178**, 361–366
10. Maguire, G. A., Docherty, K., and Hales, C. N. (1983) *Biochem. J.* **212**, 211–218
11. Bird, S. J., Forster, S., and Lloyd, J. B. (1987) *Biochem. J.* **245**, 929–931
12. Bird, S. J., Forster, S., and Lloyd, J. B. (1988) *Biochem. J.* **252**, 623
13. Iveson, G. P., Bird, S. J., and Lloyd, J. B. (1989) *Biochem. J.* **261**, 451–456
14. Maguire, G. A., Kay, J. D. S., and Hales, C. N. (1988) *Biochem. J.* **252**, 621–623
15. Lloyd, J. B. (1969) *Biochem. J.* **115**, 703–707
16. Hales, C. N., Docherty, K., and Maguire, G. A. (1982) *Biochem. Soc. Trans.* **10**, 14–15
17. Pisoni, R. L., Thoene, J. G., and Christensen, H. N. (1985) *J. Biol. Chem.* **260**, 4791–4798
18. Pisoni, R. L., Thoene, J. G., Lemons, R. M., and Christensen, H. N. (1987) *J. Biol. Chem.* **262**, 15011–15018
19. Pisoni, R. L., Flickinger, K. S., Thoene J. G., and Christensen, H. N. (1987) *J. Biol. Chem.* **262**, 6010–6017
20. Ohsumi, Y., Ishikawa, T., and Kato, K. (1983) *J. Biochem. (Tokyo)* **93**, 547–556
21. Bame, K. J., and Rome, L. H. (1985) *J. Biol. Chem.* **260**, 11293–11299
22. Baur, H., and Heldt, H. W. (1977) *Eur. J. Biochem.* **74**, 397–403
23. Barrett, A. J. (1972) in *Lysosomes, A Laboratory Handbook* (Dingle, J. T., ed) pp. 72–87, Elsevier Scientific Publishing Co., Amsterdam
24. Jadot, M., Wattiaux-De Coninck, S., and Wattiaux, R. (1989) *Biochem. J.* **262**, 981–984
25. Craik, J. D., and Elliott, K. R. F. (1980) *Biochem. J.* **192**, 373–375
26. Elliott, K. R. F., and Craik, J. D. (1982) *Biochem. Soc. Trans.* **10**, 12–13
27. Elbrink, J., and Bihler, I. (1975) *Science* **188**, 1177–1184

28. Kono, T. (1988) *Vitamins Hormones* **44**, 103–154
29. Johnson, L. W., and Smith, C. H. (1980) *Am. J. Physiol.* **238**, C160–C168
30. Pisoni, R. L., and Thoene, J. G. (1989) *J. Biol. Chem.* **264**, 4850–4856
31. Danon, M. J., Shin, J. D., DiMauro, S., Manaligod, J. R., Eastwood, A., Naidu, S., and Schliselfeld, L. H. (1981) *Neurology* **31**, 51–57
32. Byrne, E., Dennet, X., Crotty, B., Trounce, I., Sands, J. M., Hawkins, R., Hammond, J., Anderson, S., Haan, E. A., and Pollard, A. (1986) *Brain* **109**, 523–536
33. Ullrich, K., von Bassewitz, D., Shin, J., Korinthenberg, R., Sewell, S., and von Figura, K. (1989) *Prog. Clin. Biol. Res.* **306**, 163–171
34. Suzuki, K., and Kono, T. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 2542–2545
35. Cushman, S. W., and Wardzala, L. J. (1980) *J. Biol. Chem.* **255**, 4758–4762
36. Simpson, I. A., and Cushman, S. W. (1986) *Annu. Rev. Biochem.* **55**, 1059–1089
37. Garvey, W. T., Hueckstead, T. P., and Birnaum, M. J. (1989) *Science* **245**, 60–63
38. Kahn, C. R., and Goldstein, B. J. (1989) *Science* **245**, 13
39. Zorzano, A., Wilkinson, W., Kotliar, N., Thoidis, G., Wadzinski, B. E., Ruoho, A. E., and Pilch, P. F. (1989) *J. Biol. Chem.* **264**, 12358–12363

**Publication V**

Sialic acid storage diseases.
A multiple lysosomal transport defect for acidic monosaccharides.
*J. Clin. Invest.* 87:1329-1335 (1991)

# Sialic Acid Storage Diseases

## A Multiple Lysosomal Transport Defect for Acidic Monosaccharides

Grazia M. S. Mancini,* Cecile E. M. T. Beerens,* Pertti P. Aula,‡ and Frans W. Verheijen*
*Department of Cell Biology and Genetics, Erasmus University, 3000 DR Rotterdam, The Netherlands; and
‡Department of Medical Genetics, University of Turku, Turku, Finland

### Abstract

A defective efflux of free sialic acid from the lysosomal compartment has been found in the clinically heterogeneous group of sialic acid storage disorders. Using radiolabeled sialic acid (NeuAc) as a substrate, we have recently detected and characterized a proton-driven carrier for sialic acid in the lysosomal membrane from rat liver. This carrier also recognizes and transports other acidic monosaccharides, among which are uronic acids. If no alternative routes of glucuronic acid transport exist, the disposal of uronic acids can be affected in the sialic acid storage disorders. In this study we excluded the existence of more than one acidic monosaccharide carrier by measuring uptake kinetics of labeled glucuronic acid ([$^3$H]GlcAc) in rat lysosomal membrane vesicles. [$^3$H]GlcAc uptake was carrier-mediated with an affinity constant of transport ($K_t$) of 0.3 mM and the transport could be *cis*-inhibited or *trans*-stimulated to the same extent by sialic acid or glucuronic acid. Human lysosomal membrane vesicles isolated from cultured fibroblasts showed the existence of a similar proton-driven transporter with the same properties as the rat liver system ($K_t$ of [$^3$H]GlcAc uptake 0.28 mM). Uptake studies with [$^3$H]NeuAc and [$^3$H]GlcAc in resealed lysosome membrane vesicles from cultured fibroblasts of patients with different clinical presentation of sialic acid storage showed defective carrier-mediated transport for both sugars. Further evidence that the defective transport of acidic sugars represents the primary genetic defect in sialic acid storage diseases was provided by the observation of reduced, half-normal transport rates in lymphoblast-derived lysosomal membrane vesicles from five unrelated obligate heterozygotes. This study reports the first observation of a human lysosomal transport defect for multiple physiological compounds. (*J. Clin. Invest.* 1991. 87:1329–1335.) Key words: glucuronic acid • carrier • proton cotransport • heterozygotes • Salla disease

### Introduction

Sialic acid storage diseases represent a clinically heterogeneous group of inherited disorders, characterized by abnormal accumulation of free (unbound) sialic acid in urine and in lysosomes of different tissues. They must be distinguished from other forms of sialuria without lysosomal storage (1). The two main phenotypes of lysosomal sialic acid storage are the "Finnish" Salla disease, presenting with mental retardation of early onset, ataxia, and near-normal life span (2, 3), and an infantile form without any ethnical prevalence, presenting severe visceral involvement, dysostosis multiplex, psychomotor retardation, and early death (4–10). In addition, some patients have been described with intermediate phenotypes between these two extremes (11–15). Impaired efflux of sialic acid (*N*-acetylneuraminic acid, NeuAc)[1] from lysosome-rich subcellular fractions of fibroblasts from patients with all the different clinical forms of the disease has suggested a defect of a putative sialic acid transport system (16–20). Recently, we have detected and characterized a proton-driven carrier specific for sialic acid and many other acidic monosaccharides, including glucuronic acid, in rat liver lysosomal membrane vesicles (21). Glucuronic acid is normally present in the lysosomes as a degradation product of glycosaminoglycans. Sialic acid storage diseases therefore may represent a genetic transport defect of a carrier with wide substrate specificity. Consequently, storage of different compounds may be involved in the pathogenesis of the disorder, unless it is demonstrated that more than one carrier exists for the lysosomal disposal of different acidic sugars. Biochemically distinct lysosomal carriers with partially overlapping substrate specificity have been described for amino acids (22, 23). Evaluation of the number and properties of the human lysosomal acidic monosaccharide transport mechanisms is mandatory for understanding the metabolic defect in sialic acid storage diseases. In this study, we have further investigated the transport kinetics of D-glucuronic acid (GlcAc) and NeuAc in rat liver lysosomal membranes and extended the studies to resealed lysosomal vesicles from cultured human fibroblasts and lymphoblasts. Transport activity was also determined in cell lines from patients and obligate heterozygotes of various types of sialic acid storage disorders. The results provide strong evidence that the impaired transport mechanism for different acidic monosaccharides is the primary genetic defect of these diseases.

### Methods

*Cell cultures.* Fibroblasts were grown in Ham's F10 medium supplemented with 10% FCS and antibiotics. Fibroblast and lymphoblast cell lines from patients with Salla disease and their relatives were provided by one of the authors (P. Aula). Fibroblast cell line 9015 is from a fetal skin biopsy of an interrupted pregnancy after a positive prenatal diagnosis for Salla disease (Renlund, Dr. M., personal communication).

This work has been reported in part at the Fifth International Congress on Inborn Errors of Metabolism, 1–5 June 1990, in Pacific Grove, CA.

Address reprint requests to Dr. Grazia M. S. Mancini, Department of Cell Biology and Genetics, Erasmus University, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands.

*Received for publication 27 August 1990 and in revised form 14 November 1990.*

J. Clin. Invest.

0021-9738/91/04/1329/07 $2.00
Volume 87, April 1991, 1329–1335

1. *Abbreviations used in this paper:* GlcAc, D-glucuronic acid; $K_t$, affinity constant of transport; MES, 2-(*N*-morpholino)ethanesulfonic acid; NeuAc, sialic acid (*N*-acetylneuraminic acid).

Cells from patient A. Z. (5) are from Dr. E. Vamos, Dept. of Medical Genetics, University of Brussels; cells of patient E. P. (14) are from Prof. A. Fois, Dept. of Pediatrics, University of Siena; cell line D. R. is from a patient observed by Prof. F. van Hoof, I. C. P. University of Louvain; and cell line E. B. is from a patient observed by Dr. F. G. I. Jennekens, Dept. of Neurology, University of Utrecht (manuscript in preparation). Lymphoblast strains were established by EBV infection of peripheral blood lymphocytes and grown in $CO_2$ atmosphere with RPMI medium supplemented with 20% FCS and glutamine. The transformation procedure does not seem to interfere with later isolation of lysosomes and integrity of the lysosomal membrane (24).

*Lysosomal membrane vesicles.* Rat liver was used as a source of lysosomal membrane vesicles as described earlier (21). Proton-driven sialic acid uptake was only observed in fractions highly enriched for lysosomal membrane markers and not in less pure fractions obtained during the purification procedure. For fibroblast lysosomal membrane vesicles, cells from 12 to 15 850-$cm^2$ roller bottles were harvested by trypsinization and homogenized with 30 strokes of a Potter Elvejem glass-Teflon type of homogenizer in 0.25 M sucrose, 0.2 M KCl, 10 mM NaHepes pH 7.4 plus a cocktail of protease inhibitors as used for rat liver isolations. Differential centrifugation steps (650 *g* and 11,000 *g*) produced a lysosomal/mitochondrial pellet representing about 20% of the lysosomes in the total homogenate, 80% of which were intact, as judged by the distribution and latency of the soluble lysosomal marker enzyme β-hexosaminidase. No difference in lysosome recovery was observed between preparations from controls and patients. Afterwards, lysosomal membrane vesicles were prepared by selective hypotonic shock of this pellet according to the procedure of Ohsumi et al. (25) applied to human fibroblasts (26). Because of the hypotonic shock (2 × 30 min at 0°C in 0.02 M sucrose) and the following extensive washings, lysosomes lost their content and patient membranes obviously lost their storage products. In the final preparation, the specific activity of the lysosomal membrane marker enzyme β-glucocerebrosidase was comparable to the rat liver lysosomal membrane vesicles (about 2 μmol/h per milligram protein). The total enrichment of this marker was lower for fibroblast than for rat liver membranes (20-fold against 90-fold), the difference being caused by the high specific enzyme activity in the original fibroblast homogenate compared with the total liver tissue homogenate. The final recovery of membrane markers in fibroblast preparations was comparable to rat liver (4–8%). No difference in purity and recovery was observed between patient and control preparations, but usually better yields were obtained from fetal fibroblasts (cell lines 9015, 53, 698). Lysosomal membranes were also prepared from 1–2 g (wet wt) transformed lymphoblasts following the same procedure used for fibroblasts. Although the specific activity of β-glucocerebrosidase in lymphoblasts is much lower than in fibroblasts (~ 5%), the enrichment of this marker in the final preparation was similar to fibroblasts. The membrane vesicles were suspended in 20 mM NaHepes pH 7.4 plus 0.01 mM EDTA at a protein concentration of 3–5 mg/ml for both fibroblast and lymphoblast preparations and were kept frozen at −70°C before use. Frozen lysosomal membranes from any source retained normal transport activity for at least 4 mo. Each preparation was sufficient to perform duplicate determinations of marker enzymes, of GlcAc/NeuAc transport and glucose *trans*-stimulation. For each cell line transport parameters were assayed in at least two separate membrane preparations.

