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Impaired Degradation of Glycosphingolipids in lysosomal storage diseases

Study on fibroblast cell cultures with α-galactosidase A, arylsulphatase A and prosaposin deficiencies

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I declare that I have worked on this study independently under supervision of Befekadu Asfaw, Ph.D. and that I have listed all references.

In Prague, 18th April 2003

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Contents

List of abbreviations

ARMS	Amplification refractory mutation system
ASA	Arylsulphatase A
BSA	Bovine serum albumin
CBE	Conduritol B epoxid
CCM	Chemical cleavage of mismatches
Cer	Ceramide
CHO	Chinese hamster ovary
CT	Computer tomography
DEAE	Diethylaminoethyl
DMEM	Dulbecco´s Minimum Essential Medium
DNA	Deoxyribonucleic acid
dpm	Disintegration per minute
FA	Fatty acid
FCS	Fetal calf serum
Gal	Galactose
GalCer	Galactosylceramide
Gb_3Cer	Globotriaosylceramide, Gal(α1→4)Gal(β1→4)Glc(β1→1´)Cer
Gb_4Cer	Globotetraosylceramide, globoside
	GalNAc(β1→3)Gal(α1→4)Gal(β1→4)Glc(β1→1´)Cer
GlcCer	Glucosylceramide
GM1	GM1 ganglioside,
	Gal(β1→3)GalNAc(β1→4)[NeuNAc(α2→3)]Gal(β1→4)Glc(β1→1)Cer
GM2	GM2 ganglioside,
	GalNAc(β1→4)[NeuNAc(α2→3)]Gal(β1→4)]Glc(β1→1)Cer
GM2AP	GM2 activator protein
GSL	Glycosphingolipids
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography

kDa	KiloDalton
K_m	Michaelis constant
LacCer	Lactosylceramide
LDL	Low density lipoprotein
M6P	Mannose-6-phosphate
MLD	Metachromatic leukodystrophy
MRI	Magnetic resonance imaging
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PBS	Phosphate buffered saline
PCR/RFLP	Polymerase chain reaction / restriction fragment lenght polymorphism
PL	Phospholipids
SAP	Sphingolipids activator protein
SGalCer	Galactosylceramide 3-sulphate
TLC	Thin layer chromatography
SDS	Sodium dodecyl sulphate
Apo E	Apolipoprotein E
Apo B	Apolipoprotein B

Introduction

Sphingolipids comprise a group of complex lipids known as characteristic integral components of mammalian plasma membrane. They are important for various biological processes (e.g. cell recognition and signaling, differentiation, etc.). Their degradation occurs in lysosomes where they are degraded in acidic environment by the sequential action of specific hydrolases. The deficiency of an enzyme results in block in the catabolism, that leads to the accumulation of its substrates and as a consequence to a lysosomal storage disease, e.g. Fabry disease (defect of α-galactosidase A) or metachromatic leukodystrophy (defect of arylsulphatase A). For degradation of glycosphingolipids with short oligosaccharide headgroups small glycoprotein cofactors are important. Their absence results in deposition of several substrates.

Structure and nomenclature of glycosphingolipids

Glycosphingolipids are amphipathic compounds which consist of the nonpolar part called ceramide (N-acylsphingosine) (Fig. 1) that is covalently attached to carbohydrate group by glycosidic linkage between the reducing end of saccharide and the terminal hydroxyl group of the ceramide. Sphingosines are a group name for long-chain aliphatic amino alcohol sphingenine (or sphingosine; D-erythro-1,3-dihydroxy-2-amino-4,5-trans-octadecen), its homologs, stereoisomers, hydroxyderivatives and unsaturated derivatives. Structural variations in fatty acids, sphingosines, and carbohydrates result in a great number of molecular types of glycosphingolipids.

The glycosphingolipids can be subdivided as follows:
Neutral glycosphingolipids:

- mono-, oligo- and polyglycosylceramides

Acidic glycosphingolipids

- sialoglycosphingolipids (gangliosides containing one or more sialic acid residues)
- sulphoglycosphingolipids (containing one or more carbohydrate-sulphate ester groups)

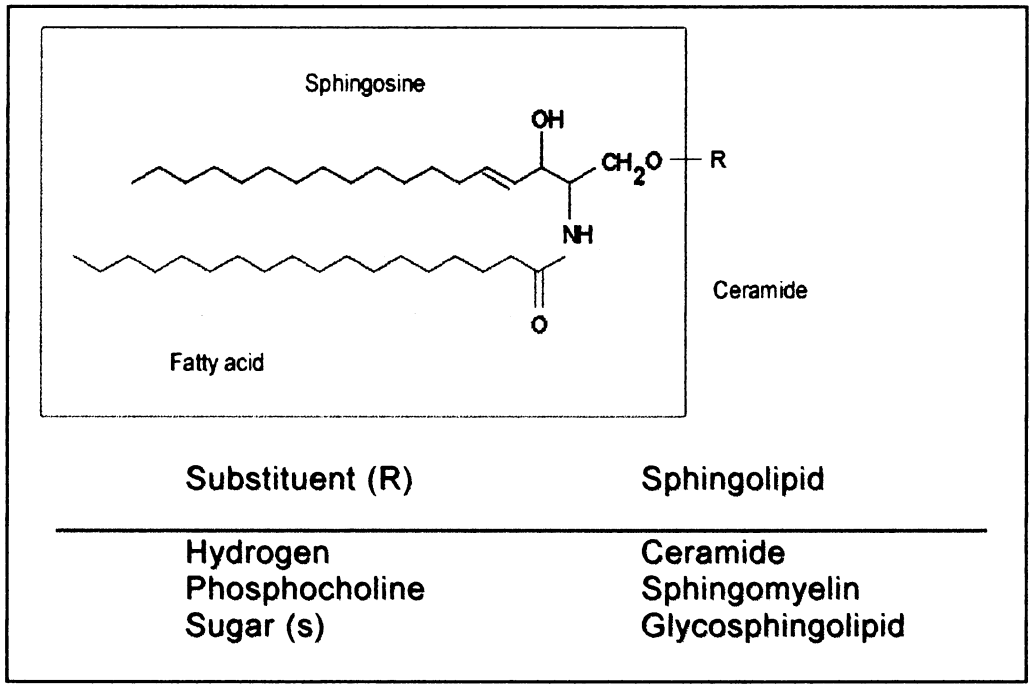


Fig.1: General sphingolipid structure

Neutral glycosphingolipids (monoglycosylceramides, diosylceramides) may be named systematically, e.g. β-D-galactosyl-(1→4)β-D-glucosyl-(1↔1)-ceramide. However, it is often more convenient to use a trivial name of the disaccharide and call the structure given above lactosylceramide (LacCer). Neutral glycosphingolipids with longer oligosaccharide chain use semisystematic names in which trivial names for "root" structures are used as a prefix (e.g. lacto-, neolacto-, globo-). The name of a given glycosphingolipid is then composed of (root name)(root size) osylceramide (Table 1). For example: III^2-α-fucosylglobotriaosylceramide or Fuc(α1→2)Gal(α1→3)Gal(β1→4)Glc(β1→1)Cer [1].

Gangliosides are named as N-acetyl- (or N-glycoloyl) neuroaminosyl(X)osylceramides where (X) stands for the root name of the oligosaccharide. A monosaccharide that carries the residue is designated in the name of the compounds by Roman numerals, counting from the ceramide end, with an Arabic superscript indicating the position to which the residue is attached. Sulphoglycosphingolipids may be named as sulphate esters of the neutral glycosphingolipids. For example: II^3-sulpho-LacCer or lactosylceramide II^3-sulphate [1].

Table 1. Glycosphingolipid series and abbreviations of corresponding oligosaccharide moieties

Glycolipid structure	*Oligosaccharide*		
	Trivial name[a]	*Symbol*[b]	*Short symbol*
Gal(α1→4)Gal(β1→4)GlcCer	Globotriaose	$GbOse_3$	Gb_3
GalNAc(β1→3)Gal(α1→4)Gal(β1→4)GlcCer	Globotetraose	$GbOse_4$	Gb_4
Gal(α1→3)Gal(β1→4)GlcCer	Isoglobotriaose	$iGbOse_3$	iGb_3
GalNAc(β1→3)Gal(α1→3)Gal(β1→4)GlcCer	Isoglobotetraose	$iGbOse_4$	iGb_4
Gal(β1→4)Gal(β1→4)GlcCer	Mucotriaose	$McOse_3$	Mc_3
Gal(β1→3)Gal(β1→4)Gal(β1→4)GlcCer	Mucotetraose	$McOse_4$	Mc_4
GlcNAc(β1→3)Gal(β1→4)GlcCer	Lactotriaose	$LcOse_3$	Lc_3
Gal(β1→3)GlcNAc(β1→3)Gal(β1→4)GlcCer	Lactotetraose	$LcOse_4$	Lc_4
Gal(β1→4)GlcNAc(β1→3)Gal(β1→4)GlcCer	Neolactotetraose	$nLcOse_4$	nLc
GalNAc(β1→4)Gal(β1→4)GlcCer	Gangliotriaose	$GgOse_3$	Gg_3
Gal(β1→3)GalNAc(β1→4)Gal(β1→4)GlcCer	Gangliotetraose	$GgOse_4$	Gg_4
Gal(α1→4)GalCer	Galabiose	$GaOse_2$	Ga_2
Gal(β1→4)Gal(α1→4)GalCer	Galatriaose	$GaOse_3$	Ga_3

[a]Name of glycosphingolipid is formed by converting ending –ose to –osyl, followed by ceramide, e.g. globotriaosylceramide

[b] Should be followed by Cer for the glycolipid, e.g. $McOse_3Cer$, Mc_4Cer

Properties and functions of glycosphingolipids

Glycosphingolipids are generally known to be anchored in the outer leaflet of the membrane bilayer. The two hydrocarbon tails of the ceramide component are inserted into the hydrophobic interior of the membrane [2]. The intramolecular hydrogen bond between the NH groups of the long chain base and the glycosidic oxygen forces the molecule to display an L schape with the oligosaccharide chain lying parallel to the membrane surface [3]. Together with membrane glycoproteins and proteoglycans the glycosphingolipids contribute to the glycocalyx which covers the cell surface with a carbohydrate wall [4].

The bioactivities of glycosphingolipids are of great interest and depend on their structure:

- Membrane stabilization and protection from inappropriate degradation, particularly during formation and maintenance of highly curved membranes (e.g. exocytosis, endocytosis, pore formation and membrane shedding) [2]
- Ion binding (e.g. gangliosides abundant on neuronal cells contribute to the brain function as the modulators of neuronal calcium)
- Signal transduction (ceramide and sphingosine-1-phosphate play a role as signal messengers) [5]
- Extracellular recognition (cell-to-cell recognition and communication, specific adhesion, growth control, immune response, differentiation, embryogenesis, etc.)[4, 6, 7]

Glycosphingolipids form cell-type specific patterns of the surface of eukaryotic cells which change with cell growth, differentiation, viral transformation and oncogene. On the cell surface, they can interact with toxins [8], viruses [9] or bacteria [10].

Metabolism of glycosphingolipids

Glycosphingolipid biosynthesis

Biosythesis of sphingolipids is catalyzed by a group of membrane-bound transferases mainly associated with the membranes of the endoplasmic reticulum and the Golgi apparatus. Presumably by vesicle flow sphingolipids reach the plasma membrane. In the first step, the condensation of the amino acid L-serine with a fatty acyl coenzyme A (usually palmitoyl coenzyme A) to 3-ketosphinganine is catalyzed by the enzyme 3-ketosphinganine synthetase. In a subsequent NADPH dependent reaction, 3-ketosphinganine is reduced to D-erythro-sphinganine by the enzyme 3-ketosphinganine reductase. Sphinganine is acylated to N-acyl-sphinganine by the enzyme sphinganine A-acyltransferase. In the next reaction catalyzed by N-acyl-sphinganine dehydrogenase ceramide is produced. Ceramide (N-acyl-sphingosine) is transported to the Golgi apparatus where on the luminal site the glycosyltranferases utilize nucleotide activated sugars and introduce sugar residues to (glyco)sphingolipid molecule (Fig.2), thus building the whole structure of the glycosphingolipid [2, 4].