*Transport studies.* Uptake of the acidic monosaccharides GlcAc and NeuAc was assayed in the presence and absence of an inward directed proton gradient ($pH_{out}$ 5.5 < $pH_{in}$ 7.4, or $pH_{in}$ = $pH_{out}$ = 7.4) generated with impermeant buffers as described (21), except that for fibroblast and lymphoblast lysosomal membranes about 20–40 μg protein was used per each assay and incubations were performed at 37°C. Briefly, 20-μl vesicles, prepared in 20 mM Na- or K-Hepes buffer pH 7.4 plus 0.01 mM valinomycin, were incubated with 10 μl radiolabeled sugar plus 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) free acid, to give a final concentration of 33 mM MES, 13 mM Hepes, and an extravesicular pH of 5.5. Reactions were stopped by diluting the sample with 70 μl ice-cold incubation buffer, and vesicles were filtered and eluted with 1 ml buffer on a small Sephadex G50 (fine) column (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) run at 0.3 ml/min at 4°C as we described earlier. Vesicle-associated radioactivity was counted by liquid scintillation. Uptake at $pH_{in}$ = $pH_{out}$ = 7.4 (no proton gradient) was assayed in 20 mM Na- or K-Hepes pH 7.4 present inside and outside the vesicles. *Trans*-stimulation of [$^3$H]GlcAc uptake was studied at $pH_{in}$ = $pH_{out}$ = 5.5 in the presence of the ionophore monensin, as previously described (21). *Trans*-stimulation of [$^3$H]D-glucose uptake was followed at pH 5.5 after preloading the vesicles with 100 mM unlabeled D-glucose, essentially as described for the rat liver system (27), except that the assay was performed at 37°C using about 30 μg membrane protein. Details for different assay conditions are indicated in the figure legends. Experiments were performed under controlled osmotic conditions and uptake of NeuAc and GlcAc was usually done in the presence of equimolar intra- and extravesicular $K^+$ and 0.01 mM valinomycin, to eliminate membrane potentials that could artificially affect movements of the charged sugars. As blanks, vesicles exposed to ice-cold buffer containing radiolabeled substrate were filtered, without incubation, through a Sephadex column. This blank was subtracted from all the determinations (about 200 dpm). Each assay was performed as duplicate or triplicate determination and the accepted variation from the mean was 10%. Kinetic parameters, obtained from the experimental data, were calculated using the computer program Enzfitter, from Elsevier-Biosoft, Cambridge, UK.

*Miscellaneous.* Activities of β-hexosaminidase and latency measurements were performed as described (16). β-glucocerebrosidase activity in the presence of Triton X-100 and taurocholate was assayed with a synthetic substrate at pH 5.3 as described (28). Radiolabeled D[1-$^3$H]GlcAc (sp act 1.8 Ci/mmol) and D[2-$^3$H]glucose (sp act 23 Ci/mmol) were prepared by Amersham International, Amersham, UK, and [9-$^3$H]NeuAc (sp act 10 Ci/mmol) was purchased from American Radiolabeled Chemicals, St. Louis, MO. Osmolarity measurements were done with a freezing point-depression osmometer (Advanced Instruments, Inc., Needham Heights, MA). The unlabeled sugars NeuAc, GlcAc, and D-glucose, the ionophore monensin, the buffers Hepes and MES were obtained from Sigma Chemical Co., St. Louis, MO. Valinomycin was from Boehringer Mannheim GmbH, Mannheim, Germany.

## Results

*Glucuronic acid transport in rat liver lysosomal membranes.* In previous studies we demonstrated that, in the rat liver, the lysosomal carrier for sialic acid also recognizes and transports glucuronic acid (21). However, using radiolabeled NeuAc as a substrate, we could not exclude the existence of alternative routes of transport for uronic acids. We therefore investigated the kinetics of [$^3$H]GlcAc transport in rat liver membranes in the presence and absence of an inward-directed proton gradient, by manipulating the intra- and extravesicular pH with impermeant buffers. As shown in Fig. 1, similarly to what we observed for sialic acid, initial uptake rates for glucuronic acid were strongly stimulated above equilibrium level (overshoot) in the presence of the proton gradient ($pH_{out}$ 5.5 < $pH_{in}$ 7.4), which is the driving force of this cotransport mechanism. Saturability of the process at increasing substrate concentrations, typical of carrier-mediated transport, was observed. The affinity constant of transport ($K_t$) of this process, calculated by a Michaelis-Menten equation, was 0.3 mM ($n$ = 2), close to that previously measured for sialic acid (0.24 mM). When the data from a representative experiment were applied to the Lineweaver-Burk and Scatchard analysis, the process linearized (Fig. 2). This indicates that, in this concentration range, only one mechanism of transport is responsible for the uptake. To confirm this observation, we could demonstrate that the *cis*-in-

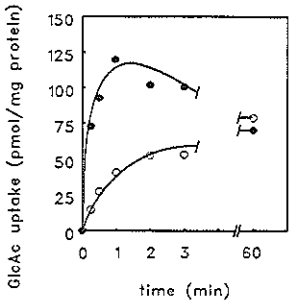


*Figure 1.* Time curve of glucuronic acid uptake in rat liver lysosomal membrane vesicles. 20-$\mu$l membrane vesicles in 7 mM $Na^+$, 13 mM $K^+$, 20 mM Hepes pH 7.4 plus 0.01 mM valinomycin were incubated at 20°C with 10 $\mu$l containing 2 $\mu$Ci [$^3$H]GlcAc (0.1 mM final total concentration) in 100 mM MES free acid, when uptake was followed in the presence of an inward-directed proton gradient ($pH_{out}$ 5.5 < $pH_{in}$ 7.4) (●—●); when assays were performed without the pH gradient ($pH_{out}$ = $pH_{in}$ = 7.4), vesicles were incubated with 10 $\mu$l radiolabeled substrate in 20 mM NaHepes pH 7.4 (○—○). Reactions were stopped with 70 $\mu$l ice-cold buffer and vesicles were filtered at 4°C as described in Methods.

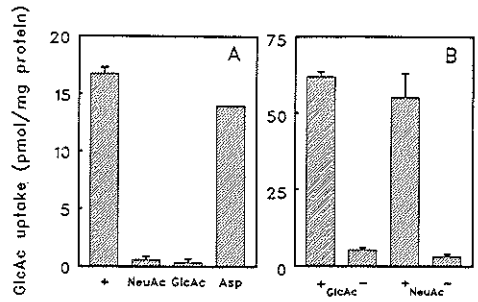


*Figure 3.* Substrate specificity of the acidic monosaccharide carrier in rat liver lysosomal membranes. (*A*) *Cis*-inhibition. Uptake of 0.02 mM [$^3$H]GlcAc was assayed for 30 s at 20°C in the presence of an inward-directed proton gradient ($pH_{out}$ 5.5 < $pH_{in}$ 7.4) plus $K^+$ and valinomycin as described in Fig. 1 without further addition (+), or upon addition of 7 mM unlabeled sialic acid (*NeuAc*), or glucuronic acid (*GlcAc*), or aspartic acid (*Asp*). All the acidic compounds were titrated with NaOH. Results are expressed as picomoles of radiolabeled GlcAc/30 s per milligram protein. (*B*) *Trans*-stimulation. 15 $\mu$l lysosomal membrane vesicles in 7 mM $Na^+$, 13 mM $K^+$, 20 mM Hepes, 33 mM MES pH 5.5 plus 0.01 mM monensin, and 0.01 mM valinomycin were preincubated 60 min at 20°C in the presence (+) or absence (−) of 1 mM unlabeled sialic acid (*NeuAc*) or glucuronic acid (*GlcAc*). The samples were then diluted 6.7-fold in the same prewarmed buffer containing 2 $\mu$Ci [$^3$H]GlcAc. In the samples that were preincubated without unlabeled sugar (−), 0.15 mM unlabeled NeuAc or GlcAc was added together with radiolabeled substrate at the start of the assay to give the same extravesicular substrate concentration in both experiments.

hibition of [$^3$H]GlcAc uptake achieved in the presence of 7 mM unlabeled GlcAc was identical to that obtained with unlabeled NeuAc. In contrast, other anionic compounds not recognized by the sialic acid carrier, like aspartic acid, did not produce significant inhibition (Fig. 3 *A*). Moreover, the same extent of *trans*-stimulation of [$^3$H]GlcAc transport was achieved by preloading the vesicles either with 1 mM NeuAc or GlcAc (Fig. 3 *B*). *Trans*-stimulation commonly proves the symmetrical function of the carrier, and that the compounds are actually transported across the membrane. Our present data in the rat liver lysosomal membranes therefore prove there is only one lysosomal carrier for the investigated acidic monosaccharides.

*Glucuronic acid and sialic acid transport across the human lysosomal membrane.* To investigate if human cells possess a similar transport mechanism for acidic sugars as so far demonstrated in rat liver, we measured transport of [$^3$H]GlcAc in resealed lysosomal membrane vesicles obtained from human cultured fibroblasts. When the uptake of [$^3$H]GlcAc was studied in the presence and absence of a proton gradient we found that the human lysosomal membrane indeed possesses a proton-dependent transport mechanism for [$^3$H]GlcAc. The transport could be inhibited to the same extent by the addition of either unlabeled GlcAc or NeuAc, but not by neutral sugars like glucose (Fig. 4). Similar experiments using [$^3$H]NeuAc showed comparable rates of proton-dependent transport and substrate inhibition pattern (data not shown). Therefore, we conclude that in human lysosomal membranes also no residual routes of glucuronic acid transport exist alternative to the sialic acid-inhibitable one. In another set of experiments, we observed that the proton-driven uptake disappears upon addition of monensin. This ionophore, in the presence of $Na^+$, shunts the proton gradient across the membrane (21, 29), confirming that the pH effect is strictly linked to the presence of a cross-membrane gradient (data not shown). Since, similarly to rat liver membranes, 30-s incubations showed clear proton-dependent stimulation, we considered these conditions as representative for zero-*trans* uptake. Proton-driven [$^3$H]GlcAc transport, assayed for 30 s as a function of the substrate concentration, showed saturability; the $K_t$ of this process was about 0.28 mM (Fig. 5). Uptake of 0.017 mM [$^3$H]GlcAc could be twofold *trans*-stimulated when fibroblast lysosomal vesicles were pre-

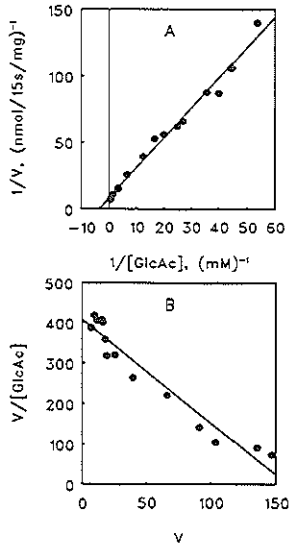


*Figure 2.* Kinetics of glucuronic acid transport in rat liver lysosomal membrane vesicles. 20 $\mu$l lysosomal membrane vesicles (80 $\mu$g protein) in 7 mM $Na^+$, 13 mM $K^+$, 20 mM Hepes pH 7.4 plus 0.01 mM valinomycin were incubated with 10 $\mu$l 100 mM MES free acid plus 1.5 $\mu$Ci [$^3$H]GlcAc and increasing concentrations of unlabeled sodium glucuronate. Duplicate incubations were performed for 30 s at 20°C. The results were fitted to a Michaelis-Menten equation for calculation of the kinetic parameters. Lineweaver-Burk (*A*) and Scatchard (*B*) transformations of the experimental data are shown.

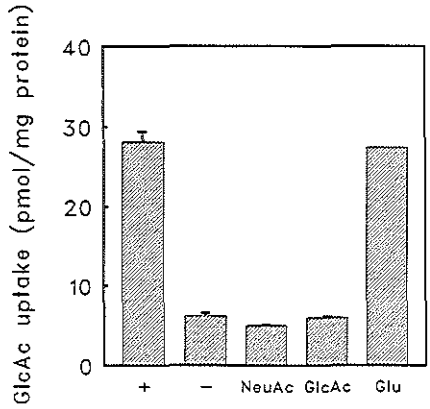


*Figure 4.* Substrate specificity of glucuronic acid transport in lysosomal membrane vesicles from human fibroblasts. Uptake of 0.046 mM [$^3$H]GlcAc (2.5 $\mu$Ci) at 37°C for 30 s was studied in lysosomal membrane vesicles from control fibroblasts (20 $\mu$g membrane protein) in the presence of a proton gradient ($pH_{out}$ 5.5 < $pH_{in}$ 7.4) (+) or at $pH_{out}$ = $pH_{in}$ = 7.4 (−), as described in Fig. 1. Proton-driven uptake ($pH_{out}$ 5.5 < $pH_{in}$ 7.4) was also followed upon addition of 7 mM unlabeled sialic acid (*NeuAc*), or glucuronic acid (*GlcAc*), or D-glucose (*Glu*) to the incubation medium. In all the assays $K^+$ and valinomycin were present. Results are presented as average of separate determinations with the same vesicle preparation, plus standard deviations.

loaded with either 2 mM unlabeled GlcAc or NeuAc at pH 5.5 (in = out) (uptake without preloading 4.3 pmol/30 s per milligram protein; after preloading with GlcAc 10.5, and after preloading with NeuAc 9.1). Altogether, these data reveal that an acidic monosaccharide carrier, with identical properties to the rat liver system, is present in the human lysosomal membrane.