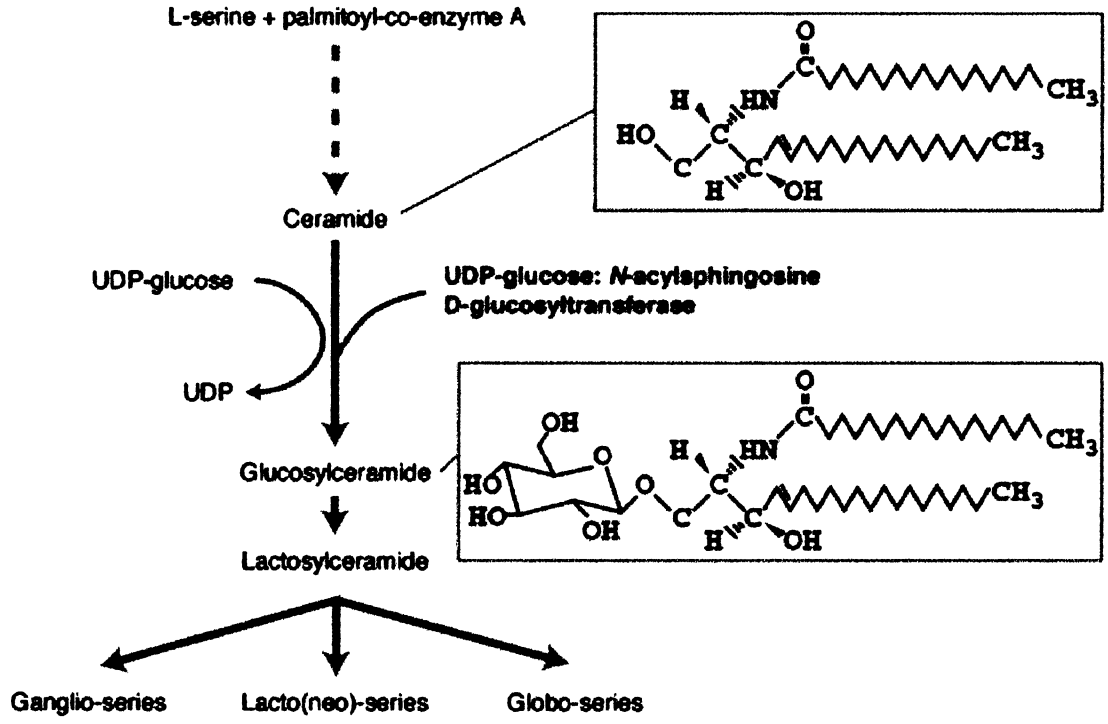


Fig.2: Elementary scheme of glycosphingolipids biosynthesis

Glycosphingolipid degradation

The constitutive degradation of glycosphingolipids occurs in the acidic compartments of the cells, the lysosomes. The composition of sphingolipids entering the lysosomes depends on the cell type. Glycosphingolipids are degraded by a series of hydrofilic lysosomal hydrolases that sequentially remove the respective terminal sugar residues before the ceramide backbone is hydrolyzed to sphingosine and a free fatty acid (Fig. 3). These processes take place at the water-lipid interface because water-soluble enzymes act on membrane-bound substrates. Glycosphingolipids with long carbohydrate moieties are easily accessible for the hydrolases because the terminal sugar is situated far enough from the lipid bilayer. In contrast, the degradation of glycosphingolipids with short carbohydrate chains depends on the presence of the activator proteins, which mediate the interaction between the enzymes and their membrane-bound substrates. Four of the five known proteins of this class, called saposins (SAP-A,-B,-C and -D), are necessary for the catabolism of simple sphingolipids (e.g. glucosylceramide, sulphatide and ceramide). The fifth protein, which shares in degradation of gangliosides GM2 is called GM2 activator protein (GM2AP). Inherited defects of either lysosomal hydrolases or activator proteins are the cause of GSL storage diseases [11].

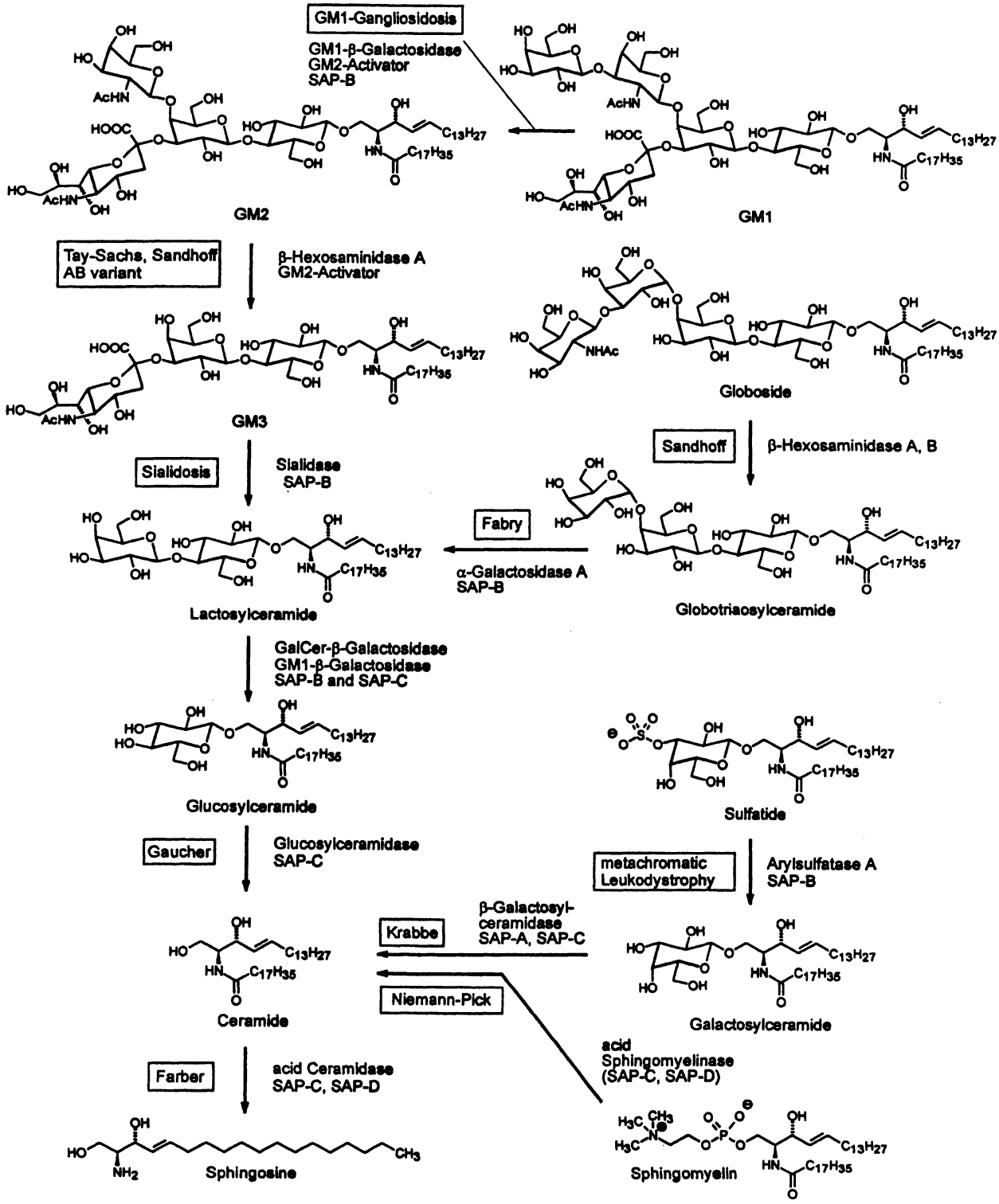


Fig. 3: Degradation schema of glycosphingolipids with names of enzymes catalyzing indicated reaction and names of diseases corresponding to the defect of indicated reaction

Sphingolipidoses

Sphingolipidoses are inherited lysosomal disorders characterized by the storage of sphingolipids in nervous tissue and visceral organs, especially in liver, spleen, and kidney. They are caused by a block on catabolic pathways of sphingolipids leading to systematic deposition of sphingolipid substrates in lysosomes of many cell types of patient's tissues. Storage process can be clearly demonstrated by histochemical and ultrastructural analysis or by biochemical analysis of undegraded substrates.

Diagnosis in clinically suspected cases is confirmed by the demonstration of a deficient activity of a specific hydrolase in cells (e.g. peripheral white blood cells, cultured cells, serum, plasma or biopsied tissues) [12]. Saposin deficiencies are proved by the immunohistochemical demonstration of protein activator absence, by the proof of storage of specific sphingolipids and by loading experiments if cultured cells are available [13].

Fabry disease

Fabry disease is an X-linked recessive lysosomal storage disorder that is caused by a deficient activity of the lysosomal enzyme α-galactosidase A (E.C.3.2.1.22.). The enzymatic defect leads to the systemic deposition of glycosphingolipids with terminal α-galactosyl moieties, predominantly globotriaosylceramide and, to a lesser extent, galabiosylceramide and blood group B, B1, and P1 substances in lysosomes of endothelial, perithelial and smooth muscle cells of blood vessels [14].

The native α-galactosidase A from human sources is a protein of approximately 101 kDa and consists of two essentially identical subunits of 46 kDa, produced in post-translational steps from a 50 kDa precursor peptide [15]. Like other lysosomal enzymes, α-galactosidase A is synthesized in the endoplasmic reticulum. The α-galactosidase A molecule is transported to the lysosomal compartments by binding of its mannose-6-phosphate (M6P) to M6P receptors in the Golgi compartment. Enzymes that escape this routing system and are secreted can bind to M6P receptors on the cell surface and be returned to lysosomes by the endocytic pathway [16, 17].

α-Galactosidase A isolated from normal tissues and fluids is a relatively heat-labile glycoprotein. Maximal activity of α-galactosidase A with the artificial substrate 4-methylumbelliferyl-α-D-galactopyranoside is obtained at pH 4,6. The K_m of the reaction with this substrate is approximately 2 mM [18].

The enzyme deficiency in Fabry disease results from mutations in the α-galactosidase A gene, which has been localized to band 22 of the long arm of the X chromosome (Xq22.1). It is 12 kb long and consists of seven exons. Because Fabry disease is transmitted as an X-linked trait, the mutant gene is transmitted through heterozygous females. Theoretically, half of female heterozygote's sons will be affected hemizygotes and half of her daughters will be heterozygous carriers. The daughter of an affected male is an obligate carrier because she inherits the mutated X chromosome from her father [19].

The full lenght human complementary DNA (cDNA) encoding α-galactosidase A has been isolated and sequenced, allowing the investigation of the type and frequency of mutations causing Fabry disease. To date, over 200 genetic mutations have been identified. Most of the mutations consist of small deletions or insertions and numerous single-base substitutions (missence or nonsense mutations) [20].

Fabry disease is a progressive complex clinical disorder with a spectrum of clinical manifestation such as angiokeratomas, hypohidrosis, corneal dystrophy and pain crises [21]. The first symptoms in individuals with very low residual activity (0-3 %) develop in childhood or adolescence [18].

Table 2. Major clinical manifestations in Fabry disease hemizygotes [18]

Vascular Glycolipid Deposition	Manifestation
Skin	angiokeratomas, oedema
Neurological	painful crises, acroparesthesias, hypohidrosis
Heart	ischaemia, infarction and angina
Brain	ischemic attack, strokes
Kidney	renal failure

Diagnosis of Fabry disease include clinical, pathological, biochemical and molecular genetic analyses. Diagnosis is proved by demonstrating deficient α-galactosidase A activity in plasma, serum, leukocytes, or cultured fibroblasts and/or by documented Gb_3Cer accumulation in plasma or urine sediment [22].

In males activity of α-galactosidase A is always almost zero, however, in female heterozygotes α-galactosidase A activity ranges from zero to normal values. Thus, molecular genetic analysis is necessary to confirm the clinical diagnosis in females. Prenatal diagnosis in the families with risk of Fabry disease can be performed by demonstrating α-galactosidase A deficiency in fetal cells obtained by amniocentesis or chorionic villi sampling [23].

The genotype of the suspected heterozygotes can be established by simple PCR methods such as PCR/RFLP or ARMS. Another method based on chemical cleavage of mismatches (CCM) and adapted to fluorescence-based detection systems has been developed for the identification and precise localization of novel α-galactosidase A gene mutations. This method has proven to serve as a useful tool for identifying heterozygous carriers when biochemical methods failed [24].

The treatment is generally nonspecific and symptomatic. Recently causal treatment of Fabry disease with recombinant α-galactosidase A was shown to be efficient [25, 26]. α-Galactosidase A with metabolic activity is produced by recombinant DNA technology using mammalian Chinese Hamster Ovary (CHO) cell culture [27] or human fibroblast culture [28]. The results of enzyme replacement studies and the lack of neuropathology make Fabry disease an ideal candidate disease for gene replacement. Recombinant adenoviral vectors expressing the α-galactosidase A cDNA were constructed and injected intravenously into Fabry knockout mice. This treatment caused an increase of the α-galactosidase A activity in all tissues [18].

Metachromatic leukodystrophy

Metachromatic leukodystrophy is an autosomal recessive inborn error of metabolism caused by a deficiency of the enzyme arylsulphatase A (ASA, E.C.3.1.6.1.) [29]. Arylsulphatase A is a lysosomal sulphatase, which catalyzes the hydrolysis of galactosylceramide 3-sulphate (galactocerebroside sulphate, sulphatide) and a variety of other lipid sulphate esters. Desulphation of sulphatide depends on the combined action of arylsulphatase A and SAP-B, a nonenzymatic activator protein [29, 30]. Defect in degradation caused by either deficiency of ASA or SAP-B results in the lysosomal accumulation of the sulphatide throughout the central and peripheral nervous systems and also in some visceral organs (kidney, gallbladder). The storage mainly affects the nervous system leading to a progressive demyelination.

Arylsulphatase A is a housekeeping enzyme, it is found ubiquitously and has been purified from various sources, including liver [31], placenta [32] and urine [33]. Recently ASA has been crystallized and its three-dimensional structure resolved [34]. At the acidic pH of lysosomes, ASA forms a homooctamer, which is composed of dimers. Multiple molecular forms of ASA have been demonstrated in various tissues by electrophoretic separation and isoelectric focusing. These variations can be attributed to charge heterogeneity of the oligosaccharide side chains [35].