*Transport of acidic sugars in lysosomal membrane vesicles from cultured cells of patients.* The standardized assay system that we developed for fibroblast lysosomal membrane vesicles was used to investigate the transport of acidic sugars in cell lines from various types of sialic acid storage disorders. To check the integrity of the lysosomal membrane from fibroblasts of the patients, we measured carrier-mediated transport of [$^3$H]D-glucose. Glucose and other neutral monosaccharides are not recognized by the sialic acid carrier, but are instead transported by a distinct lysosomal transporter for neutral monosaccharides (27). We found conditions under which human lysosomal membrane vesicles show carrier-mediated *trans*-stimulation of [$^3$H]D-glucose uptake, upon preloading with unlabeled D-glucose (Fig. 6). As shown in Table I, lysosomal membranes from patients with sialic acid storage diseases demonstrate normal rates of [$^3$H]D-glucose *trans*-stimulation, proving the integrity of the lysosomal vesicles in patient preparations. The following assay system was chosen to study essential parameters of acidic monosaccharide transport in patient cell lines. The uptake of [$^3$H]NeuAc was measured after 30 s incubation in the presence of an inward-directed proton gradient ($pH_{out}$ 5.5 < $pH_{in}$ 7.4) and compared with the inhibitory effect of unlabeled NeuAc on such proton-driven uptake and with the uptake without a proton gradient ($pH_{out}$ = $pH_{in}$ = 7.4) (Fig. 7, *A* and *B*). The cell line from the patient E. P. (14) was initially used in this experiment and showed clear deficiency of proton-driven sialic acid transport. In the control cell line, the proton-mediated uptake could be inhibited by addition of 7 mM sialic acid. Since the residual uptake upon inhibition paralleled the non-proton-driven uptake, we assumed that this residual component is apparently not carrier-mediated. Accordingly, the residual uptake in the patient could not be further inhibited. The same assay was applied to the study of [$^3$H]GlcAc transport. As shown in Fig. 7, *C* and *D*, lysosomal membranes from the same patient showed comparable deficient carrier-mediated transport of glucuronic acid. We extended our study to a larger number of patient cell lines, using the same experimental conditions. The results are shown in Table II. Carrier-mediated transport activity is here expressed by the difference between the uptake in the presence and absence of the proton gradient. Each patient's cell line showed a total deficiency of lysosomal transport for both NeuAc and GlcAc. As separately tested, in no case was the residual uptake further inhibitable by 7 mM GlcAc or NeuAc, nor did it differ significantly in patient and control preparations.

*Transport of acidic sugars in cell lines from obligate heterozygotes.* To confirm that the transport defect directly reflects the genetic mutation, we measured transport activity in cell lines from parents of Salla disease patients. Extended pedigree studies indicated that the disease is inherited as an autosomal recessive trait (2, 30), and therefore half maximal activities are expected for obligate heterozygotes. We used transformed lym-

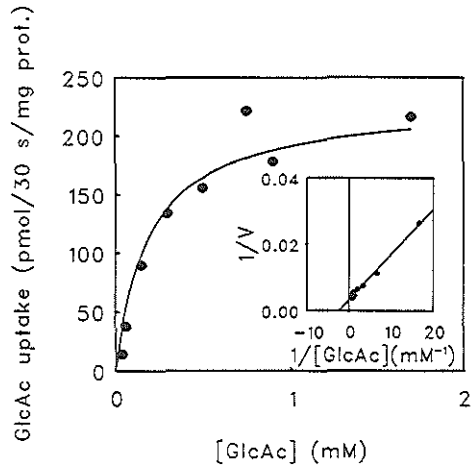


*Figure 5.* Concentration dependence of proton-driven glucuronic acid transport in human lysosomal membrane vesicles. Lysosomal membrane vesicles from control fibroblasts (27 $\mu$g protein per assay) were incubated with 2.5 $\mu$Ci [$^3$H]GlcAc plus increasing concentrations of unlabeled sodium glucuronate at 37°C for 30 s in the presence of an inward-directed proton gradient ($pH_{out}$ 5.5 < $pH_{in}$ 7.4), and in the absence of a proton gradient ($pH_{out}$ = $pH_{in}$ = 7.4). After subtraction of the residual uptake at pH 7.4, the data relative to the proton-driven uptake were fitted to a Michaelis-Menten equation. (*Inset*) Lineweaver-Burk transformation of the data.

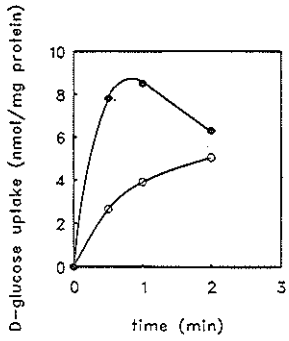


*Figure 6. Trans*-stimulation of [$^3$H]D-glucose uptake in fibroblast lysosomal membranes. Vesicles from control fibroblasts (32 μg protein) in 20 mM NaMES pH 5.5 were preincubated with 100 mM D-glucose for 45 min at 20°C, and the uptake of 6 μCi [$^3$H]D-glucose was followed at 37°C after diluting the sample 6.7-fold in 70 mM NaMES pH 5.5 (•—•). In control experiments (o—o), membranes were preincubated with 20 mM NaMES plus 50 mM NaCl instead of glucose and the uptake of radiolabeled glucose was studied after diluting the sample in MES buffer containing 15 mM D-glucose, to obtain the same final external substrate concentration in both experiments.

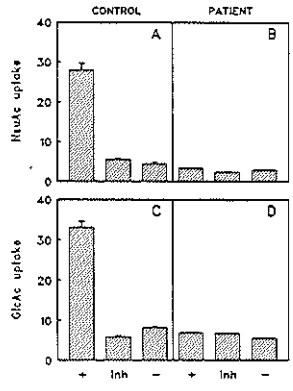


*Figure 7.* Determination of sialic acid and glucuronic acid transport rates in patient and control lysosomal membranes. Uptake of 0.046 mM [$^3$H]NeuAc or [$^3$H]GlcAc (2.5 μCi) was assayed in the presence of an inward-directed pH gradient generated with impermeant buffers (+), or in the presence of a proton gradient plus 7 mM unlabeled NeuAc or, respectively, GlcAc (*Inh*), or in the absence of a pH gradient (pH 7.4, in = out) (–), as specified for the experiments of Fig. 4. Lysosomal membrane vesicles from the control and the patient contained, respectively, 20 μg and 28 μg protein per assay. Results are averages of triplicate determinations plus standard deviations and are expressed as picomoles acidic sugar/30 s per milligram protein. (*A*) and (*C*): control; (*B*) and (*D*): patient E.P.

phoblasts as a source of lysosomal membrane vesicles because of the ease of growing the large quantities of material needed for the membrane isolations. Lysosomal membrane vesicles from lymphoblasts were obtained according to the same procedure as for rat liver and human fibroblasts. In control material, proton-driven transport of glucuronic acid was present, comparable to fibroblast membranes, and defective uptake was found in vesicles of Salla disease patients (Fig. 8 *A*). When transport studies were performed on lysosomal vesicles from parents of Salla disease patients, we found that cells from five unrelated obligate heterozygotes exhibited reduced rates of [$^3$H]GlcAc lysosomal transport, compared with controls (Fig. 8 *A*); mean of control cell lines 42 ($n$ = 4), heterozygotes 26 ($n$ = 5), and patients 8 pmol/30 s per milligram protein ($n$ = 2). The residual activity could still be inhibited by unlabeled substrate, confirming that it represents carrier-mediated transport (Fig. 8 *B*). Similar results were obtained from two heterozygote cell lines when [$^3$H]NeuAc uptake rates were measured (control:21 pmol/30 s per milligram protein; Salla disease patient:2.0; heterozygote 900015:7.8; heterozygote 891070:7.8; $n$ = 2).

*Table I. Trans-stimulation of [$^3$H]D-glucose Transport in Fibroblast Lysosomal Membrane Vesicles from Controls and Patients with Sialic Acid Storage Diseases*

| Cell line | Percentage of basal uptake |
|---|---|
| Control 698 | 378 |
| Control 540 | 288 |
| Control 53 | 231 |
| Patient A.Z. | 314 |
| Patient D.R. | 265 |
| Patient S.P. | 282 |
| Patient 9015 | 283 |
| Patient E.P. | 271 |
| Patient E.B. | 224 |

Uptake of 6 μCi [$^3$H]D-glucose was followed for 30 s at 37°C after preloading with 100 mM unlabeled D-glucose, as described in the legend to Fig. 6. Results are reported as percentage of increased uptake after preloading, compared with the basal transport rates without glucose preloading (100%). Results are from duplicate determinations in two or three different membrane vesicle preparations per each cell line.

## Discussion

Evidence for saturability, cotransport with ions, *cis*-inhibition and *trans*-stimulation are general parameters of carrier-mediated transport in biological systems (31, 32). The advantage of using resealed lysosomal membrane vesicles for transport studies lies in the absence of the intralysosomal content and the ease of performing uptake studies under close-to-normal physiological conditions. Although many lysosomal transport defects can be expected (33), only cystinosis has so far been proven to be caused by a defective carrier-mediated transport mechanism (34). The transport defect involves a single amino-acid, cystine. Significant biochemical and clinical improvement of the disease can be achieved upon treatment with cysteamine (35, 36). This compound specifically depletes lysosomes of cystine (37, 38), making cystinosis the only pharmacologically treatable lysosomal storage disease.

Various studies have previously suggested a transport defect for sialic acid in patients with Salla disease (16–18) and other forms of sialic acid storage disease (16, 19, 20), but direct evidence for saturability or other parameters has not been provided to support the involvement of a lysosomal carrier-mediated transport mechanism. The detection of a transporter for different acidic monosaccharides in rat liver lysosomal membrane vesicles pointed out the possible physiological mechanism of sialic acid transport in mammalian lysosomes (21). This carrier apparently performs a secondary active transport

*Table II. Proton-driven Transport of Sialic Acid and Glucuronic Acid in Fibroblast Lysosomal Membrane Vesicles from Patients with Different Clinical Forms of a Sialic Acid Storage Disease*

| Cell line | Uptake rate | |
|---|---|---|
| | *pmol/30 s/mg protein* | |
| | [$^3$H]NeuAc | [$^3$H]GlcAc |
| Control 698 | 20.9 | 24.6 |
| Control 540 | 12.6 | 17.4 |
| Control 53 | 23.6 | 35.0 |
| Infantile sialic acid storage disease | | |
| Patient A.Z. | 1.1 | 0 |
| Patient D.R. | 0.4 | 0 |
| Salla disease | | |
| Patient S.P. | 0 | 0 |
| Patient 9015 | 0 | 0 |
| Intermediate non-Finnish phenotype | | |
| Patient E.P. | 0.5 | 1.4 |
| Patient E.B. | 0.7 | 0 |

Uptake of 0.046 mM [$^3$H]GlcAc and [$^3$H]NeuAc was assayed in the presence and absence of an inward-directed proton gradient, as described in Fig. 7. The difference between the two rates was considered as representative for carrier-mediated transport and is reported. In the control cell lines, the ratio between proton-stimulated and proton-independent uptake was about 5:1 for NeuAc and 4:1 for GlcAc. The residual uptake rates at pH 7.4 (in = out) did not significantly differ between controls and patients.

with protons, linked to the physiological pH gradient present across the membrane (32). Here we have employed resealed lysosomal membrane preparations to the clarification of a putative human lysosomal transport defect. Experiments on GlcAc transport kinetics enabled us to demonstrate that only one lysosomal carrier disposes polysaccharides-derived acidic sugars. Evidence is provided by the linear kinetics found at the Lineweaver-Burk and Scatchard analysis for [$^3$H]GlcAc transport and also by the total interchangeable possibility to use either GlcAc or NeuAc to *cis*-inhibit or *trans*-stimulate [$^3$H]GlcAc uptake. Since these properties were observed in human fibroblasts as well, we could theoretically exclude the possibility that a sialic acid transport defect could coexist with a normal glucuronic acid transport activity, unless a mutation in the same transport protein would affect the recognition site for only one sugar (39). The data from patients' material demonstrate that a lysosomal carrier function is impaired in sialic acid storage disorders. The transport defect involves multiple physiological substrates. Defective transport for sialic acid and glucuronic acid, but not for glucose, was found both in patients with classic Salla disease and in patients with a severe infantile form or with a milder intermediate form of the disease.