The ASA gene is localized on chromosome 22 band q13. To date, more than 60 different MLD-causing mutations have been identified in the ASA gene. Many of these mutations have been found only in single patients, demonstrating the genetic heterogeneity of the disease [36, 37].

The genotype-fenotype correlation of the different clinical forms of MLD with residual enzyme activity was established. In the late-infantile form (onset between 0 and 2 years of age) enzyme activity is virtually zero, in the juvenile (between 3 and 16 years of age) and adult (>16 years) forms, some residual enzyme activity is measurable [38, 39]. In all forms of MLD, sulphatide is excreted in the urine in large amounts.

Diagnosis of MLD is based on clinical symptoms and laboratory findings caused by the demyelination and on demonstration of the deficiency of ASA or saposin B. The most useful clinical laboratory tests are brain CT or MRI, measurements of nerve conduction velocity, and potencial studies [29]. The critical diagnostic parameter for MLD is the deficiency of ASA

measured in leukocytes or/and cultured skin fibroblasts, combined with the proof of massive excretion of sulphatides in urine [40-42].

4-Nitrocatechol sulphate and the 4-methylumbelliferyl sulphate are commercially available and the most commonly used synthetic substrates for colorimetric and fluorometric determination of ASA activity, respectively. In MLD patients with a saposin B deficiency, ASA activity determined with a synthetic substrate is normal [29].

1-2 percent of the population display a substantial deficiency of ASA activity, are clinically normal, have no signs of sulphatide storage or urinary excretion, and do not appear to have a preclinical stage of MLD. These individuals have about 5-15 percent of normal ASA activity. The phenomenon of ASA deficiency without clinical consequences has been termed pseudodeficiency [43-45].

Bone marrow transplantation has been introduced as a therapeutic option for MLD, but is mostly recommended in juvenile and adult forms of the disease [29]. Prevention of the disease is restricted to the possibility of prenatal diagnosis and carrier identification in families with known risk or in populations with a high incidence of MLD . Prenatal diagnosis is possible by determination of ASA activity in cultured amniotic fluid cells or chorionic villi [46, 47].

Prosaposin and sphingolipid activator deficiencies

The lysosomal degradation of sphingolipids with short oligosaccharide chains depends on small glycosylated non-enzymatic sphingolipid activator proteins (SAPs, saposins). In its activity as a sphingolipid hydrolase facilitator it has an acidic pH optimum and is associated with the lysosomal compartment of the cell [48].

A total of five SAPs are known, and they are encoded by only two genes. One gene consisting of 15 exons and 14 introns is on chromozome 10 and codes for the SAP precursor, prosaposin. The second gene is localized on chromozome 5, consists of 4 exons and 3 introns and codes for the GM2 activator protein (GM2AP). The prosaposin is proteolytically processed to four highly homologous proteins, saposins SAP-A, -B, -C, and –D. High concentrations of SAP – precursor are found in brain, semen and other human secretory fluids, whereas the individual SAPs are mainly present in liver, kidney and spleen [49].

The SAP precursor (prosaposin), with a total of 524 amino acids and five N-glycosylation sites, contains 4 homologous domains, each of about 80 amino acids. Six different mutations have been identified in the stretch of the SAP precursor gene coding for SAP B. The mature human GM2 activator consists of a polypeptide chain of 162 amino acids and carries one N-linked carbohydrate chain on Asn 32. Four point mutations have been identified so far in the GM2 activator gene. An accumulation of GM1 is not observed in either case [50, 51].

SAP-C activates the degradation of glucosylceramide by β-glucocerebrosidase (E.C.3.2.1.45) and probably participates in degradation of galactosylceramide by β-galactocerebrosidase (E.C.3.2.1.46). Its deficiency causes a juvenile variant of Gaucher disease. SAP-D was shown to be essential for the degradation of ceramide. Like SAP-C, SAP-A stimulates the degradation of galactosylceramide by β-galactocerebrosidase as it was recently proved on mice model [52].

SAP-B is important for the degradation of sulphatides, globotriaosylceramide, digalactosylceramide and ganglioside GM3 by specific lysosomal enzymes. The clinical findings in SAP-B deficiency are similar but not identical to those in juvenile metachromatic leukodystrophy (arylsulphatase A deficiency). In addition to sulphatide the glycosphingolipid lactosylceramide, globotriaosylceramide and digalactosylceramide are excreted in increased amounts in urine of such patients [48, 51].

Diseases caused by a specific defect in one of the saposins are known for SAP-B, -C and GM2AP only. No isolated defects have been reported for SAP-A or SAP-D. A total defect of all four SAPs A-D caused by a mutation in the start codon of the SAP-precursor gene has been described [13].

The complete absence of the SAP precursor results in a generalized accumulation of ceramide, glucosylceramide, galactosylceramide, sulphatide, lactosylceramide, digalactosylceramide, ganglioside GM3, and so forth [49]. A deficiency in GM2AP leads to the AB variant of GM2 gangliosidoses which is characterized by an accumulation of ganglioside GM2 and glycolipid GA2. [30]

Cell cultures

In different areas of biomedical research, tissue culture methods were developed. Many disease processes are investigated on cell cultures, e.g. hepatocytes, amniotic cells, skin fibroblasts, etc. [53].

Cultured skin fibroblasts are widely used for metabolic studies *in situ*. They are popular for their relative resistance to environmental shocks and ease of graft withdrawal. Skin fibroblasts are grown as monolayer cultures derived from small skin biopsies [54, 55]. In the research of lysosomal storage diseases, feeding radiolabelled glycolipids to cell cultures has been used for various purposes, e.g. investigation of general metabolic pathways, diagnostic purposes such as discrimination between genuine metachromatic leukodystrophy and arylsulfatase A pseudodeficiency, determination of residual activities in enzyme-deficient cells, or identifying deficiencies of SAPs, including prosaposin deficiency.

One of the technical difficulties in cell culture dynamic experiments is the efficient transport of substrate through plasmatic membrane. To increase the uptake of the lipid substrate, cells are "starved" in lipid-free medium before the application of the substrate, which leads to the increased internalization of lipid substrate into the cell.

Radioactively labelled substrates are incorporated into the plasma membrane via micelle or liposomes. The substrate in the form of liposomes can be presented to the cells in the mixture with apolipoprotein Apo-E which is recognized by specific LDL receptors on the cell surface and its interaction with the receptors can increase the uptake of some substrates significantly (e.g. from 3 to 15 %) [56].

Endogenous cholesterol biosynthesis can be blocked by inhibiting the committed step in the biosynthesis of isoprenoids and sterols catalyzed by 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR). Drugs, known as statins, are inhibitors of HMGR and effectively lower serum cholesterol with inhibition constant values of nanomolar range [57].

Application of statins in cell cultures may theoretically cause similar phenomenon to "starvation" of cells for cholesterol and possibly for other lipids. Consequently, receptors for lipoproteins may be upgraded on the surface of cell membranes and could be used for increased internalization of exogenous lipid substrates. If this assumption is proved to be true in practice, it will serve as a novel method for efficient transport of lipid substrates in commonly used cell culture media.

After a period of incubation the analysis of degradation products is evaluated by appropriate methods [58, 59]. The cellular system reflects better the conditions *in vivo* than studies of enzyme activity *in vitro*.

Recombinant α-galactosidases A

Enzyme replacement therapy for the treatment of Fabry disease is currently available in the form of two products: E 1 and E 2. For experiments *in vivo* both these a two recombinant forms of α-galactosidase A were used. Both recombinant enzymes are glycoproteins with identical aminoacid sequences.

The aims of the diploma thesis

This work deals with two main objectives, based on dynamic metabolic studies with sphingolipid substrates on cultured skin fibroblasts.

These objectives are as follows:

I. To perform degradation studies on cells with deficiencies of lysosomal α-galactosidase A (Fabry disease), arylsulphatase A (metachromatic leukodystrophy, MLD) and prosaposin deficiency (defect of precursor of activator proteins, saposins).

II. To provide evidence on the efficiency of two recombinant human α-galactosidases A to degrade Gb_3Cer accumulated in tissues of patients with Fabry disease.

To fulfill these objectives the following steps were planned.

I. 1. Test of the quality of glycolipid substrates, [^{3}H]-sulphatide and [^{3}H]-globoside, previously isolated in our laboratory.

I. 2. Using these glycolipids in loading experiments on fibroblast cultures from patients with arylsulphatase A, α-galactosidase A and prosaposin deficiencies

II. 1. Optimalization of *in vitro* assay for recombinant α-galactosidases A

II. 2. Internalization of recombinant α-galactosidases A into fibroblast cell line

II. 3. Degradation study of pre-loaded Gb_3Cer by recombinant α-galactosidases A in cultured fibroblasts from Fabry patients

Materials and methods

Materials

4-methylumbelliferon (Calbiochem, San Diego, U.S.A.)

4-methylumbelliferyl-α-D-galactopyranoside (Sigma, Saint Louis, U.S.A.)

AC-2 Viable colostrum-based serum replacement media without lipoproteins – (Valio Bioproducts, Finland)

Apolipoprotein Apo-E (a gift from Dr. Schindler, Dept. Human genetics, Biocentrum, Wuerzburg, Germany)

Conduritol B-epoxide (Sigma, Saint Louis, U.S.A.)

Cultured skin fibroblasts (Fabry, metachromatic leukodystrophy, prosaposin deficiency patients and control cell lines, the cell culture bank of the Institute of Inherited Metabolic Diseases)

Dulbecco's Minimum Essential Medium – DMEM (Sevapharma a.s., Praha, ČR)

Fetal calf serum – FCS (PAA laboratories, Linz, Austria)

Gentamicin (Lek Pharmaceutical and Chemical Company, Ljubljana, Slovenia)

HPTLC plates (Silica gel Merck, Germany)

$NaHCO_3$ (Sevapharma a.s.,Praha, ČR)

Recombinant α-galactosidases A were gifts from TKT and Genzyme companies

Scintillation coctail (Biodegradable counting scintillant, Amersham Corporation,Arlington Heigts,U.S.A.)

Trypsin (PAA laboratories, Linz, Austria)

All chemicals were of reagent-grade quality.

Organic solvents were redistilled before use.

Methods

Isolation of glycosphingolipids

Glycosphingolipids were isolated in our laboratory of Institute for Inherited Metabolic Diseases.

Sulphatides were isolated and subsequently purified from a total lipid extract (chloroform-methanol, 2:1, v/v) of human brain. Neutral and acidic glycolipids were separated by ion-exchange chromatography on a DEAE-Sephadex column. Alkali-labile lipids were removed by mild alkaline hydrolysis and dialysis. Mixture of sulphatides and gangliosides were separated by silicic acid chromatography. Final purification of sulphatides was performed by HPLC with elution by gradient of solvents composed of chloroform and methanol-water (95:5, v/v) in various ratios [60-62].

Globoside (Gb_4Cer) was prepared from human erythrocyte concentrate. Total lipids from erythrocyte membranes were extracted with 83 % aqueous ethanol and chloroform-methanol-water (60:40:9, v/v/v). Alkali-labile lipids were removed by mild alkaline methanolysis and extensive dialysis. Neutral and acidic sphingolipids were separated by ion-exchange chromatography on a DEAE-Sephadex column. Globoside was separated from the crude neutral glycosphingolipid fraction by HPLC on silica gel column with linear gradient of solvents chloroform and methanol-water [58].

Radioactive labelling

Radioactive labelling of isolated glycosphingolipids with tritium was performed in The Central Isotope Laboratory, The First Faculty of Medicine by Dr. B. Černý. Glycosphingolipids were labelled by catalytic hydrogenation of double bond at the ceramide moiety with gaseous $[^3H]_2$. The apparatus for microhydrogenation was used. The glycosphingolipid, the catalyst (10 % $Pd/BaSO_4$) and 0.5 ml of solvent (dioxane-methanol (2:1, v/v)) were mixed together with tritium gas under 80 kPa pressure. For 1 mg of glycosphingolipid approximately 10 mg of catalyst and 1 ml of solvent were used. After 60 min the solvent was removed by lyophilisation. Then the residue was redissolved in dioxane-methanol (2:1, v/v), the catalyst removed by centrifugation and the supernatant obtained was freeze-dried. The samples were purified by HPLC [58].

Test of the quality of radiolabelled glycolipid substrates

Before experiments the purity of both [^{3}H]-globoside and [^{3}H]-sulphatide were tested on HPTLC plates. The chromatogram was developed in a system chloroform-methanol-water (70:30:05, v/v/v). After complete drying chromatogram was evaluated by TLC-Linear Radioactivity Analyzer (Raytest, Germany).

Liposome preparation

Two different types of liposomes were prepared.

Neutral liposomes used for loading of [^{3}H]-globoside were prepared as a mixture of organic solutions of 28.8 μmol 1,2-dioleoyl-sn-glycero-3-phosphocholine (in chloroform), 0.6 μmol α-tocopherol (in chloroform-methanol, 2:1), and 0.6 μmol phosphatidic acid (Na salt).