Strong evidence for the idea that the transport defect is indeed the primary genetic mutation is the finding that lymphoblast lysosomal membrane vesicles from obligate heterozygotes revealed intermediate transport rates. In this respect, lymphoblast lysosomal membrane vesicles proved to be a suitable and sensitive system to discover the deficient function of a membrane transport system. The use of other techniques did not allow earlier demonstration of half-maximal lysosomal sialic acid efflux in heterozygotes (19). Our findings suggest that the wide clinical heterogeneity of this disease is not based on differences between defective transport or disposal of one substrate instead of another. Nevertheless, our assay did not allow us to discriminate for residual transport activities, which would explain lower levels of storage in the milder clinical forms. Blom et al. (40) have recently found accumulation of glucuronic acid in fibroblast homogenates from infantile sialic acid storage disease, which accounted for only 5% of the cellular sialic acid storage. In the same study, glucuronic acid storage in Salla disease was even lower (sometimes undetectable) than in the infantile form, resembling the difference in sialic acid storage usually observed between these two forms of the disease. The similarity of the $K_t$ for GlcAc and NeuAc makes it unlikely that a difference in substrate affinity determines the threshold for the storage of one or the other compound. Rather, the physiological rate of intralysosomal production of these sugars might play a role and should therefore be investigated. Urinary excretion or accumulation in tissues of glucuronic acid in patients with lysosomal sialic acid storage has not been reported. This may reflect methodological difficulties in glucuronic acid determination. The role of glucuronic acid (and perhaps of other acidic sugars as well) in the pathogenesis of these diseases needs to be reanalyzed, not only for full understanding of the disease mechanisms, but also for possible therapeutical approaches (41). The substrate affinity of the affected transporter indicates that a drug must be designed that can deplete lysosomes of acidic monosaccharides in general and not only of sialic acid.

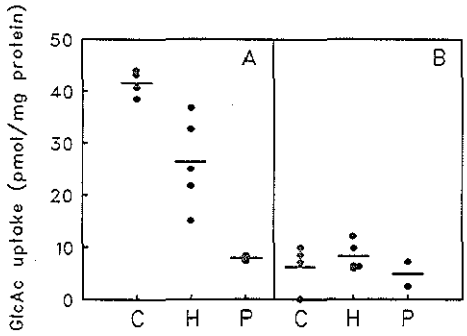


*Figure 8.* Transport rates of glucuronic acid in lysosomal membrane vesicles from human lymphoblasts. (*A*) Lysosomal membrane vesicles from controls, from patients with Salla disease, and from five unrelated parents of Salla disease patients were assayed for transport capacity of 0.055 mM [$^3$H]GlcAc (3 $\mu$Ci) for 30 s at 37°C at $pH_{out}$ 5.5 < $pH_{in}$ 7.4 or at $pH_{out}$ = $pH_{in}$ = 7.4, as already described for fibroblast lysosomal vesicles. Proton-driven transport is reported after subtraction of the residual proton-independent uptake. (*B*) The same experiment was performed in the presence of 7 mM unlabeled sodium glucuronate and the difference between proton-driven and proton-independent uptake is presented. *C*, controls; *H*, heterozygotes; *P*, patients.

## Acknowledgments

We thank Prof. H. Galjaard for support and advice in the preparation of the manuscript. We acknowledge the collaboration of Drs. M. Renlund, A. Fois, E. Vamos, F. Van Hoof, W. J. Kleijer, F. G. I. Jennekens, and F. A. Beemer, for making patient cell lines available. We thank Drs. J. W. Gratama and R. E. W. van Leeuwen, Dept. of Immunology, Daniel den Hoed Hospital, and Dr. N. G. J. Jaspers, Dept. Cell Biology, Erasmus University, Rotterdam, for assistance with the lymphoblast cultures, and for providing control material. We are indebted to J. Lokker and R. Boucke for secretarial assistance.

## References

1. Weiss, P., F. Tietze, W. A. Gahl, R. Seppala, and G. Ashwell. 1989. Identification of the metabolic defect in Sialuria. *J. Biol. Chem.* 264:17635–17636.

2. Aula, P., S. Autio, K. O. Raivio, J. Rapola, C. J. Thoden, S. L. Koskela, and I. Yamashina. 1979. "Salla disease." A new lysosomal storage disorder. *Arch. Neurol.* 36:88–94.

3. Renlund, M. 1984. Clinical and laboratory diagnosis of Salla disease in infancy and childhood. *J. Pediatr.* 104:232–236.

4. Horwitz, A. L., L. W. Hancock, G. Dawson, and M. M. Thaler. 1981. Generalized sialic acid storage disease. *Pediatr. Res.* 15:363.

5. Tondeur, M., J. Libert, E. Vamos, F. van Hoof, G. H. Thomas, and G. Strecker. 1982. Infantile form of sialic acid storage disorder: clinical, ultrastructural and biochemical studies in two siblings. *Eur. J. Pediatr.* 139:142–147.

6. Stevenson, R. E., M. Lubinsky, H. A. Taylor, D. A. Wenger, R. J. Schroer, and P. M. Olmstead. 1983. Sialic acid storage disease with sialuria: clinical and biochemical features in the severe infantile type. *Pediatrics.* 72:441–449.

7. Paschke, E., G. Trinkl, W. Erwa, M. Pavelka, I. Hutz, and A. Roscher. 1986. Infantile type of sialic acid storage disease with sialuria. *Clin. Genet.* 29:417–424.

8. Cooper, A., I. B. Sardahrwalla, M. Thornley, and K. P. Ward. 1988. Infantile sialic acid storage disease in two siblings. *J. Inherited Metab. Dis.* 11(Suppl. 2):259–262.

9. Cameron, P. D., V. Dubowitz, G. T. N. Besley, and A. H. Fensom. 1990. Sialic acid storage disease. *Arch. Dis. Child.* 65:314–315.

10. Sperl, W., W. Gruber, J. Quatacker, L. Monnens, W. Thoenes, F. M. Fink, and E. Paschke. 1990. Nephrosis in two siblings with infantile sialic acid storage disease. *Eur. J. Pediatr.* 149:477–482.

11. Baumkötter, J., M. Cantz, K. Mendla, W. Baumann, H. Friebolin, J. Gehler, and J. Spranger. 1985. *N*-acetylneuraminic acid storage disease. *Hum. Genet.* 71:155–159.

12. Ylitalo, V., B. Hagberg, J. Rapola, J. E. Mansson, L. Svennerholm, G. Sanner, and B. Tonnby. 1986. Salla disease variants. Sialolyaciduric encephalopathy with increased sialidase activity in two non-Finnish children. *Neuropediatrics.* 17:44–47.

13. Echenne, B., M. Vidal, I. Maire, J. C. Michalski, P. Baldet, and J. Astruc. 1986. Salla disease in one non-Finnish patient. *Eur. J. Pediatr.* 145:320–322.

14. Fois, A., P. Balestri, M. A. Farnetani, G. M. S. Mancini, P. Borgogni, M. A. Margollicci, M. Molinelli, C. Alessandrini, and R. Gerli. 1987. Free sialic acid storage disease. A new Italian case. *Eur. J. Pediatr.* 146:195–198.

15. Wolburg-Buchholz, K., W. Schlote, J. Baumkötter, M. Cantz, H. Holder, and K. Harzer. 1985. Familial lysosomal storage disease with generalized vacuolization and sialic aciduria. Sporadic Salla disease. *Neuropediatrics.* 16:67–75.

16. Mancini, G. M. S., F. W. Verheijen, and H. Galjaard. 1986. Free *N*-acetylneuraminic acid (NANA) storage disorders: evidence for defective NANA transport across the lysosomal membrane. *Hum. Genet.* 73:214–217.

17. Renlund, M., F. Tietze, and W. A. Gahl. 1986. Defective sialic acid egress from isolated fibroblast lysosomes of patients with Salla disease. *Science (Wash. DC).* 232:759–762.

18. Mendla, K., J. Baumkötter, E. Rosenau, B. Ulrich-Bott, and M. Cantz. 1988. Defective lysosomal release of glycoprotein-derived sialic acid in fibroblasts from patients with sialic acid storage disease. *Biochem. J.* 250:261–267.

19. Tietze, F., R. Seppala, M. Renlund, J. J. Hopwood, G. S. Harper, G. H. Thomas, and W. A. Gahl. 1989. Defective lysosomal egress of free sialic acid (*N*-acetylneuraminic acid) in fibroblasts of patients with infantile free sialic acid storage disease. *J. Biol. Chem.* 264:15316–15322.

20. Jonas, A. J. 1986. Studies of lysosomal sialic acid metabolism: retention of sialic acid by Salla disease lysosomes. *Biochem. Biophys. Res. Commun.* 137:175–181.

21. Mancini, G. M. S., H. R. de Jonge, H. Galjaard, and F. W. Verheijen. 1989. Characterization of a proton-driven carrier for sialic acid in the lysosomal membrane. Evidence for a group-specific transport system for acidic monosaccharides. *J. Biol. Chem.* 264:15247–15254.

22. Pisoni, R. L., K. S. Flickinger, J. G. Thoene, and H. N. Christensen. 1987. Characterization of carrier-mediated transport systems for small neutral amino acids in human fibroblast lysosomes. *J. Biol. Chem.* 262:6010–6017.

23. Stewart, B. H., E. J. Collarini, R. L. Pisoni, and H. N. Christensen. 1989. Separate and shared lysosomal transport of branched and aromatic dipolar amino acids. *Biochim. Biophys. Acta.* 987:145–153.

24. Harms, E., J. Kartenbeck, G. Darai, and J. A. Schneider. 1981. Purification and characterization of human lysosomes from EB-virus transformed lymphoblasts. *Exp. Cell Res.* 131:251–266.

25. Ohsumi, Y., T. Ishikawa, and K. Kato. 1983. A rapid and simplified method for the preparation of lysosomal membranes from rat liver. *J. Biochem. (Tokyo).* 93:547–556.

26. Barne, K. J., and L. H. Rome. 1986. Genetic evidence for transmembrane acetylation by lysosomes. *Science (Wash. DC).* 233:1087–1089.

27. Mancini, G. M. S., C. E. M. T. Beerens, and F. W. Verheijen. 1990. Glucose transport in lysosomal membrane vesicles. Kinetic demonstration of a carrier for neutral hexoses. *J. Biol. Chem.* 265:12380–12387.

28. Galjaard, H. 1980. Genetic Metabolic Disease. Early Diagnosis and Prenatal Analysis. Elsevier Science Publishers B. V., Amsterdam. 821–822.

29. Mollenhauer, H., D. J. Morre, and L. D. Rowe. 1990. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim. Biophys. Acta.* 1031:225–246.

30. Renlund, M., P. P. Aula, K. D. Raivio, S. Autio, K. Sainio, J. Rapola, and S. L. Koskela. 1983. Salla disease: a new lysosomal storage disorder with disturbed sialic acid metabolism. *Neurology.* 33:57–66.

31. Christensen, H. N. 1988. What are the requisites for a model transport analog? *Trends Biochem. Sci.* 13:40–42.

32. Gennis, R. B. 1989. Biomembranes: molecular structure and function. Springer-Verlag, New York. 270–322.

33. Editorial. 1986. Lysosomal storage diseases. *Lancet* ii:898–899.

34. Gahl, W. A., N. Bashan, F. Tietze, I. Bernardini, and J. D. Schulman. 1982. Cystine transport is defective in isolated leukocyte lysosomes from patients with cystinosis. *Science (Wash. DC).* 217:1263–1265.

35. Da Silva, V. A., R. P. Zurbrugg, P. Lavanchy, A. Blumberg, H. Suter, S. R. Wyss, C. M. Luthy, and O. H. Oetliker. 1985. Long-term treatment of infantile nephropathic cystinosis with cysteamine. *N. Engl. J. Med.* 23:1460–1463.

36. Gahl, W. A., G. F. Reed, J. G. Thoene, J. D. Schulman, W. B. Rizzo, A. J. Jonas, D. W. Denman, J. J. Schlesselmann, B. J. Corden, and J. A. Schneider. 1987. Cysteamine therapy for children with nephropathic cystinos. *N. Engl. J. Med.* 316:971–977.

37. Thoene, J. G., R. G. Oshima, J. C. Crawhall, D. L. Olson, and J. A. Schneider. 1976. Cystinosis. Intracellular cystine depletion by aminothiols in vitro and in vivo. *J. Clin. Invest.* 58:180–189.

38. Pisoni, R. L., J. G. Thoene, and H. N. Christensen. 1985. Detection and characterization of carrier-mediated cationic amino acid transport in lysosomes of normal and cystinotic human fibroblasts. Role in therapeutic cystine removal? *J. Biol. Chem.* 260:4791–4798.

39. Scriver, C. R., and O. H. Wilson. 1964. Possible locations of a common gene product in membrane transport of imino acids and glycine. *Nature (Lond.).* 202:92–93.

40. Blom, H. J., H. C. Andersson, R. Seppala, F. Tietze, and W. A. Gahl. 1990. Defective glucuronic acid transport from lysosomes of infantile free sialic acid storage disease fibroblasts. *Biochem. J.* 268:621–625.

41. Christensen, H. N. 1988. Amino acid transport systems of lysosomes: possible substitute utility of a surviving transport system for one congenitally defective or absent. *Biosci. Rep.* 8:121–124.

**Publication VI**

Functional reconstitution of the lysosomal sialic acid carrier into proteoliposomes.
(submitted)

# FUNCTIONAL RECONSTITUTION OF THE LYSOSOMAL SIALIC ACID CARRIER INTO PROTEOLIPOSOMES.

Grazia M.S. Mancini, Cecile E.M.T. Beerens, Hans Galjaard and Frans W. Verheijen.

Department of Cell Biology and Genetics. Erasmus University Medical Faculty. 3000 DR Rotterdam, the Netherlands.

## Summary

The lysosomal carrier for the acid monosaccharides sialic acid and glucuronic acid was solubilized from rat liver lysosomal membranes and reconstituted into phospholipid vesicles. Membrane proteins were extracted from lysosomal membranes with Triton X-100. Upon removal of the detergent by absorption on Amberlite XAD-2 beads, the solubilized proteins were incorporated in egg yolk phospholipids. The reconstituted proteoliposomes show proton-driven carrier-mediated uptake of acid monosaccharides. The reconstituted carrier was compared in a number of characteristics with the transporter as present in the native lysosomal membrane. Substrate affinity ($K_t$ for glucuronic acid = 0.4 mM) and specificity for acid monosaccharides are completely retained. The proteoliposomes also demonstrate *trans*-stimulation properties with both substrates sialic acid and glucuronic acid. The transporter is inhibited, both in its native and in the reconstituted state, by the sulfhydryl modifying agents *para*-chloromercuribenzoic acid, N-ethylmaleinimide and phenylisothiocyanate. In native membrane vesicles, arginine and histidine modifiers phenylglyoxal and diethylpyrocarbonate inactivated transport in a substrate-protectable manner. In reconstituted proteoliposomes similar inhibition was observed. However, protection by substrates was achieved only after treatment with phenylglyoxal. These data suggest that arginine and/or histidine residues are present at or near the substrate binding site of the carrier. Possibly, other essential histidines become exposed in the reconstituted state. The first successful functional reconstitution of the lysosomal sialic acid carrier represents an important step towards its purification and its detailed molecular characterization.

(Abbreviations: CMC: critical micelle concentration; DEP: diethylpyrocarbonate; DTT: dithiothreitol; EAI: ethylacetimidate; GlcAc: D-glucuronic acid; MES: 2-[N-morpholino]ethanesulfonic acid; NBS: N-Bromosuccinimide; NEM: N-ethylmaleinimide; NeuAc: N-acetylneuraminic acid, sialic acid; NeuGc: N-glycolylneuraminic acid; $NH_2OH$: hydroxylamine; pCMB: *para*-chloromercuribenzoic acid; PG: phenylglyoxal; PITC: phenylisothiocyanate; TNBS: trinitrobenzensulfonic acid).

## Introduction

In lysosomes several membrane transport systems have been characterized for amino acids, nucleosides, monosaccharides, vitamins and miscellaneous compounds (1-5). In general, these carriers permit the efflux of catabolites from the organelle to the cytosol, allowing their (re)utilization. So far none of these carriers has been identified and very little is known of their molecular properties. Recently we have developed a sensitive transport assay making use of vesiculated highly purified lysosomal membranes. This technique allowed us to demonstrate the existence of a proton cotransport system for anionic monosaccharides, with sialic acid and glucuronic acid as important physiological substrates (3). In sialic acid storage diseases, a heterogeneous group of inherited disorders characterized by lysosomal storage and excessive excretion of sialic acid, a specific transport defect of the lysosomal acidic monosaccharide carrier has been found (6). Sialic acid and glucuronic acid are produced by intralysosomal degradation of respectively sialylglycoconjugates and glycosaminoglycans. Because of the importance of this lysosomal carrier in the metabolism of normal and diseased states, we are interested in extending our knowledge about the function of this transporter at the molecular level. The first approach to the identification of transport proteins, in general, is the development of a functional reconstitution system by which the membrane proteins are first solubilized, and then incorporated into artificial phospholipid membranes. This system provides the functional assay to follow the fractionation and purification of the solubilized transporters. Several methods have been described in the literature for the reconstitution of transport proteins from various sources, however none of them has been successfully reported for lysosomal transporters, except for the proton pump (7).

In the present paper we present the successful solubilization and functional reconstitution of the lysosomal carrier for acidic monosaccharides, using the nonionic detergent triton X-100 and egg yolk phoshpolipids. The active reconstituted transporter is compared with the carrier in its native state with respect to several kinetic parameters, substrate specificity and some structural characteristics.

## Materials and Methods

### Solubilization and reconstitution.

Highly purified lysosomal membrane vesicles from rat liver were prepared as described (3, 8). Extraction of membrane proteins took place in ice by mixing 150 $\mu$l of membrane vesicles (in 20 mM NaHepes pH 7.4) with 150 $\mu$l of 6% triton X-100 (w/v), 200 mM KCl, 2 mM DTT, and 20 mM NaHepes pH 7.4. After 10 min, unextracted material was pelleted by centrifugation at 4°C at 150,000xg for 20 min, the supernatant was collected and kept in ice. This extract usually contained 50-60% of the original protein. Egg yolk phospholipids were freshly prepared as described, and stored under $N_2$ at -20°C (9, 10). To

prepare liposomes, 300 $\mu$l phospholipids were dried under $N_2$ and resuspended in 3 ml of 20 mM NaHepes pH 7.4, 100 mM KCl, 1 mM EDTA. The tube was sonicated in an ice bath with a Branson B15 sonifier (pulsed mode 50% duty for 60 min). Reconstitution was done in a final volume of 680 $\mu$l containing 200 $\mu$l of the detergent extract, 60 $\mu$l 10% triton X-100 (w/v), 100 $\mu$l of the sonicated liposomes, 320 $\mu$l of 24 mM NaHepes pH 7.4, 119 mM KCl, 1 mM DTT. Proteoliposomes were formed by removal of detergent on an Amberlite XAD-2 (Fluka Chemie, Buchs, Switzerland) column (0.5 x 3.6 cm), at room temperature, passing the sample 15 times through the same column, which was equilibrated with 20 mM NaHepes pH 7.4 and 100 mM KCl. Proteins were determined according to the procedure of Lowry et al. (11), after aceton precipitation to delipidate the samples and resuspension in 1% SDS.

**Transport assays.**

GlcAc transport was assayed in the presence of an inward-directed proton gradient, using D[1-$^3$H]GlcAc (Amersham, spec. act. 1.8 Ci/mmol). Uptake was initiated by adding 25 $\mu$l of proteoliposomes to 5 $\mu$l 240 mM MES acid containing 2 $\mu$Ci of D[1-$^3$H]GlcAc (37 $\mu$M final concentration) and 60 $\mu$M valinomycin at 37°C. This titrated the extravesicular pH to 5.5. After the required incubation time, uptake was stopped by adding 70 $\mu$l of ice-cold incubation buffer. The sample was immediately applied to a Sephadex G 50 (fine; Pharmacia LKB Biotechnology Inc.) column (0.5 x 5 cm), run at 4°C at 0.3 ml/min, and eluted with 1 ml of the incubation buffer. The recovered radioactivity was counted by liquid scintillation. Experiments in the absence of a pH gradient were performed at pH 7.4 in 20 mM NaHepes, 100 mM KCl and 10 $\mu$M valinomycin. For *trans*-stimulation experiments a 60% proteoliposome solution (25 $\mu$l) was pre-equilibrated for 60 min at 37°C with 40 mM MES acid, 1 mM Na-GlcAc or Na-NeuAc and 10 $\mu$M monensin (final concentrations) in an iso-osmotic solution (pH 5.5). The assay was started by adding 75 $\mu$l of an equivalent buffer containing 2 $\mu$Ci of radiolabeled GlcAc at 37°C. Control experiments were performed by pre-equilibration of the membranes with the same buffer without Na-GlcAc or Na-NeuAc, but the external final concentration was corrected as in the case of preloading (3). Assays were regularly performed in duplicate or triplicate. Kinetic parameters from the experimental results were calculated by the computer program Enzfitter, from Elsevier-Biosoft, Cambridge,UK.

**Protein side-chain modification.**

On the basis of the amino acid selectivity and of the tolerance of the carrier to the different assay conditions a choice was made among different covalent protein modifiers. Native lysosomal membranes (50 $\mu$l) were incubated with (final concentrations in 250 $\mu$l) 1 mM pCMB for 15 min at 37°C in 50 mM NaHepes pH 7.0, or with 1 mM NEM for 20 min at 20°C in 50 mM NaHepes pH 7.4, or with 8 mM PITC for 20 min at 22°C in 20 mM NaHepes pH 7.4, or with 1mM EAI or TNBS for 30 min at 20°C in 20 mM NaHepes pH 8.0, or with 4 mM NBS or 1 mM DEP for 30 min at 20°C in 30 mM NaMES pH 6.0, or with 1 mM PG for 60 min at 37°C in 50 mM NaHepes pH 7.4. All the incubations were

performed in the dark. Alternatively, the same incubations were performed in the presence of 30 mM NeuAc or GlcAc. Afterwards the membranes were washed with 25 ml of 20 mM NaHepes pH 7.4 and centrifuged at 50000xg 30 min at 4°C. The pellet was resuspended in 20 mM NaHepes pH 7.4 for direct assay of transport and protein content. Controls were run per each assay condition without modifiers in the presence and absence of excess substrate. None of these conditions significantly altered basal transport rates. The effect of 100 mM $NH_2OH$-HCl in Tris-Hepes pH 7.4 for 1 hr at 20°C was tested on washed membranes, pretreated with DEP, before proceeding to the transport assay. Reconstituted proteoliposomes were incubated with protein modifiers under appropriate conditions as described for native lysosomal membranes. The only difference was that after the preincubations the extravesicular modifier and the eventual excess substrate were removed by filtration of 200 $\mu$l samples on small (2 ml) Sephadex G50 (medium) columns equilibrated in 20 mM NaHepes pH 7.4 plus 100 mM KCl and centrifuged at 100xg for 2 min. The cycle was repeated twice. The resultant eluate was directly assayed for protein content and transport activity. All the chemicals were provided by Sigma, except for valinomycin (from Boeringher, Mannheim) and NEM (from Merck, Darmstadt).

## Results

Initial experiments focussed on developing reconstitution conditions which yielded proteoliposomes sufficiently impermeable to protons. This should be necessary to prove the activity of the proton gradient-driven acidic monosaccharide (sialic acid) carrier, after solubilization and reconstitution.

We have applied a rapid reconstitution procedure, which had been originaly developed for several mitochondrial carriers (12). This method uses the low CMC nonionic detergent triton X-100 for extraction. Mixtures of extracted lysosomal membrane proteins and egg yolk phospholipids are depleted of triton X-100 by repetitive chromatography on small columns of Amberlite XAD-2. This procedure leads to proteoliposomes which are able to transport glucuronic acid (Fig. 1). Because sialic acid and glucuronic acid are transported by one and the same lysosomal carrier for acidic monosaccharides (3, 6), we have performed all studies using radiolabeled GlcAc, which was more readily available. After reconstitution we observed that uptake of GlcAc is significantly stimulated in the presence of an inward directed proton gradient, leading to a transient overshoot. At 37 °C, initial uptake rates are linear for about 1 minute. Since the influence of membrane potentials was circumvented by the presence of valinomycin and potassium ions, these results prove that the pH gradient represents the driving force for a GlcAc-proton cotransport system.