Positive liposomes used for loading of [^{3}H]-sulphatide were prepared as a mixture of organic solutions of 28.8 μmol 1,2-dioleoyl-sn-glycero-3-phosphocholine (in chloroform), 0.6 μmol α-tocopherol (in chloroform-methanol, 2:1), and 0.6 μmol phosphatidylethanolamine.

The solvents were evaporated under a stream of N_2 and the mixtures were completely dried in a desiccator under vacuum. Then 3 ml of PBS were added and the milky suspensions sonicated for 15 min (Ultrasonic homogenizer 4710, Cole-Parmer, Chicago, Illinois, 40% output). The opalescent liposome suspensions were transferred to 2 ml Eppendorf tubes and centrifuged for 10 min in an Eppendorf centrifuge at maximal speed. The supernatants were transferred to new vials and autoclaved (20 min, 121°C) [58].

Cell culture

Cultured skin fibroblasts from patients and control subjects were grown as a monolayer according to routine procedures in DMEM supplemented with 10 % FCS, 1 % gentamicin and 2 % $NaHCO_3$ in 25 cm^2 flasks (Nunc A/S, Denmark) and cultivated at 37°C in humidified 5 % CO_2 atmosphere (incubator Jouan IGO 150, France). All cell culture procedures were carried out in the laminar flow cabinet (Biohazard, Clean Air CA/RE3, the Netherlands).

For the metabolic studies, cells were treated with trypsin, seeded in equal amounts into the 9 cm^2 6-well plates and cultured to confluency under standard conditions as described. The growth of the cells was checked by phase microscop regularly (Nikon,Diaphot 2000,

Japan). Experiments were then performed in DMEM supplemented with 5% AC-2, 1% gentamicin and 2 % $NaHCO_3$ (AC-2 culture medium).

Loading experiments

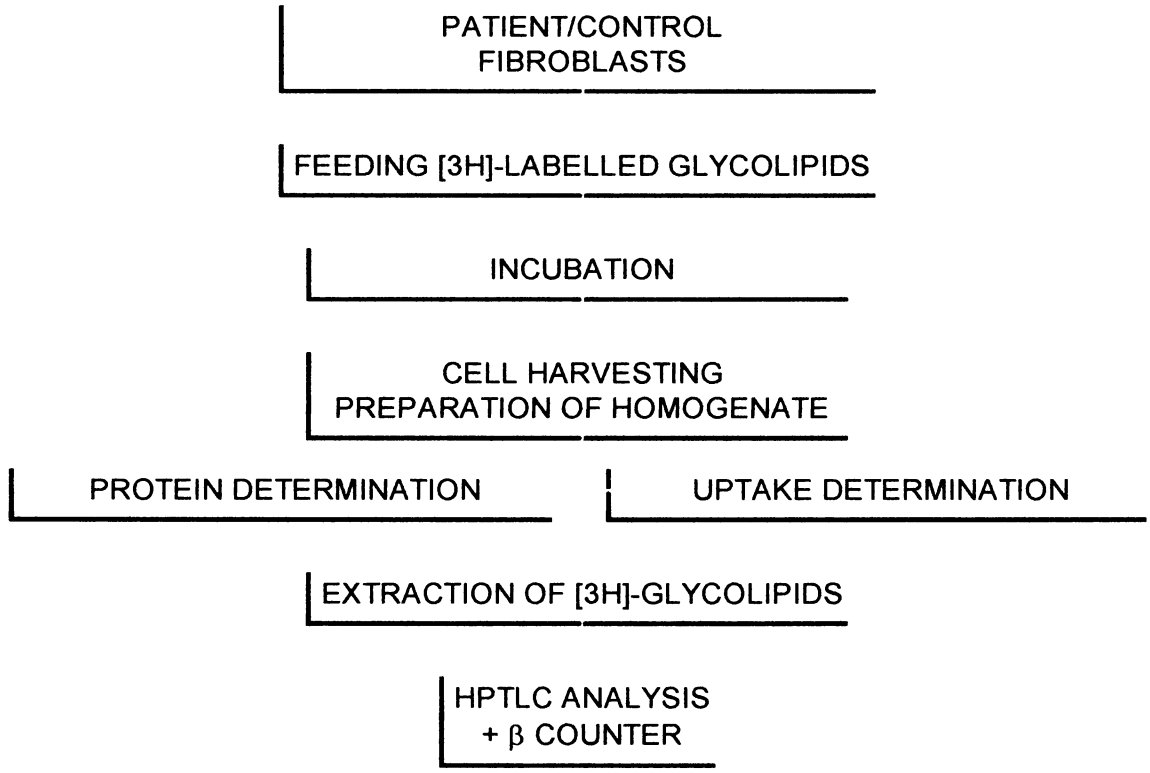


Fig. 4: Scheme of loading experiments

Following procedures are documented in Fig. 4

Feeding of [^{3}H]-sulphatide

A typical preparation of substrate for one 6-well cultivation plate was as follows: 120 µl of the stock solution of radioactively labelled sulphatide (7.5 MBq in 1 ml chloroform-methanol (2:1, v/v)) was evaporated under a stream of nitrogen. The walls of Eppendorf tube were sterilised by chloroform:methanol (2:1, v/v) and the solvent reevaporated under vacuum in a desiccator. In a laminar box 2 µl of a positively charged liposome suspension and subsequently 120 µl of AC-2 culture medium were added. The mixture was sonicated on ice in a cup-horn sonifier for 10 min. The substrate was then added to 9 ml of AC-2 culture medium, vortexed thoroughly and applied (1.5 ml per well).

Cultured skin fibroblasts from patients with metachromatic leukodystrophy, prosaposin deficiency and a control subject were used. Before the experiment the cells were grown as described above. Confluent monolayer of fibroblasts was preincubated in the low-lipid AC-2 medium for 48 hours. Then 1.5 ml of prepared substrate was applied to the cells. The cultivation then continued for 5 days under standard conditions.

Feeding of [^{3}H]-globoside

A typical preparation of substrate for one 6-well cultivation plate was as follows: 75 µl of the stock solution of radioactively labelled globoside (19 MBq in 1 ml chloroform-methanol (2:1, v/v)) was evaporated under a stream of nitrogen. The walls of Eppendorf tube were sterilised by chloroform:methanol (2:1) and the solvent reevaporated under vacuum in a desiccator. In a laminar box 3 µl of neutral liposome suspension and subsequently 300 µl of AC-2 culture medium were added. The mixture was sonicated in a cup-horn sonifier for 10 min. Then 6 µl of apolipoprotein Apo-E solution (1mg/ml) were added and mixed. The substrate was then pipetted to 9 ml of AC-2 culture medium and vortexed thoroughly. At last, conduritol B epoxide (75 µM of final concentration) as inhibitor glucosylceramidase (E.C.3.2.1.45.) was added, to limit production of soluble radioactive products. The substrate mixture was standing for 30 min before transferring to culture plate.

Cultured skin fibroblasts from patients with Fabry disease, prosaposin deficiency and a control subject were used. Experimental conditions and procedure were the same as described above for [^{3}H]-sulphatide loading.

Cell harvesting

The experiment was terminated by removing the medium. The fibroblasts were quickly rinsed twice with 1.5 ml of PBS and then incubated with 300 µl of trypsin (10 % trypsin in PBS) for 5 min at 37°C. Gentle tapping at the side of a plate helped to release the fibroblasts. Then 1 ml FCS (10 % FCS in PBS) as an inhibitor of the reaction was added and the cells were homogenized. The suspension was transferred to a centrifuge tube, the culture well was washed twice with 1.5 ml of PBS, and both washings added to the cell suspension. The cell pellet was isolated by centrifugation for 10 min at 2 000 g (table centrifuge, Jouan BR4, France) at room temperature and the pellet was transferred into an Eppendorf tube with 300 µl + 400 µl of PBS. The Eppendorf tubes were centrifuged again and the supernatant discarded [55]. The cell pellet was homogenized in 250 µl of distilled water by sonication in a cup-horn sonifier for 3x30sec (Ultrasonic homogenizer 4710, Cole-Parmer, Chicago, Illinois, 40% output). The Eppendorf tube was chilled with ice during sonication.

Determination of protein

Protein content in cell homogenates was determined by the procedure of Hartree [63] (the modification of a Lowry method). For calibration curve bovine serum albumin (BSA, 0.1 mg of BSA/ml of 0.01 M NaOH) was used as a standard. 10 µl aliquot of the cell homogenate was used. The calibration series with 0, 50, 100, 150, 200 µl of BSA standard was prepared. The samples were diluted with distilled water to 200 µl volume and treated with 200 µl of a solution A (2 % Na_2CO_3, 0.4 % NaOH, 0.16 % tartrate sodium, 1 % SDS). A blank and a standard were set up in the same way. The tubes were placed in a water bath at 50°C for 10 min, cooled to ambient temperature, and treated with 20 µl of solution B (4 % $CuSO_4.5H_2O$). The tubes were left at 25°C for at least 10 min. Finally 600 µl of Folin reagens (1 ml alkali cuprum reagens + 14 ml H_2O) were added and the sample mixed. The tubes were heated at 50°C for 10 min again and cooled to room temperature. The samples were measured by spectrometer (UV/VIS spectrometer, UNICAM UV 2-100, U.K.) at wavelenght 650 nm.

Determination of substrate uptake

10 µl aliquots of the medium, the PBS washings and the cell homogenate were mixed with 4 ml of scintillation cocktail and radioactivity was measured in each fraction (Liquid scintillation analyzer, Packard 2500 TR).

Extraction of glycolipids

The rest of the cell homogenate was extracted with 4 multiple volume of chloroform-methanol (2:1, v/v). After vortexing the mixture was centrifuged for 10 min, 1 000 g and both the upper and lower phases were collected. The interphase with precipitated proteins was reextracted with chloroform-methanol (2:1, v/v). The combined extracts were evaporated under a stream of nitrogen at 45°C [58].

HPTLC analysis

The evaporated cell lipid extract was dissolved in 50 µl of chloroform-methanol (1:1, v/v) and separated on the HPTLC plate (Silica gel Merck, Germany). The chromatogram was developed by ascending technique in a system chloroform-methanol-water (70:30:05, v/v/v). After complete drying chromatogram was evaluated by TLC-Linear Radioactivity Analyzer (Raytest, Germany).

Effect of addition of human recombinant α-galactosidases A to Fabry fibroblasts

Preparation of enzyme stock solution

Enzymes E 1 and E 2 were dissolved in distilled water according to the producer recommendations to the same final concentration of 1mg/ml in a laminar box. The protein concentration of both enzymes was checked (see Determination of protein above page 26). During all experiments enzyme solutions were kept on ice.

Cell culture

All experiments with recombinant enzymes were performed on cultured skin fibroblasts of a patient with Fabry disease. They were cultivated as described above (page 23).

Methods for characterization of the recombinant enzymes

Optimalization of conditions for in vitro enzyme activity measurement

Solutions of both enzymes were subsequently diluted in total 80 000 × in four steps by BSA (1mg/ml) solution before the measurement of activity.

For the enzyme assay the artificial substrate 4-methylumbelliferyl-α-D-galactopyranoside was dissolved in citrate buffer (pH 4.5) to final concentration of 10 mM. 4-methylumbelliferon (MU, 1 μM) was used as a standard for calibration curve. Reaction mixtures of total volume 40 μl (20 μl sample + 20 μl substrate) were incubated 30 min in 37°C in thermoblock. The reactions were terminated by addition of 600 μl of 0.2 mol/l glycine/NaOH buffer (pH 10.6). Fluorescence of released 4-MU was measured at 365/448 nm on the luminescence spectrometer (Perkin Elmer LS50B, Wellesley, U.S.A.).

Stability of enzymes in culture medium

Stability of the enzymes in the culture medium (DMEM with 10 % FCS) was tested before the experiments. E 1 and E 2 solutions were added to the culture medium, mixed thoroughly and the mixture was applied to an empty 6-well plate (without cells). Plates were incubated under standard conditions. After stated periods of time (0, 6, 12, 24 and 48 hours), the activities of the enzymes were measured in aliquots of the media.

Study of internalization of recombinant enzymes into the enzyme-deficient cell

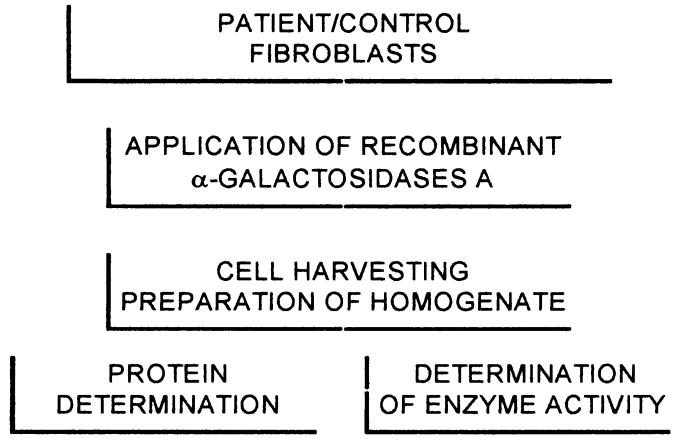


Fig. 5: Scheme of procedures used in a study of α-galactosidases A internalization

Following procedures are documented in Fig. 5

Preparation of the enzyme working solution

In sterile environment 6.2 µl of the enzyme stock solution was pipetted and thoroughly mixed with 1 ml of AC-2 medium. The volume was topped up with AC-2 medium to 9 ml and thoroughly mixed. This amount was sufficient for one 6-well plate.