We next determined if initial transport rates of proton-driven GlcAc uptake were saturable and displayed a similar affinity constant as in native lysosomal membranes. The results of this experiment confirm typical kinetics of carrier-mediated transport for the reconstituted system (Fig.2). The proteoliposomes show only one mode of uptake with an

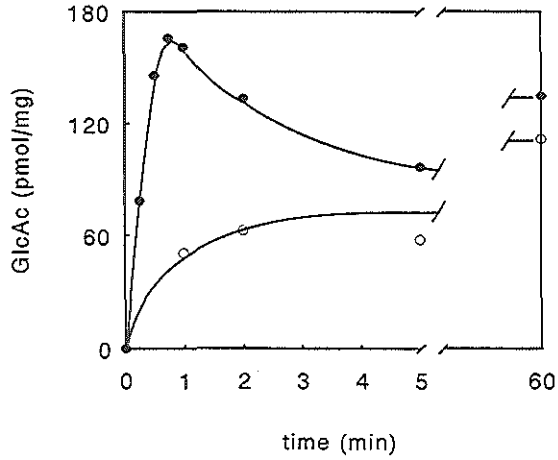


**Figure 1.** Proton gradient dependent uptake of the acidic monosaccharide GlcAc into reconstituted proteoliposomes. Lysosomal membrane proteins were solubilized from rat liver lysosomal membranes in the presence of 3 % triton X-100 and centrifuged at 150000 g for 20 min. The solubilized proteins were incorporated in liposomes and the transport of [$^3$H]GlcAc was determined at 37 °C. Proteoliposomes prepared in 20 mM NaHepes, 100 mM KCl pH 7.4 were incubated with 37 $\mu$M [$^3$H]GlcAc and 10 $\mu$M valinomycin (no proton gradient, $pH_{in}=pH_{out}=7.4$, o———o), or in the same buffer titrated to pH 5.5 with 40 mM MES free acid (with proton gradient, $pH_{in}=7.4 > pH_{out}=5.5$, ●———●).

affinity constant $K_t$ of 0.4 mM, which is similar to the affinity constant of the native carrier. No significant leakage (not carrier-mediated component) was present. Control liposomes, made in the absence of protein, did not show significant proton stimulated uptake. These results demonstrate that the sialic acid carrier polypeptide(s) is stable under the conditions used for extraction and subsequent reconstitution into liposomes. Observation of the preparations at the electronmicroscope revealed that the reconstituted proteoliposomes are homogeneous in size and unilamellar (data not shown). As measured from the equilibration point, the internal volume is about 3 $\mu$l/mg protein, slightly larger compared to the native membrane vesicles (3).

The carrier in its native membrane displays *trans*-stimulation properties. To investigate if the reconstituted carrier shows this same characteristic, the experiment of figure 3 was performed. Proteoliposomes were preloaded with concentrations above $K_t$ of either sialic acid or glucuronic acid. After four-fold dilution, uptake of trace amounts of [$^3$H]GlcAc was measured. The presence of an outward-directed gradient of unlabeled sialic acid or glucuronic acid stimulates the time-dependent uptake of radiolabeled GlcAc. This effect disappears upon dissipation of the outward gradient. Apparently the reconstituted carrier retaines its typical *trans*-stimulation properties, with both substrates.

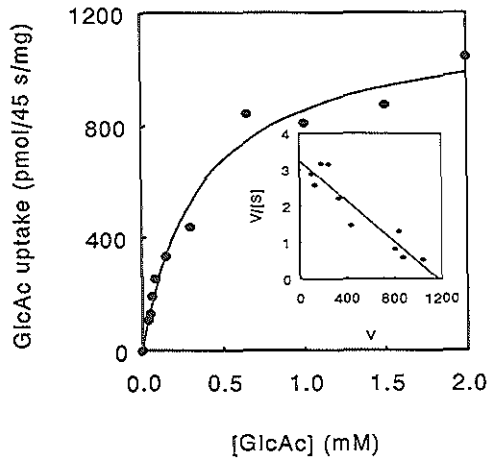


**Figure 2.** Saturable uptake of GlcAc into proteoliposomes. Proteoliposomes were incubated in the presence of a proton gradient as described in the legend of fig.1. Initial uptake rates were measured for 45 s, using different GlcAc concentrations. (*Inset*) Scatchard transformation of the experimental data.

The substrate specificity was further investigated by *cis*-inhibition experiments. Uptake of radiolabeled GlcAc was measured in proteoliposomes in the presence of high concentrations of several potential substrates (inhibitors). As shown in Table 1 the carrier retains the same substrate specificity pattern as in native membrane vesicles (3). Furthermore some interesting inhibitors are found like saccharic acid 1,4 lacton and hydroxycinnamate derivatives, which both could be useful for developing further purification steps.

To get insight into the molecular characteristics of the carrier we investigated the sensitivity of the transporter to protein modifiers. A number of modifiers has been selected both on the basis of assay conditions which do not *per se* lead to inactivation, as well as on the basis of amino acid selectivity, considering that some amino acids are more often encountered at the active site (binding site) of proteins interacting with anionic substrates (13). The results in Table 2 indicate that significant inactivation of transport in native lysosomal membrane vesicles was achieved by cystine covalent modifiers pCMB, NEM and PITC. The last two, however, can, under the employed conditions, also react with lysine residues. Though, the lysine-specific reagent TNBS and EAI did not inhibit glucuronic acid transport. In separate experiments, the apparent $K_i$ for inactivation was calculated about 0.8 mM for NEM and 6 mM for PITC, but was not calculated for pCMB. 1 mM PG and DEP produced significant inactivation of glucuronic acid transport with an apparent $K_i$ respectively of 0.67 mM and 0.46 mM. 4 mM NBS, another histidine modifier, also inactivated totally the carrier. Inhibition by DEP and PG could be completely prevented by concomitant

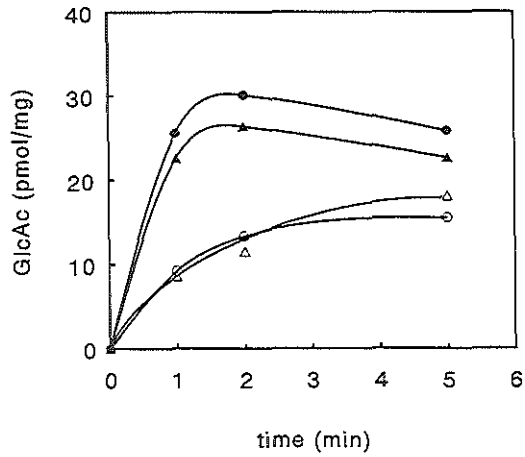


**Figure 3.** *Trans*-stimulation of [$^3$H]GlcAc uptake after preloading proteoliposomes with the unlabeled acidic monosaccharides NeuAc or GlcAc. Proteoliposomes were preincubated 60 min at 37 °C, in the presence or absence of either 1 mM unlabeled NeuAc or GlcAc, in 20 mM NaHepes, 100 mM KCl, 40 mM MES, 10 $\mu$M monensin. The transport assay was started by a four-fold dilution in pH 5.5 incubation buffer supplemented with 37 $\mu$M [$^3$H]GlcAc. With preloading NeuAc (●——●) or GlcAc (▲——▲); without preloading NeuAc (○——○) or GlcAc (△——△).

incubation with 30mM GlcAc (Table 2) or NeuAc (not shown) with identical results. Lower substrate concentrations were progressively less effective (not shown). While both PG and DEP can react with histidine residues, the assay conditions we chose would make the PG reaction exclusive for arginines (14, 15). In theory, the reactions of histidines with DEP, generating a carbetoxy group, can be reversed by treatment with hydroxylamine. We could not reverse the effect of DEP with $NH_2OH$, but control membranes, treated with $NH_2OH$ only, totally lost their transport activity (Table 2). We conclude that essential arginines and histidines are possibly involved in the binding process of acidic sugars to the lysosomal sialic acid carrier.

Interestingly, inactivation of transport was also achieved in reconstituted proteoliposomes by NEM, DEP and PG (Table 3), revealing that the effect is likely directed to the transporter polypeptide(s). However, inhibition could be prevented by the contemporary presence of substrate only in PG-treated but not in DEP-treated membranes.

## Discussion

Our recent investigations on transport of the acidic monosaccharides sialic and glucuronic acid have demonstrated the existence of a carrier in the lysosomal membrane with substrate specificity for these carboxylated sugars. This carrier was demonstrated to be defective in the sialic acid storage diseases (6). All these studies are based on transport assays

Table 1. *Cis*-inhibition of transport of GlcAc into proteoliposomes

| Inhibitor (7mM) | Transport activity (as % of uninhib.rate) |
|---|---|
| None | 100 |
| GlcAc | 16 |
| NeuAc | 17 |
| NeuGc | 18 |
| Glucose | 88 |
| N-Acetylglucosamine | 95 |
| Aspartic Acid | 105 |
| Cyano-3-hydroxycinnamic Acid | 9 |
| Cyano-4-hydroxycinnamic Acid | 10 |
| D-Saccharic Acid-1,4-lacton | 11 |
| No proton gradient | 31 |

Proteoliposomes were incubated 45 sec. at 37°C with 37 $\mu$M [$^3$H]GlcAc as described in the legend of fig.1, in the presence of an inward directed protongradient and in the presence of 7mM of the indicated compounds. Control uptake without inhibitor was 167 $\pm$7 pmol/45 s/mg protein. Uptake in presence of inhibitor is expressed as % of this control uptake without inhibitor. Results are average of two independent determinations.

with vesiculated lysosomal membranes. We have now shown that the lysosomal sialic acid carrier can be extracted from the lysosomal membrane with the detergent triton X-100 and subsequently functionally reconstituted in liposomes from crude egg yolk phospholipids, without loss of activity. The reconstitution procedure is fast and easy and can be performed on a small scale. The reconstitution assay opens the door, by using fractionated solubilized lysosomal membrane proteins, to purification and further molecular characterization of the transporter polypeptide(s).

The carrier functionally reconstituted in proteoliposomes was compared in a number of characteristics with the carrier in its native membrane. Uptake of GlcAc in proteoliposomes is also driven by an imposed proton gradient. The uptake process is saturable with an affinity constant similar to that measured in original membranes. *Trans*-stimulation and *cis*-inhibition characteristics of the carrier were also fully retained after reconstitution.

Some additional interesting inhibitors were found, which could be considered for further affinity chromatography purification steps. The first was saccharic acid 1,4 lacton, as a strong competitive inhibitor of ß-glucuronidases (16) which has been used in its immobilized state for the purification of these glucuronic acid recognizing proteins. The second are cinnamic acid derivatives, which were shown to be potent inhibitors of monocarboxylate transporters in mitochondria and have been used for their purification (17, 18).

**Table 2.** Effect of amino acid modifiers on transport activity of the sialic acid carrier in rat liver lysosomal membrane vesicles

| Reagent | Transport activity (as % of uninhib.rate) | Transport activity after protection by substrate (%) |
|---|---|---|
| No Modifier | 100 | - |
| pCMB | 0 | 0 |
| NEM | 20 | 0 |
| PITC | 10 | 18 |
| EAI | 100 | ND |
| PG | 30 | 100 |
| DEP | 20 | 95 |
| $NH_2OH$ + DEP | 9 | ND |
| $NH_2OH$ | 19 | ND |
| TNBS | 100 | ND |
| NBS | 0 | ND |

Lysosomal membrane vesicles were pretreated under the appropriate conditions for each amino acid modifier in the absence or presence of 30 mM GlcAc. After preincubation vesicles were spun down at 50000 g, 30 min and the resultant pellets were suspended in 20 mM Na-Hepes pH 7.4. 50$\mu$l vesicles were assayed for [$^3$H]GlcAc uptake as described in materials and methods. The activities after treatment (without or with protection) are expressed as % of the transport rate displayed by vesicles treated without modifier. ND: not determined.

A number of covalent side-chain protein modifiers was chosen to investigate which amino acid residues are determinant for the transport activity, in the native and reconstituted state, and which might be located at or nearby the substrate binding site. In particular cystine modifiers (NEM, PITC and pCMB) were selected because preliminary observations had suggested the importance of disulphide bridges in the native conformational state (3). Cationic amino acids, and particularly arginines, have been observed often at the substrate binding site of enzymes reacting with anionic substrates (13). Preliminary observations with side-chain modifiers on native proteins have often been confirmed after cloning of the correspondent gene and expression of *in vitro* mutagenized residues involved in the formation of the active site (19, 20). Thus, PITC (cystine- and lysine-specific), EAI and TNBS (lysine-specific), NBS, DEP and PG (arginine- and histidine-specific) were employed. In conclusion thiol modifiers inhibit glucuronic acid transport in a substrate-unprotectable manner. Although this does not exclude that cystine residues can be involved in the substrate-binding process and in the consequent conformational changes, certainly also other cystines must be kept in their native state to maintain activity. Lysines are apparently not essential for transport, since EAI and TNBS did not alter transport function. Instead, histidines and arginines are essential for the normal carrier function and apparently are present at or near

Table 3. Effect of amino acid modifiers on transport activity of the reconstituted sialic acid carrier

| Reagent | Transport activity ( % of control uptake) | |
|---|---|---|
| | after treatment | after treatment, with protection |
| No Modifier | 100 | 100 |
| NEM | 0.2 | 1.3 |
| DEP | 0.0 | 2.3 |
| PG | 15.0 | 101 |

Proteoliposomes were preincubated at the appropriate condition for each modifier, in absence or presence of 30 mM GlcAc. Afterwards extravesicular modifier and excess substrate were removed by gelfiltration. 50 $\mu$l vesicles were incubated with [$^3$H]GlcAc as described in materials and methods, in presence of a proton gradient. The transport activity is expressed as a % of proton gradient dependent uptake in control proteoliposomes similarly preincubated in the absence of protein modifiers. Results shown represent average values of two independent determinations.

the substrate-binding site. This was confirmed in reconstituted proteoliposomes, where, however, protection could be obtained only for the PG treatment. We could not definitively demonstrate by $NH_2OH$ that histidines were modified by DEP. On the other hand, the pH of the PG reaction should only select arginines for covalent modification. Taken together, the present results suggest that in the native state both arginines and histidines are involved in substrate recognition. Upon solubilization and reconstitution it is possible that other essential histidines are exposed which are not involved in substrate binding. In membrane spanning regions of integral membrane proteins it seems that histidines can be "buried" with low energy expenditure (21). We hypothesize that such a mechanism is responsible for histidines exposure upon solubilization and reconstitution of the lysosomal acidic sugar carrier.