Cell culture

Confluent culture of fibroblasts of a patient with Fabry disease were used. They were cultivated in a 6-well plate in DMEM and 10 % FCS under standard conditions.

Application of enzyme

The enzyme solution in AC-2 medium was applied to the cells (1.5 ml per well) and incubated in CO_2 incubator. For each time period (0, 6, 12, 18, 24 and 48 hours) one 6-well plate was prepared.

Cell harvesting

The incubation was terminated by removing the medium. The fibroblasts were quickly rinsed twice with 1.5 ml of PBS and then incubated with 300 µl of trypsin (10 % trypsin in PBS) for 5 min at 37°C. Gentle tapping at the side of plate helped to release the attached

fibroblasts. Then 1 ml FCS (10 % FCS in PBS) as an inhibitor of the reaction was added and the cells were homogenized. The suspension was transferred to a centrifuge tube, the culture well was washed twice with 1.5 ml of PBS, and the washing was added to the cell suspension. The cell pellet was isolated by centrifugation for 10 min at 2 000 g at room temperature and the pellet was transferred into an Eppendorf tube with 300 µl + 400 µl of PBS. The Eppendorf tubes were centrifuged again and the supernatant was discarded. The cell pellet was homogenized in 250 µl of distilled water by sonication in a cup-horn sonifier for 3x30sec (Ultrasonic homogenizer 4710, Cole-Parmer, Chicago, Illinois, 40% output). The Eppendorf tube was chilled with ice during sonication.

Determination of protein

Protein content in cell homogenates was determined by the procedure of Hartree [63] as described in details above (page 26). Aliquot 25 µl of cell homogenate per analysis was used.

Determination of enzyme activity in cultured fibroblasts

Enzyme activities of α-galactosidases A were assayed in all medium samples and cell homogenates. The artificial substrate 4-methylumbelliferyl-α-D-galactopyranoside was dissolved in citrate buffer (pH 4.5) to final concentration of 5mM. Fot the measurement of activities in homogenate, α-N-acetyl-D-galactosaminide as inhibitor of α-N-acetylgalactosaminidase (E.C. 3.2.1.49.) was added. For calibration curve 4-methylumbelliferon (MU, 1µM) was used as a standard. Reaction mixtures of total volume of 40 µl (20 µl sample + 20 µl substrate) were incubated for 30 min at 37°C in thermoblock. The reactions were terminated by addition of 600 µl of 0.2 mol/1 glycine/NaOH buffer (pH 10.6). Fluorescence of released 4-MU was measured at 365/448 nm on the luminescence spectrometer.

Studies of degradation of [^{3}H]-globoside by recombinant α-galactosidases A

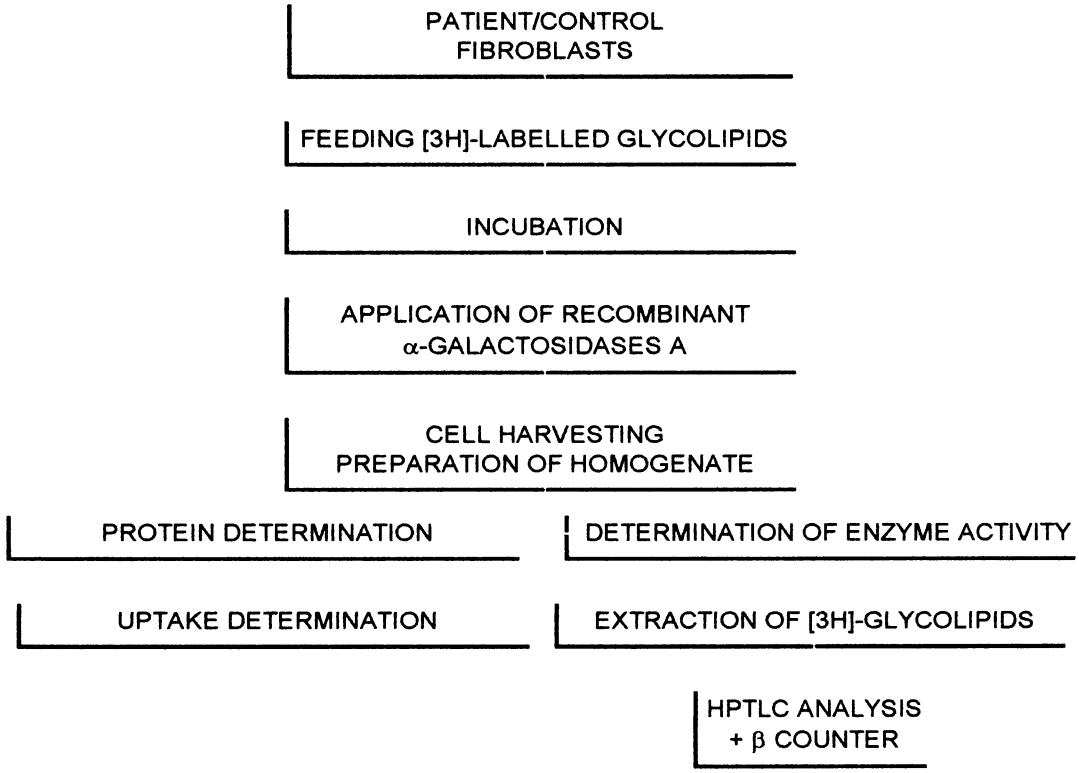


Fig. 6: Scheme of procedures used in function studies of recombinant α-galactosidases A

Following procedures are documented in Fig. 6

Preparation of [^{3}H]-globoside containing medium

For one 6-well plate 75 µl of the stock solution of radioactively labelled globoside (19 MBq in 1 ml chloroform-methanol (2:1, v/v)) was evaporated under a stream of nitrogen. The walls of an Eppendorf tube were sterilised by chloroform-methanol (2:1, v/v) and the solvent reevaporated under vacuum in a desiccator. In a laminar box 3 µl of liposome suspension and subsequently 300 µl of AC-2 culture medium were added. The mixture was sonicated in a cup-horn sonifier for 10 min. Then 6 µl of apolipoprotein Apo-E solution (1mg/ml) was added and mixed. Finally, conduritol B epoxide as an inhibitor of glucosylceramidase was added, to limit the production of soluble radioactive products. The substrate mixture was standing for 30 min at room temperature before transferring to culture plate.

Preparation of the enzyme working solution

In sterile environment 6.2 µl of the stock solution of the enzyme was pipetted and thoroughly mixed with 1 ml of AC-2 medium. The volume was topped up with AC-2 medium to 9 ml and thoroughly mixed. This amount was sufficient for one 6-well plate.

Feeding experiment

Confluent fibroblasts of a patient with Fabry disease were preincubated with low-lipid AC-2 medium for 48 hours before [^{3}H]-globoside was applied. The incubation with [^{3}H]-globoside continued for another 3 days. Afterwards, the medium was changed for fresh AC-2 medium containing either E 1 and E 2 (1.5 ml per well). The cells were incubated at 37°C in CO_2 incubator and after a period of 0, 9, 14, 19 and 24 hours the medium was collected and the cells harvested. The enzyme activity was determined both in media and cell homogenates and the analysis of degradation of the substrate ([^{3}H]-globoside) was performed.

Uptake determination

10 µl aliquots of the medium, the PBS washings and the cell homogenate were mixed with 4 ml of scintillation cocktail and radioactivity was measured in each fraction (liquid scintillation analyzer, Packard 2500 TR).

Extraction of glycolipids and HPTLC analysis

Extraction and analysis of glycolipids in the cell homogenates were performed as described in the paragraph Loading experiments (page 27).

Results

Test of quality of radiolabelled glycolipid substrates

It was observed that some radiolabelled glycolipids are susceptible to degradation during prolonged storage. The purity of both [^{3}H]-sulphatide and [^{3}H]-globoside were tested before the experiments were started. The results (Fig. 7) showed that there were no impurities possibly interfering with the degradation products formed in loading experiments.

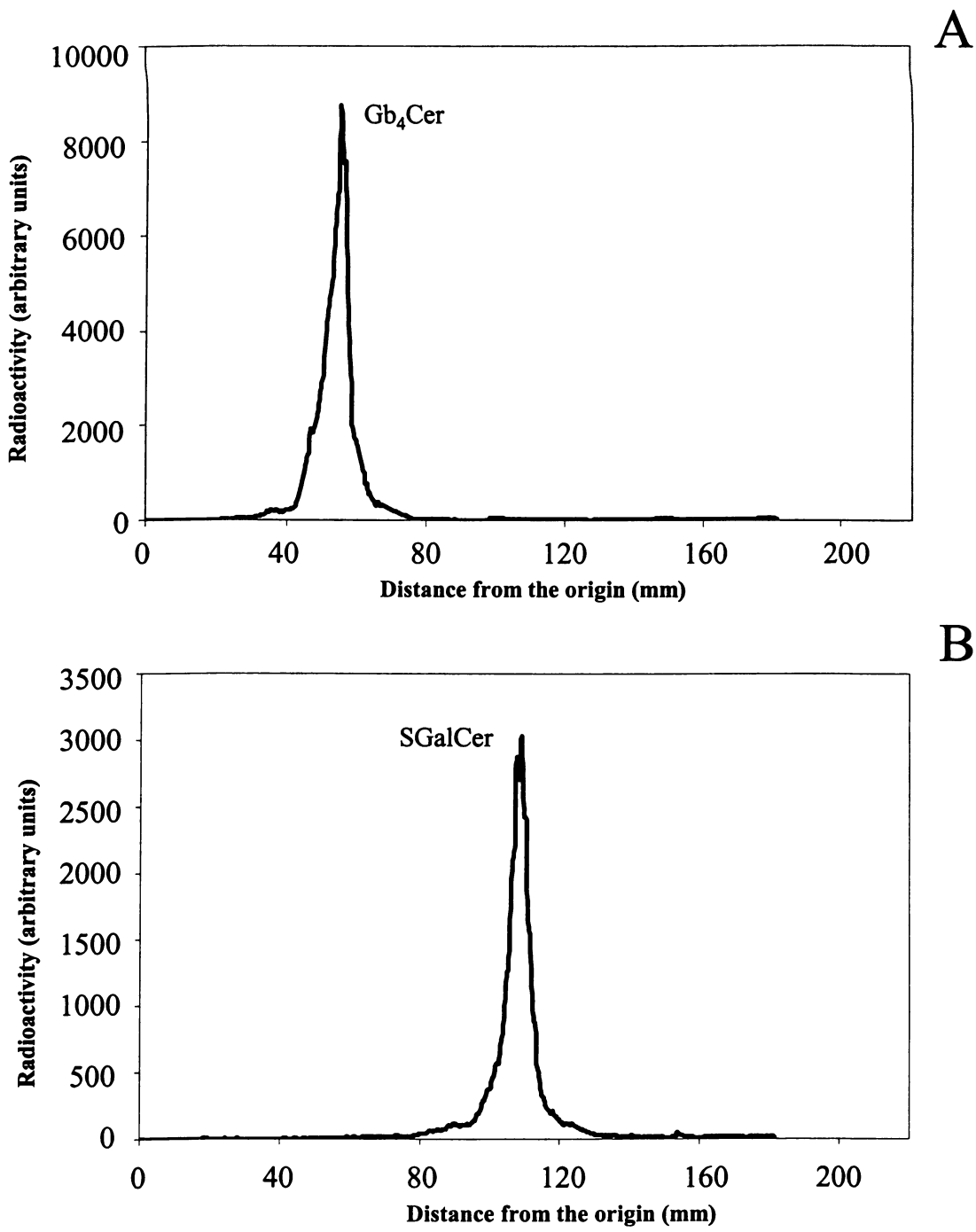


Fig. 7: Test of quality of radiolabelled glycolipid substrates

Tritium labelled substrates were separated by HPTLC (ascending technique in a system chloroform-methanol-water (70:30:05, v/v/v)). In both cases only single peaks of labelled substrates were observed.

A: [^{3}H]-globoside substrate

B: [^{3}H]-sulphatide substrate

Loading experiments

[^{3}H]-sulphatide loading

Analysis of the metabolic defect in metachromatic leukodystrophy and prosaposin deficiency cells was carried out in culture of skin fibroblasts of patients and controls. Radioactively labelled galactosylceramide 3-sulphate was used as a substrate for feeding the cells.

In metachromatic leukodystrophy (late infantile form) cell line, defect in degradation of sulphatides was clearly evident (Fig. 8A). In prosaposin deficiency cells, degradation profile of the substrate resembles to MLD, e.g. a massive accumulation of sulphatide with minute production of degradation products (Fig. 8B). Normal fibroblasts metabolized sulphatide to the products expected from lysosomal metabolism. The quantified results of product formation reflect the real situation, the capacity of normal and pathological cells to degrade the substrate (Fig. 8C). Furthermore, it seems that the degradation of other degradation products, GalCer and Cer, is also hindered, although their production is lowered to minimum due to the block in degradation of SGalCer.