REFERENCES


1. Forster, S. and Lloyd, J.B. (1988) *Biochim. Biophys. Acta* 947:465-491.
2. Pisoni, R.L. and Thoene, J.G. (1989) *J. Biol. Chem.* 264:4850-4856.
3. Mancini, G,M.S., de Jonge, H.R., Galjaard, H. and Verheijen, F.W. (1989) *J. Biol. Chem.* 264:15247-15254.
4. Mancini, G.M.S., Beerens, C.E.M.T. and Verheijen, F.W. (1990) *J. Biol. Chem.* 265:12380-12387.
5. Jonas, A.J. and Idriss, J.M. (1991) *J. Biol. Chem.* 266:9438-9441.
6. Mancini, G.M.S., Beerens, C.E.M.T., Aula, P.P. and Verheijen, F.W. (1991) *J. Clin. Invest.* 87:1329-1335.
7. D'Souza, M.P., Ambudkar, S.V., August, J.T. and Maloney, P.C. (1987) *Proc. Natl. Acad. Sci. USA* 84:6980-6984.

8. Bame, K. and Rome, L.H. (1985) *J. Biol. Chem.* 260:11293-11299.
9. Dawson, R.M.L. (1963) *Biochem. J.* 88:414-423.
10. Lynch, A.M. and McGivan, J.D. (1987) *Biochem. J.* 244:503-588.
11. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193:265-275.
12. Krämer, R. and Palmieri, F. (1989) *Biochim. Biophys. Acta* 974:1-23.
13. Riordan J.F., McElvany, K.D. and Borders, C.L.Jr. (1977) *Science* 195:884-886.
14. Takahashi, K. (1977) *J. Biochem.* 81:395-402.
15. Riordan, J.F. (1979) *Mol. Cell. Biochem.* 26:71-92.
16. Levvy, G.A. (1952) *Biochem.* 52:464-472.
17. Halestrap, A.P. (1975) *Biochem. J.* 148:85-96.
18. Bolli, R., Nalecz, K.A. and Azzi, A. (1989) *J. Biol. Chem.* 264:18024-18030.
19. Wedland, M., Waheed, A., von Figura, K. and Pohlmann, R. (1991) *J. Biol. Chem.* 266:2917-2923.
20. Bateman, R.C.Jr., Jackson, D., Slaughter, C.A., Unnithan. S., Chai, Y.G., Moomaw, C. and Hersh, L.B. (1989) *J. Biol. Chem.* 264:6151-6157
21. Singer, S.J. (1971) The molecular organization of biomembranes. In "Structure and functions of biomembranes". Ed. L.I. Rothfield, pp.145-222. New York Academic Press.

## General discussion and future perspectives

Several human diseases have been related to metabolic defects of membrane transport. However, only in one case the genetic mutation of a membrane carrier has been demonstrated to be the cause of a monogenetic disease. In fact, a mutation in the gene encoding for the intestinal $Na^+$/glucose cotransporter has been recently diagnosed in patients with the rare form of glucose/galactose malabsorption (Turk et al., 1991).

To date twentytwo different transport systems (twenty carriers, one pump, one channel) have been described in the lysosomal membrane. Congenital defects of those which are essential for the cellular homeostasis can be the cause of a predictable new disease. Recent reports reviewing the clinical implications of lysosomal transport suggest that new lysosomal transport defects must be searched for among patients with unknown lysosomal storage diseases (Gahl, 1989; Jonas et al., 1989; Editorial, 1986). Although some clinical signs are common to many lysosomal storage disorders, which have as a constant feature the presence of lysosomal storage detectable at microscopical level, the clinical presentations and the biochemistry of lysosomal transport defects warn against the generalization that any transport defect represents a disease with evident lysosomal storage.

Table V summarizes the lysosomal transport defects in relation to the clinical syndromes by which they manifest.

TABLE V

| Lysosomal transport defects | Clinical presentations | Lysosomal morphology | Ref. |
|---|---|---|---|
| Cystinosis | Fanconi tubulopathy | cystine crystals | Gahl, 1989 |
| Sialic acid storage diseases | Glycoproteinoses | empty, swollen vacuoles, fine granulation | Gahl, 1989 |
| Cbl F mutation | Organic acidemia, SIDS | normal | Rosenblatt, 1986<br>Shih, 1989 |
| Niemann-Pick type C | Sphingolipidosis | lamellar inclusions, foamy cells | Spence, 1989 |

As shown, the clinical pictures of transport disorders are quite polymorphic and the microscopical alteration of the lysosomal morphology in patient tissues is totally lacking in the case of Cbl F mutation. Conversely, the histologic evidence of lysosomal storage in the absence of an enzyme deficiency is not a sufficient parameter to suspect a transport defect (as in case of deficiencies of activator proteins for lysosomal enzymes). Lysosomal transport defects can lack the progressivity (Salla disease), the neurological dysfunctions (cystinosis), the visceromegaly or skeletal dysostosis (Salla disease, Cbl F, cystinosis) typical of most lysosomal storage diseases. On the other hand they can manifest as Fanconi renal tubulopathy (cystinosis), non progressive brain damage recalling cerebral palsy (Salla disease), poor feeding, vomiting and failure to thrive, typical of organic acidurias and sudden infant death syndrome (Cbl F). It seems, in general, that detectable lysosomal storage can be expected when the metabolite involved is produced in the lysosome at a relatively high rate and/or it represents an abundant structural component, rather than trace components like a vitamin or a hormone. Also, the compound might need to display a strong osmotic property, like hydrophilic solutes with absolute inability to freely diffuse through the lipid bilayer, in order to lead to organelle swelling and detectable vacuolation.

It is theoretically possible that the genetic mutation of a transporter for a substrate which can escape by alternative routes, like proline which can be transported by two distinct carriers (Pisoni et al., 1987), would not generate a clinical disease, unless each of these carriers fulfils another yet undiscovered essential function. Moreover, for some hydrophobic compounds, simple diffusion could also contribute to the efflux *in vivo*, as it seems to be the case for glucose in intact lysosomes (Iveson et al., 1989). On the other hand, mutations affecting transport systems which are essential for the existence and proper function of the lysosome, like the proton pump, are perhaps not compatible with life.

Biochemical studies have not been able so far to explain the wide clinical heterogeneity of SASD. In our studies, lysosomal proton-dependent transport rates of sialic acid and glucuronic acid were deficient in the cells from different patients. As we said, the lysosomal sialic acid carrier shows *trans*-stimulation properties (proton gradient-independent) as well as proton-driven cotransport. For similar $H^+$-cotransport systems in bacteria, two distinct sites on the same polypeptide are respectively involved in proton or substrate binding. A mutation of the $H^+$ binding site will affect $H^+$-cotransport, but it will not affect *trans*-stimulated transport and *vice versa* (Kaback, 1987). If Salla disease represents a mutation of the $H^+$ binding site of the sialic acid carrier, it is possible that the carrier can still have a residual proton-independent activity *in vivo*. A residual carrier function in these patients might explain the milder symptoms and the lesser extent of storage. This hypothesis should be tested with *trans*-stimulation experiments in lysosomal membrane preparations from SD patients, and, if proven, can open new therapeutical perspectives.

Many general questions remain open after the completion of this research. Some of

them, already mentioned before, seem particularly important and will be summarized here briefly.

*Multiple transport defect: what does this imply for the evolution of the disease?*

Sialic acid storage disorders are the first lysosomal transport defect for multiple compounds. It has been demonstrated that both sialic acid and glucuronic acid cannot efflux from the patient lysosomes. An important problem to be addressed in the near future is to define the extent of accumulation of glucuronic acid in patient tissues and to clarify its role in the pathogenesis.

*Lysosomal transport defect: does this always lead to a lysosomal storage disease?*

Some transport defects, like Cbl F mutation, do not present clinically and microscopically as a lysosomal storage disease. As mentioned before, in the clinicians'mind lysosomal transport defects and vacuolation should not represent synonyms.

*Lysosomal transport systems: how can they supply for one another?*

In general, therapeutical perspectives of lysosomal transport defects are more hopeful than for other lysosomal diseases caused by enzyme deficiencies. For example, cystinosis is the only lysosomal disorder which can be treated pharmacologically. Though the observation of the therapeutical role of thiols in this disease was almost serendipitous, studies on the amino acid transport mechanisms have provided the explanation for the success of this treatment and the possibility to design improved drugs (Christensen, 1988). As mentioned above, the definition of the molecular defect in SASD might open new therapeutical possibilities.

*Lysosomes: only "end point" or also "starting point" for cellular metabolism?*

Studies about the multiple functions of the lysosome membrane have also contributed to understand how lysosomes actively participate into complex cellular mechanisms. Their role in information storage (via transport of calcium and neurotransmitters) is one of the most exciting perspectives . Vitamins are made available in an active form by the lysosome (biotin and cobalamin). Lysosomal transporters can be target of hormonal regulation (TSH on tyrosine transport) and can dispose hormones themselves ($T_3$ and $T_4$ by the tyrosine carrier). A passage through lysosomes is one of the steps involved in the antigen realization in immunocompetent cells (antigenic peptides devoted to MHC-II complex formation). Intriguingly, substrate degradation by the relevant hydrolase is not the rule, but recycling of undegraded material can take place (for example glucosylceramide; Trinchera et al., 1990 and 1991). Integral lysosomal membrane proteins of unknown function can be expressed on the plasma membrane under special metabolic circumstances (LEP100, CD63 antigen, LIMP).

The definition of lysosomes as a point of no return of the intracellular catabolism, still formulated a couple of years ago (Kornfeld and Mellman, 1989), may be too restrictive today.

REFERENCES


* Christensen, H.N. (1988) Amino acid transport systems of lysosomes: possible substitute utility of a surviving transport system for one congenitally defective or absent. *Biosci. Rep.* 8:121-124.
* Editorial (1986) Lysosomal storage diseases. *Lancet* 2:898-899.
* Gahl, W.A. (1989) Lysosomal membrane transport in cellular nutrition. *Annu. Rev. Nutr.* 9:39-61.
* Iveson, G.P., Bird, S.J., Lloyd, J.B. (1989) Passive diffusion of non-electrolytes across the lysosome membrane. *Biochem. J.* 261:451-456
* Jonas, A.J., Speller, R.J., Conrad, P.B., Dubinsky, W.P. (1989) Transport of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine by rat liver lysosomes. *J. Biol. Chem.* 264:4953-4956
* Kaback, H.R., (1987) Use of site-directed mutagenesis to study the mechanism of a transmembrane transport protein. *Biochemistry* 26:2071-2076
* Kornfeld, S. and Mellman, I. (1989) The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483-525.
* Pisoni, R.L., Flickinger, K.S., Thoene, J.G. and Christensen, H.N. (1987) Characterization of carrier-mediated transport systems for small neutral amino acids in human fibroblast lysosomes. *J. Biol. Chem.* 262:6010-6017
* Trinchera, M., Ghidoni, R., Sonnino, S. and Tettamanti, G. (1990) Recycling of glucosylceramide and sphingosine for the biosynthesis of gangliosides and sphingomyelin in rat liver. *Biochem. J.* 270:825-820.
* Trinchera, M., Carrettoni, D. and Ghidoni, R. (1991) A part of glucosylceramide formed from exogeneous lactosylceramide is not degraded to ceramide but recycled and glycosylated in the Golgi apparatus. *J. Biol. Chem.* 266:9093-9099
* Turk, E., Zabel, B., Mundlos, S., Dyer, J. and Wright, E.M. (1991) Glucose\galactose malabsorption caused by a defect in the $Na^+$/glucose cotransporter. *Nature* 350:354-356.

## Summary

Lysosomes are intracellular acid organelles with a major function in the catabolism of intracellular and extracellular components. In the last decade, a large number of transporters has been characterized in the lysosomal membrane for the facilitated transport of amino acids, sugars, nucleosides, inorganic ions, vitamins and other miscellaneous compounds. This has clarified that the products of catabolism efflux from the lysosome via specialized mechanisms, regulated by transport proteins. Biochemically, they function as channels, pumps or carriers. Their function can be regulated by nucleotides (ATP), by membrane potentials, by hormones, or by ion gradients. The substrate specificity is often extended to a category of compounds with similar chemical characteristics, which compete with each other for transport.