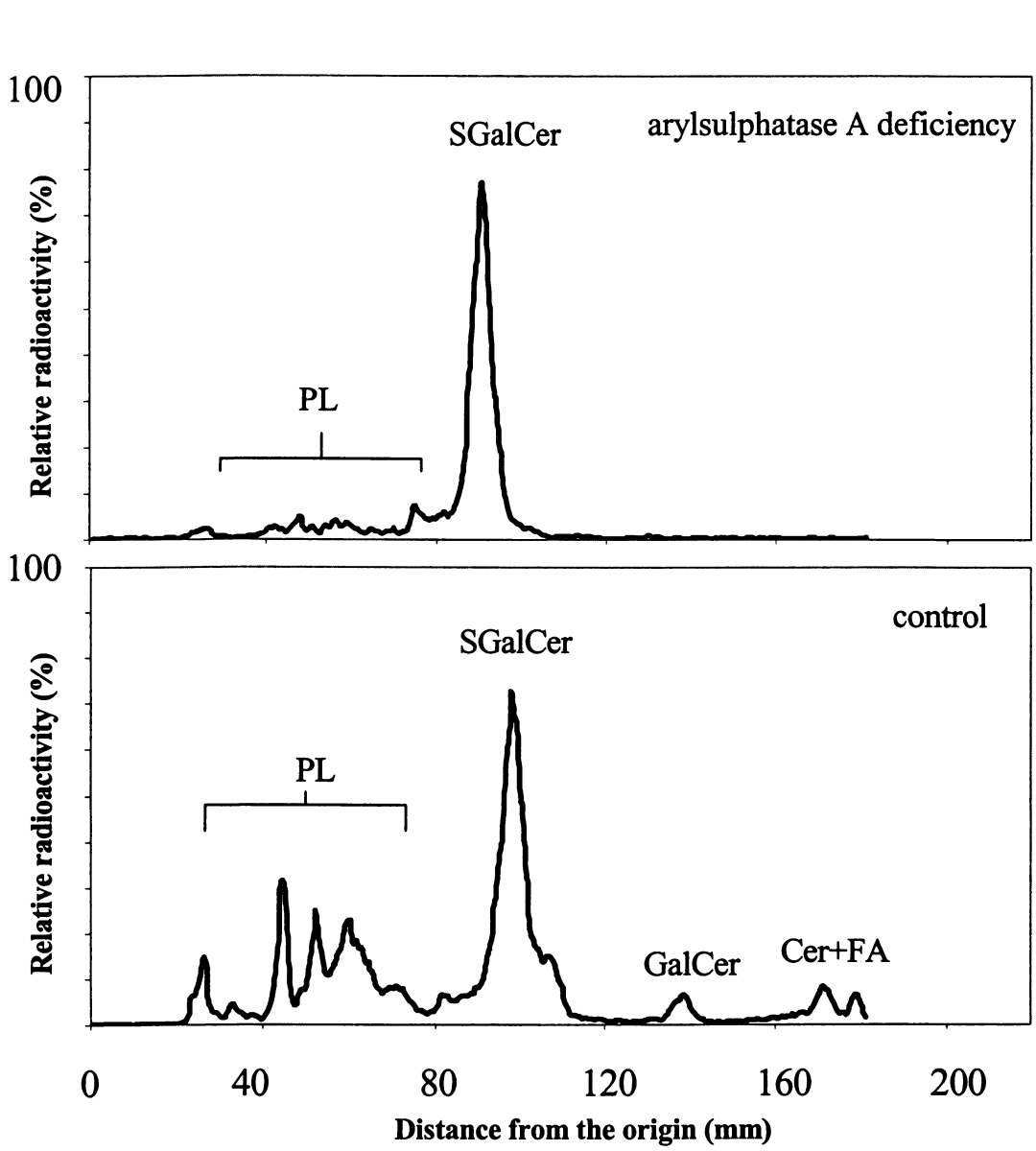
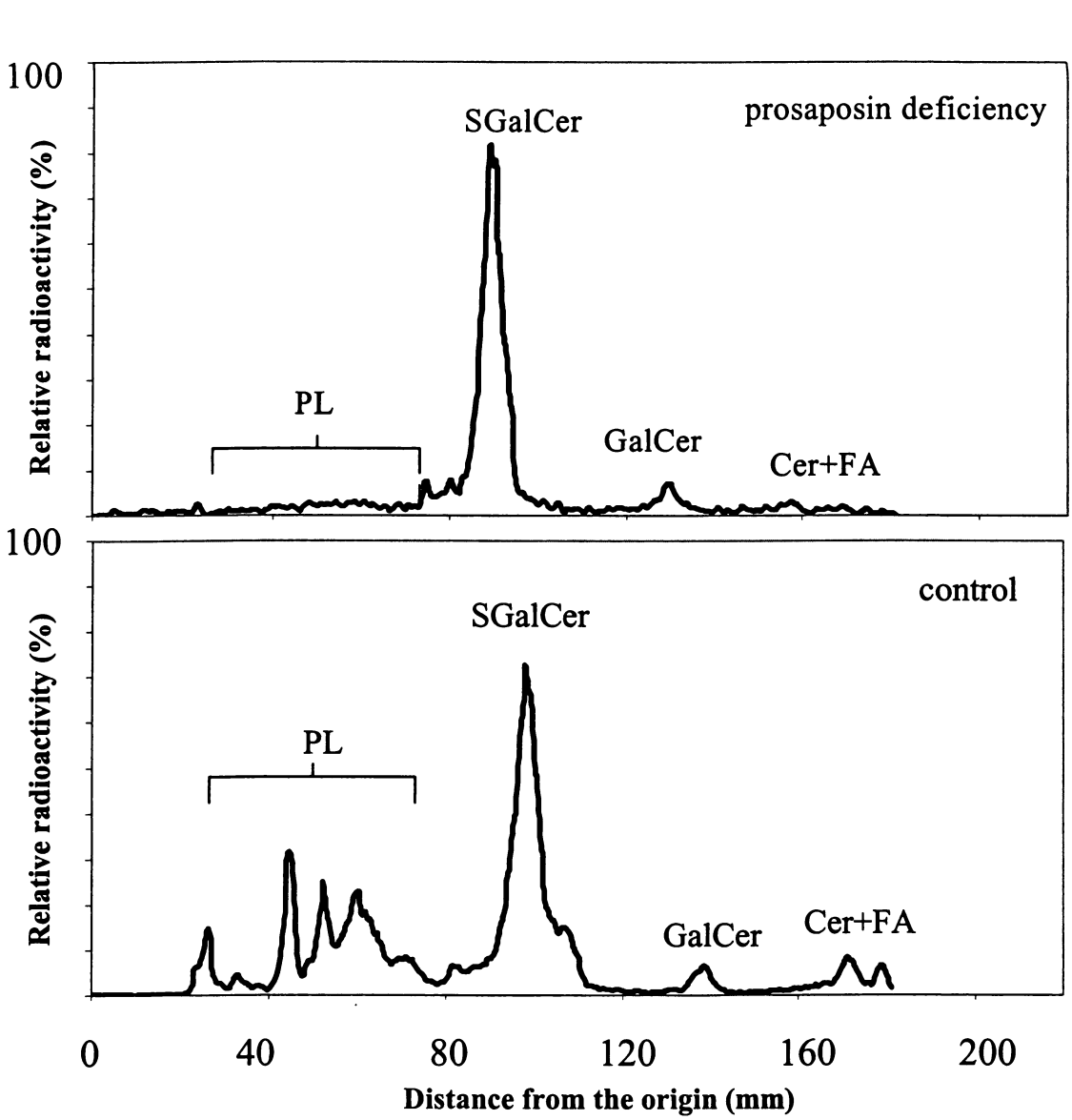


Fig. 8: Degradation of [^{3}H]-sulphatide by skin fibroblasts from patients with arylsulphatase A and prosaposin deficiencies and a control. Tritium labelled sulphatide (5×10^6 dpm) incorporated into liposomes, was added into the culture medium as described in Methods. After 5 days cells were harvested, the lipids extracted and separated by HPTLC (ascending technique in a system chloroform-methanol-water (70:30:05, v/v/v)).

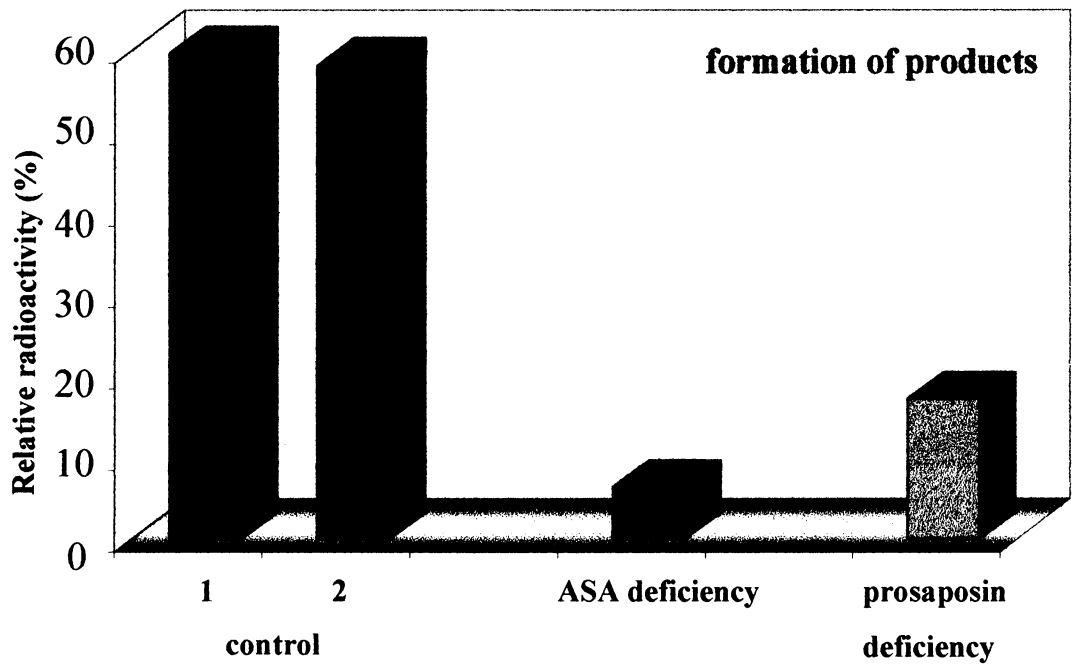
A: Degradation of [^{3}H]-sulphatide by cultured skin fibroblasts with arylsulphatase A deficiency and of a control.



B: Degradation of sulphatide by cultured skin fibroblasts with prosaposin deficiency and by control cells.

PL – phospholipids, **SGalCer** – galactosylceramide-3-sulphate, **GalCer** – galactosylceramide, **Cer** – ceramide, **FA** – fatty acids

C



C: Quantification of products (a sum of all products) formed by arylsulphatase A deficient, prosaposin deficient, and control cells. Values are average of at least three experiments.

[^{3}H]-globoside loading

Radioactively labelled globoside (precursor of critical substrate Gb_3Cer) was used as a substrate for feeding of the fibroblasts from patients with Fabry disease and prosaposin deficiency. Conduritol B epoxide serves as an inhibitor of glucosylceramidase and as such was added to all cultures in order to limit formation of resynthesis products (Fig. 9).

In the α-galactosidase A deficient cells globoside was first degraded to Gb_3Cer (substrate of α-galactosidase A). In the second step of degradation profound defect in catabolism of Gb_3Cer in the α-galactosidase A deficient cell lines was evident (Fig. 9A). In the prosaposin deficient cells, the degradation of Gb_4Cer was also normal as in control cells (Fig. 9B) and thus we conclude that none of the saposins formed from prosaposin precursor participates in the first step of degradation of this glycolipid. However in the next step, accumulation of Gb_3Cer occured similarly as in Fabry cells. Furthermore, we have observed (inspite of almost complete block in degradation of Gb_3Cer) small amount of its degradation product LacCer. This result shows that the degradation of both glycolipids is dependent on the presence of saposins.

The difference in a quantity of products (Fig. 9C, patients 1, 2, 3) is caused by different residual activities of α-galactosidase A in these patients (the residual activity of a patient with cardiac variant of Fabry disease was the highest). The residual activities in variants of Fabry disease (classical versus cardiac) correlated with clinical status of these patients.