Some human genetic disorders have been ascribed to the defective function of a lysosomal transport system. The classical example of this situation is represented by nephropathic cystinosis, where the lysosomal carrier for the disulfide cystine is affected.

In this thesis the biochemical and clinical aspects of patients with different types of sialic acid storage disorders (SASD) have been studied with the conclusion that these disorders represent the second example of a genetic error of a lysosomal transport protein.

SASDs are inborn errors of metabolism characterized by excessive sialuria and cellular storage of free sialic acid in the lysosomal compartment.

In publication I a patient with sialic acid storage disease is described. His phenotype resembles that of Salla disease (SD) in many respects. Salla disease is an eponym used for the form of the disease encountered in the northern Finnish population with an estimated heterozygote frequency of 1:40. However the patient from publication I is of Italian ancestry and more severely affected than most of SD patients. This demonstrates a wide phenotypical variability between SD and the infantile severe form of this disorder (ISSD).

Previous studies showed that all the enzymes involved in the metabolism of sialic acid are normal in SASD and the hypothesis of a transport defect for sialic acid out of the lysosomal compartment had been proposed. In publication II a preliminary study of lysosome-rich fractions from patient fibroblasts indicated that the efflux of sialic acid is defective in lysosomes from patients with different clinical forms of SASD.

Subsequently we characterized the biochemical mechanism of transport for sialic acid in highly purified lysosomal membrane vesicles from rat liver (publication III). The lysosomal membrane was found to contain a carrier for the transport of a category of acidic monosaccharides. Among these sialic acid and glucuronic acid are physiologically important, since they both represent intralysosomal products of macromolecule degradation. The carrier works as a secondary-active proton cotransporter, so that the efflux of lysosomal acid sugars is driven by the physiological pH gradient existing across the lysosomal membrane.

While studying the lysosomal mechanism of transport for other sugars in rat liver lysosomal membranes, we discovered another protein involved in the transport of neutral

sugars. In publication IV the biochemical evidence for carrier-mediated transport of neutral monosaccharides, among which D-glucose, D-galactose, D-mannose and D- and L-fucose, is presented. This carrier does not recognize pentoses, nor acid monosaccharides, nor their lactones, and it is inhibited by cytochalasin B and phloretin, and seems biochemically distinct from already known glucose transporters.

Further investigations of the functions of both these carriers in human fibroblast lysosomal membranes led to the conclusion that, in all patients with different forms of SASD tested, the carrier-mediated transport of sialic acid and glucuronic acid is defective, while neutral hexoses are normally transported (publication V). These studies also introduced the concept that lysosomal transport defects can involve multiple physiological substrates, raising the question about the contribution of each of these substrates to the pathogenesis.

Intermediate (half-maximal) transport rates were found in lymphoblast preparations from five unrelated parents of SD patients, who are obligate heterozygotes of this autosomal recessive disease. This proves that the transport defect indeed represents the primary genetic defect.

Up to now, none of the functionally and biochemically defined lysosomal transporters has been purified and characterized. As a first step to achieve this goal, it was essential to develop a functional reconstitution assay for the solubilized carrier in proteoliposomes. The first successful reconstitution of a lysosomal carrier has been summarized in publication VI. All the biochemical characteristics shown by the carrier for acidic monosaccharides in the native membrane, are maintained after solubilization in Triton X-100 and subsequent reconstitution in egg yolk liposomes. The availability of an assay system for the solubilized protein will enable the purification and characterization of the transport protein in the near future.

## Samenvatting

Lysosomen zijn intracellulaire zure organellen met een belangrijke rol in de afbraak van stofwisselingsproducten. Gedurende de laatste 10 jaar zijn in het lysosomale membraan een groot aantal transport eiwitten gevonden die betrokken zijn bij het transport van aminozuren, suikers, nucleosiden, inorganische ionen, vitaminen en allerlei andere chemische producten. Dit heeft aangetoond dat de afbraakproducten het lysosoom verlaten via speciale membraan-transportsystemen. Deze eiwitten functioneren als kanalen, pompen of transporteurs. Hun functie kan gereguleerd worden door nucleotiden, zoals ATP, door hormonen of door iongradiënten. De substraat specificiteit omvat vaak groepen van gelijksoortige chemische producten die getransporteerd kunnen worden.

Een aantal erfelijke afwijkingen van de mens wordt veroorzaakt door het niet goed functioneren van een lysosomaal transportsysteem. Het klassieke voorbeeld is cystinose, waarbij het lysosomale transport eiwit voor het disulfide aminozuur cystine is aangedaan.

In dit proefschrift zijn de biochemische en klinische aspecten van siaalzuur stapelingsziekten (SASD) bestudeerd. De conclusie is dat deze ziekten het tweede voorbeeld zijn van een erfelijke afwijking in een lysosomaal transport eiwit.

SASD's zijn aangeboren metabole afwijkingen met als verschijnselen een overmaat vrij siaalzuur in de urine en in de lysosomen van verschillende celtypen.

In publicatie I wordt een patiënt met siaalzuur stapelingsziekte beschreven. Zijn klinische beeld lijkt veel op dat van de ziekte van Salla (SD). Deze ziekte komt vooral in noord-Finland voor met een dragersfrequentie van 1:40. De patiënt beschreven in publicatie I is echter van Italiaanse afkomst en hij heeft ernstiger symptomen dan de meeste patiënten. Dit laat zien dat er een grote fenotypische variatie bestaat tussen SD en de infantiele ernstige vorm (ISSD).

Eerder onderzoek heeft aangetoond dat alle enzymen, betrokken bij het metabolisme van siaalzuur, normaal functioneren in SASD en daarom werd een mogelijk transport defect van siaalzuur uit het lysosoom als hypothese naar voren gebracht. In publicatie II is gevonden, gebruik makend van lysosomale fracties geïsoleerd uit fibroblasten van patiënten, dat het transport van siaalzuur uit de lysosomen niet goed functioneert in de verschillende klinische vormen van SASD.

We hebben het biochemische mechanisme van het transport van siaalzuur in sterk gezuiverde lysosomale membraan vesicles uit rattelever beschreven in publicatie III. Het lysosomale membraan bevat een transport eiwit noodzakelijk voor het transport van een groep van zure monosacchariden. Uit deze groep zijn siaalzuur en glucuronzuur van fysiologische betekenis, want zij vertegenwoordigen producten die in het lysosoom ontstaan uit de afbraak van macromoleculen. Het transport eiwit werkt als een secundair geactiveerde zgn. cotransporteur, waarbij het transport van zure monosacchariden uit het lysosoom gedreven wordt door de fysiologisch aanwezige pH gradiënt over het lysosomale membraan.

Verdere studies aan het transport van suikers over het lysosomale membraan hebben

geleid tot de ontdekking van een ander eiwit betrokken bij het transport van neutrale suikers. In publicatie IV wordt een transport eiwit beschreven voor neutrale monosacchariden zoals D-glucose, D-galactose, D-mannose en D- en L-fucose. Dit eiwit transporteert geen pentoses, geen zure monosacchariden noch hun lactonvormen, en wordt geremd door cytochalasine B en phloretine, en lijkt biochemisch verschillend te zijn van andere al bekende glucose transporteurs.

Verder onderzoek aan de werking van deze beide transport eiwitten in lysosomale membranen verkregen uit huidfibroblasten heeft aangetoond dat in alle patiënten met verschillende vormen van SASD de transporteur voor siaalzuur en glucuronzuur niet goed functioneert. Daarentegen is het transport van neutrale suikers normaal. Deze studies laten ook zien dat een lysosomaal transport defect meerdere fysiologische substraten kan omvatten die aan het verloop van het ziekteproces mee zouden kunnen doen.

In lymfoblasten van vijf verschillende ouders van SD patiënten werd intermediair (half-maximaal) transport gemeten. Dit bewees dat het transport defect het gevolg is van een primaire genetische mutatie van een recessief allel.

Tot dusverre is geen van de functioneel en biochemisch beschreven lysosomale transport eiwitten gezuiverd en moleculair gekarakteriseerd. Als eerste stap om dit doel te bereiken, was het nodig om de functie van het uit de membranen geëxtraheerde eiwit te kunnen bepalen. In publicatie VI wordt de eerste succesvolle reconstitutie van een lysosomaal transport eiwit beschreven. Het eiwit werd met het detergens Triton X-100 uit de membranen geëxtraheerd en vervolgens in een artificieel liposoom ingebouwd. Alle biochemische eigenschappen van de transporteur voor zure monosacchariden zijn in dit proteoliposoom identiek aan die gevonden in het natieve membraan. Dit reconstitutie systeem zal de zuivering en de verdere moleculaire karakterisering vergemakkelijken.

**Acknowledgments**

Tutti quelli che mi conoscono sanno che cosa abbia significato per me venire a Rotterdam e quanto devo alle persone che mi hanno accolto.
I like to mention some of the people who have contributed to the realization of this work.
Frans, without you I would not be here writing this thesis: words are not enough.
Professor Dr. H. Galjaard, my promotor, thank you for your hospitality and the full trust and freedom which I have had in developing a new project with Frans: I realize that this has been a privilege.
Professor A. Fois, grazie alla sua apertura i suoi collaboratori possono avere le occasioni che ho avuto io. Rosario, Paolo, Mariangela, Gabriella, Antonietta, Rossella, Renato, Maddalena: si stava bene con voi.
Professor Dr. P. Aula, dear Pertti and Sirkka, we have had a nice time with you in Rotterdam and in Finland: kiitos paljon!
Arthur, professor Dr. M. Niermeijer, professor Dr. E. Sachs, you gave me a warm welcome and helped me to get started in Holland: I won't forget it.
Hedwig, since the beginning you have been a good host, a skillful guide and finally a dear friend: thank you.
Bert, Cecile, Rita, open colleagues and dear friends, I hope we will continue to have fun together.
Cecile, your accurate work and sense of responsibility (and the patience to stand the boy's humour) have been essential to the success of this work!
Wim, Otto, Henk, Marja, Diana, Ruud and all the "prenatal" group: besides science I learned with you my first dutch (unforgettable) words. Certainly, our collaboration will continue with reciprocal satisfaction.
Andrè, the time spent with you, Silvia, Giancarlo and Frans in the "small" lab has been unique.
Hugo, Bob, Jan, Ben, Pieter, Andrè, Elly, Joke, Martina, Maarten, and all the "CF" group, thank you for teaching me that transport has nothing to do with air pollution and traffic between compartments is a good system for communication.
Arnold, Marian, An, if needed, you are always very helpful: thank you.
Rein, Piet, Jan, Mieke, Nelly, Jeannette, Rita, Jopie, Elly, Joke, Joop, Tom and Mirko: you are the backbone of the department.
Thank you Sjozef, for your help with the computer (the infernal machine).
Thank you Rob, your collaboration may be "microscopical", but technically perfect!
Thank you, Ed Lansbergen for your help with the rats.
I wish to thank Hans van Pelt, professor Dr. H. Kamerling and professor Dr. Dr. Brossmer for their collaboration and suggestions in early steps of our work.
I also thank all the members of the academical committee for the constructive advices.
Ik wil mijn schoonouders bedanken voor "het altijd klaar staan", en voor de steun en de

positieve kijk op mijn problemen.
Il contributo dei miei genitori e di tutta la mia famiglia e dei miei amici più cari è stato direttamente proporzionale all'entusiasmo con cui hanno sempre sostenuto le mie scelte: miei cari, non mi siete mancati perchè so che ci siete!

## Curriculum vitae et studiorum

The writer of this thesis was born in San Severo (Italy) on April 26, 1957.
Her education and professional positions are listed.
1970-1975: Education at the "Liceo Classico M. Tondi" of San Severo.
1975-1981: Student at the Medical School, University of Siena (Italy).
November 1981: Medical degree cum laude.
December 1981: Qualification as practitioner in Italy (national examination).
December 1981 - October 1985: Specialization in Pediatrics at the Medical School of Siena, Department of General Pediatrics (prof. Dr. A. Fois).
March 1984 - July 1985: Fellowship at the Department of Clinical Genetics (Stichting Klinische Genetica) and Cell Biology, Erasmus University, Rotterdam (prof. Dr. H. Galjaard). In this period she contributed to the development of biochemical techniques for prenatal diagnosis of metabolic disorders in chorionic villi and to the investigation of the metabolic fate of radiolabeled gangliosides in cultured human fibroblasts.
October 85: Degree in Pediatrics cum laude at the Medical School of Siena.
October 1985 - July 1986: Consultant pediatrician at the Medical School of Siena, Department of Pediatrics.
August 1986 - december 1991: Research associate at the Department of Cell Biology, Erasmus University, Rotterdam (prof. Dr. H. Galjaard).
She is coauthor of 18 papers published in international journals.
The experimental work on the molecular aspects of sialic acid storage diseases was carried out under the supervision of Dr. F.W. Verheijen.