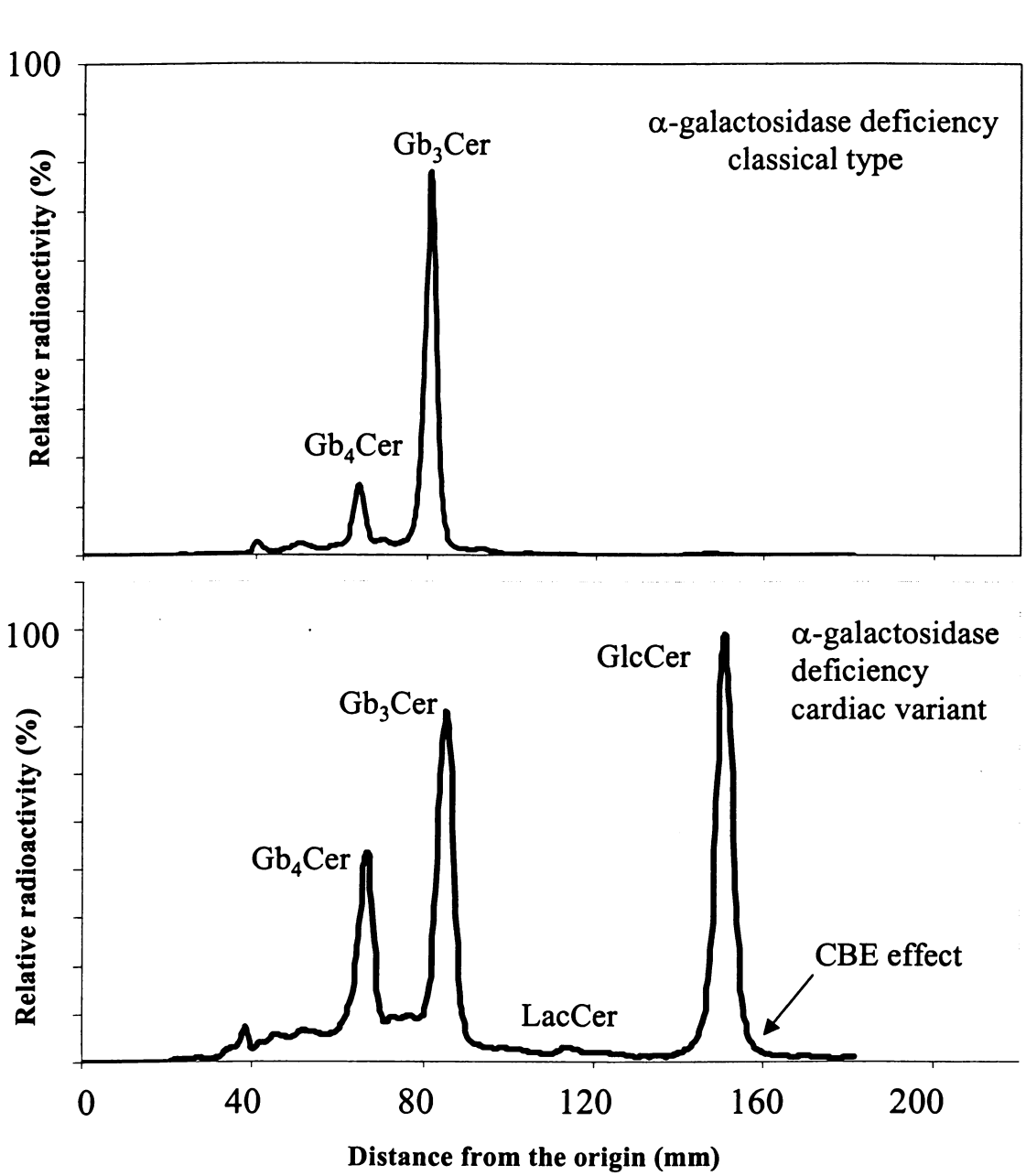
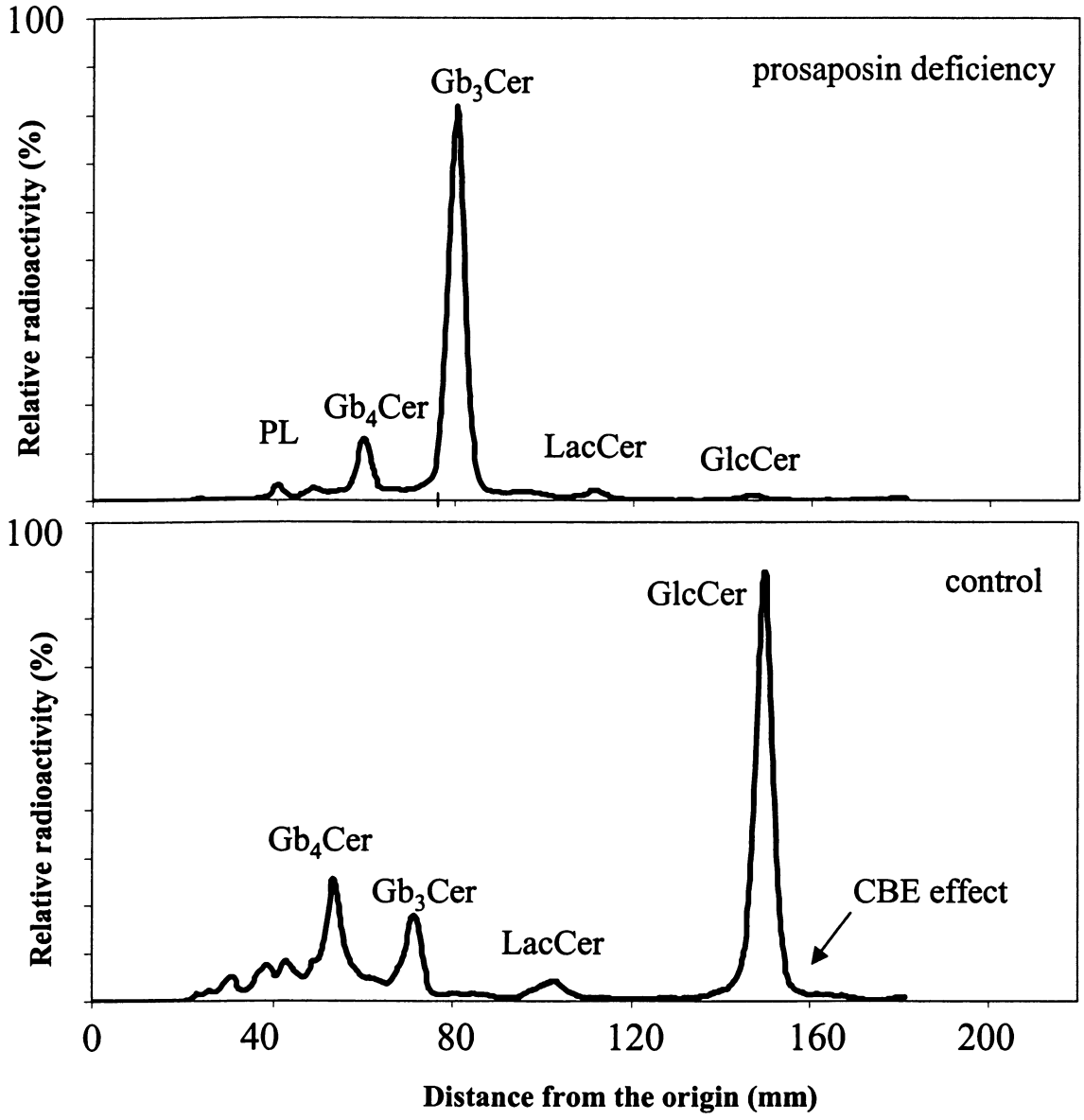


Fig. 9: Degradation of [^{3}H]-Gb_3Cer by skin fibroblasts from patients with α-galactosidase A and prosaposin deficiencies and from a control in presence of CBE.. Tritium labelled globoside (5×10^6 dpm) incorporated into the liposomes coated with apolipoprotein Apo-E, was added into the culture medium as described in Methods. After 5 days cells were harvested, the lipids extracted and separated by HPTLC (ascending technique in a system chloroform-methanol-water (70:30:05, v/v/v)).

A: Degradation of [^{3}H]-Gb_3Cer by cultured skin fibroblasts from a classical Fabry patient and a patient with cardiac variant of the disease.

B



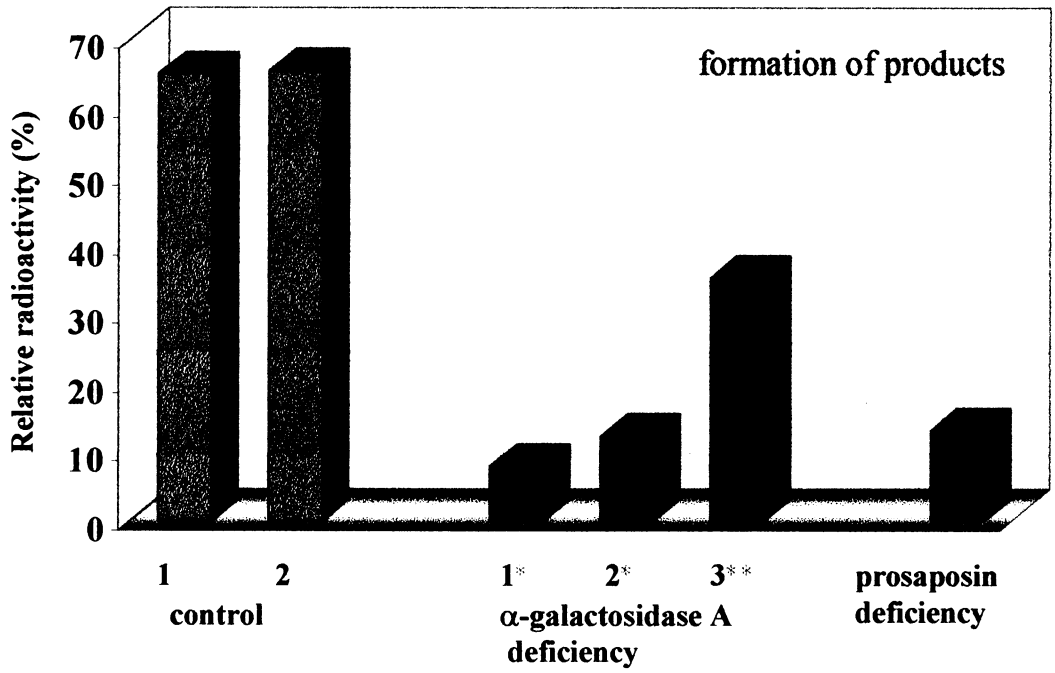
B: Degradation of Gb_3Cer by cultured skin fibroblasts with prosaposin deficiency and of a control.

PL – phospholipids, **Gb_4Cer** – globoside, **Gb_3Cer** – globotriaosylceramide,

LacCer – lactosylceramide, **GlcCer** – glucosylceramide, **Cer** – ceramide, **FA** – fatty acids,

CBE – conduritol B epoxide

C



* Classical type of Fabry disease ** Cardiac variant of Fabry disease

C: Quantification of products (a sum of all products) formed by α-galactosidase A deficient, prosaposin deficient, and control cells. Values are average of at least three experiments.

Studies of effects of human recombinant α-galactosidase A

Preparation of enzyme stock solution

Enzymes E 1 and E 2 were dissolved to the same final concentration according to the manufacturers´ recommendation. In both solutions the protein concentration was verified. The concentration in E 2 was determined to 1.06 mg/ml and in E 1 to 1.17 mg/ml.

Characterization of recombinant enzymes

Optimal experimental conditions for in vitro enzyme activity measurement

Optimal dilution of the stock solutions for enzyme assays had to be determined to measure the enzyme activity in these solutions. Dilution 80 000x was finally found to be optimal. The enzymes were diluted in BSA solution (1mg/ml). BSA was used to maintain the optimal protein concentration for stability of the enzyme during the assay [64]. Dilution of the stock solutions in PBS was found unsuitable for enzyme assays (results not shown). BSA solution was verified to have zero α-galactosidase A activity. The activity of an E 2 stock solution was 2.779 $mmol.mg^{-1}.hr^{-1}$ and of an E 1 2.845 $mmol.mg^{-1}.hr^{-1}$.

Stability of enzymes in culture medium

Stability of recombinant α-galactosidases A in the culture medium (DMEM with 10 % FCS, without cells) under defined condition (37°C, humidified atmosphere, 5 % CO_2) was studied. The activity of both enzymes decreased significantly under the experimental conditions, after 48 hours the residual activity of both α-galactosidases A was approximately 15 % of the initial activity (Fig. 10). On the basis of these results, the periods of incubations in experiments that followed were set to the maximum of 48 hours.

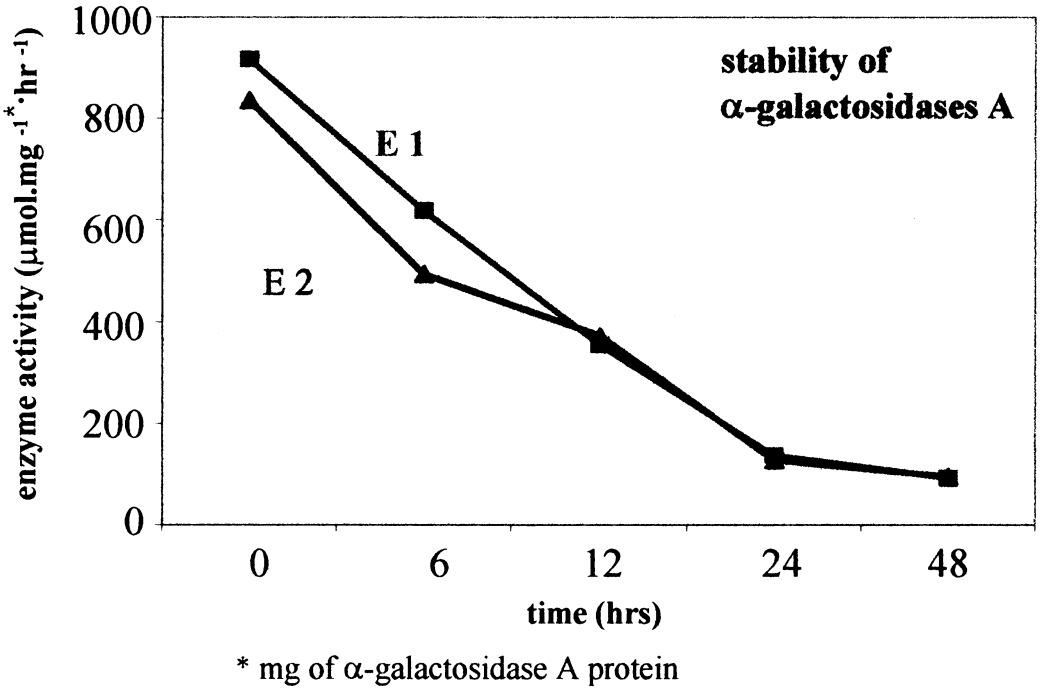


Fig. 10: **Time dependent *in vitro* activity of the recombinant α-galactosidases A (E-2 and E-1) in culture medium.** Recombinant enzymes were added to the medium (DMEM with 10 % FCS) at final activities of 1.896 $\mu mol.ml^{-1}.hr^{-1}$ and 1.852 $\mu mol.ml^{-1}.hr^{-1}$ for E 1and E 2, respectively, and incubated at 37°C. The activity of the enzymes were assayed after 0, 6, 12, 24 and 48 hours as described in Methods. Values are average of three measurements.

Study of internalization of recombinant enzymes into the enzyme-deficient cell

The ability of recombinant α-galactosidases A to be taken up through plasma membrane of target cells was investigated. It was proved by *in vitro* determination of enzyme activity in α-galactosidase A deficient cells that the transport of E 1 through the membrane is more efficient. At the maximum difference after 18 hours of incubation the activity of E 1 in cells was 1.96 $\mu mol.mg^{-1}.hr^{-1}$ while in E 2 was 0.31 $\mu mol.mg^{-1}.hr^{-1}$ (approximately 6× lower)(Fig. 11A). The quantitative difference in internalization of these enzymes through cell membranes may also reflect their rate of degradation of Gb_3Cer preloaded into the Fabry fibroblasts. Following experiments intended to proved this assumption.

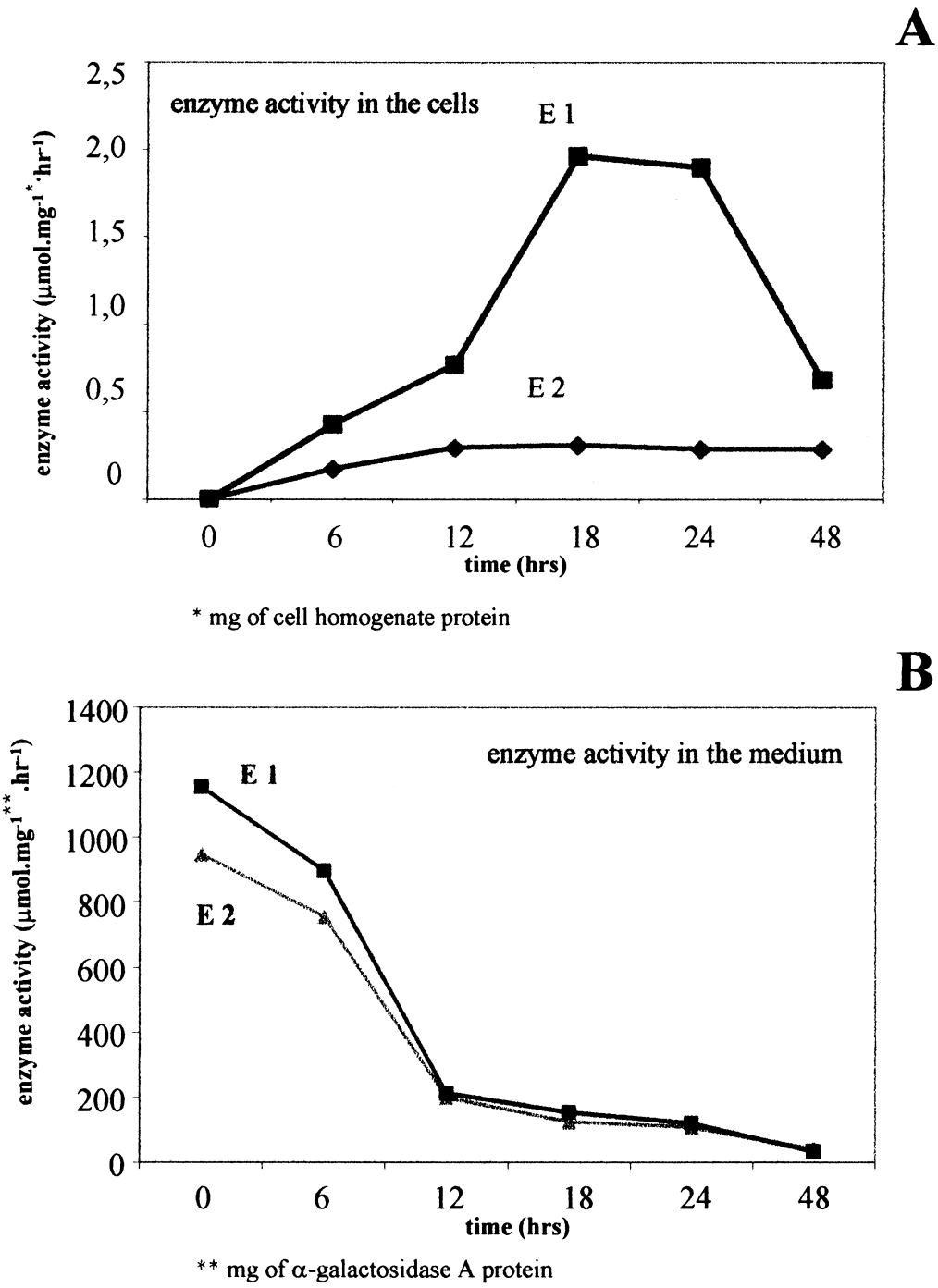


Fig.11: Internalization of recombinant enzymes (E 1 and E 2) by skin fibroblast cells with Fabry disease. Prepared recombinant enzymes were applied into the cell line with α-galactosidase A deficiency and incubated under standard conditions. After 0, 6, 12, 18, 24 and 48 hours medium was removed and cells were harvested as described in Methods. Enzyme activity in media and cell homogenates was measured.

A: Activity of recombinant enzymes in the cells during 48 hours.

B: Activity of recombinant enzymes in the medium during 48 hours.

Studies of degradation of [^{3}H]-globoside by recombinant α-galactosidases A

The recombinant forms of α-galactosidase A were added to the media of cultured skin fibroblasts from a patient with Fabry disease preloaded with [^{3}H]-globoside. The ability of the exogenously added enzyme to be transported to the cells and to catabolize the accumulated Gb_3Cer substrate was clearly demonstrated (Fig. 12A, B.). Degradation of Gb_3Cer to less polar products was iniciated by both E 1 and E 2. Although the experimental conditions were identical for both enzyme preparations, quantified results of product formation showed less lipid products of Gb_3Cer degradation in experiments with E 2 than with E 1 (Fig. 12C, D).

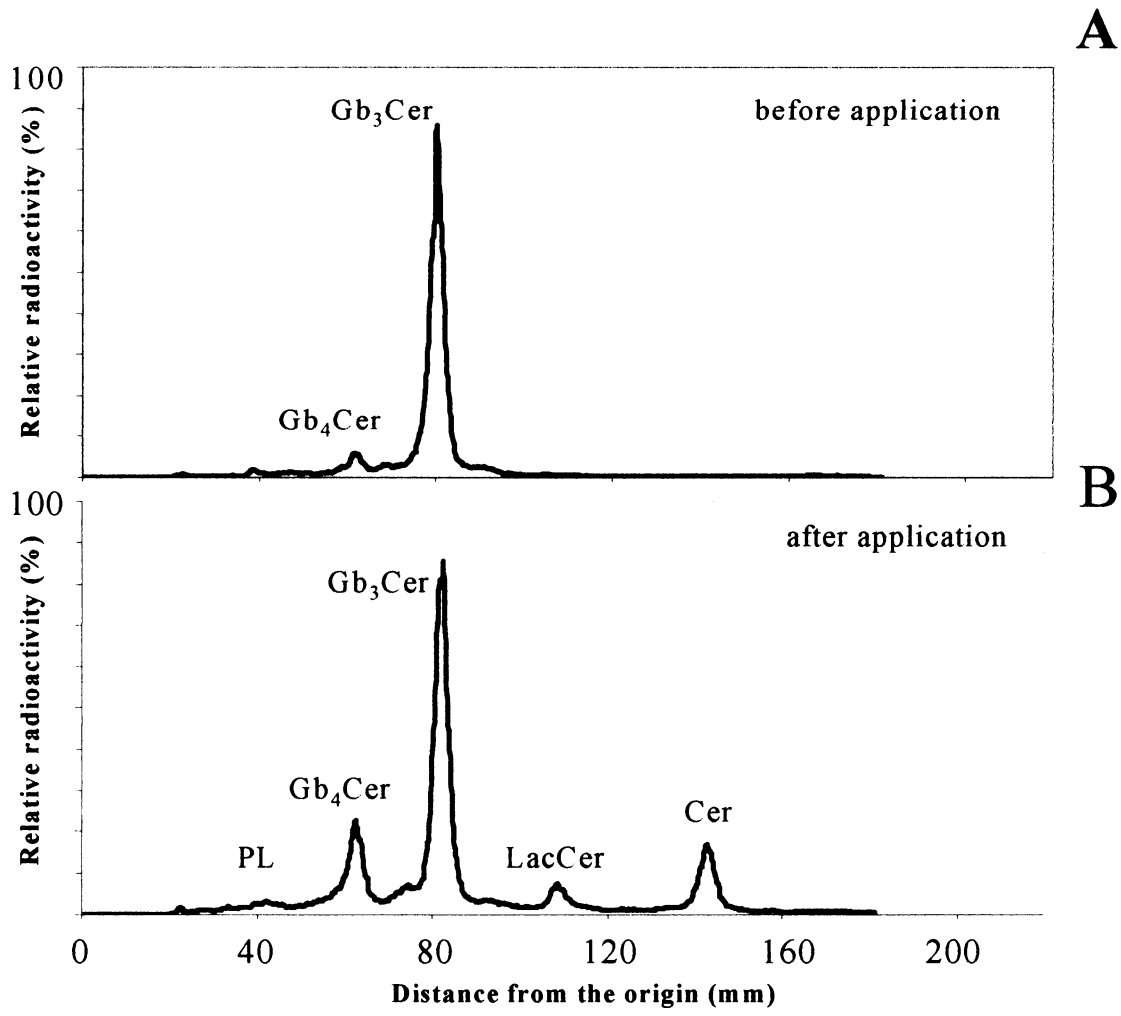
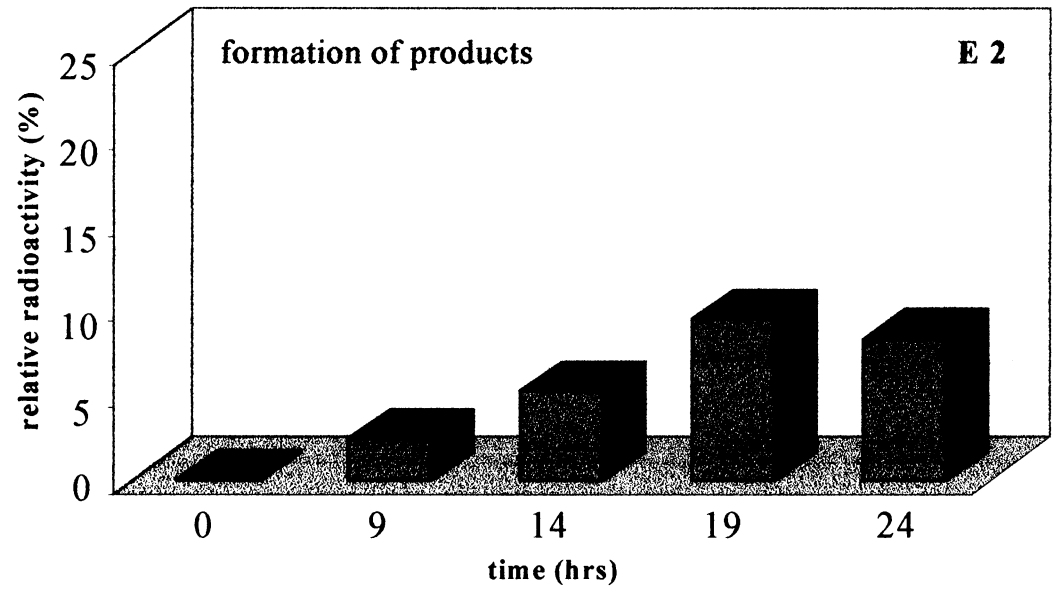


Fig. 12: Degradation of [^{3}H]-Gb_3Cer by skin fibroblasts from patients with Fabry disease loaded with [^{3}H]-globoside before (A) and after (B) application of recombinant α-galactosidase A. Tritium labelled globoside (5×10^6 dpm) incorporated into the liposomes coated with Apo E, was added to the culture medium of skin fibroblasts as described in Methods. After 3 days of incubation the recombinant α-galactosidase A solutions were applied at final activities of 1.896 $\mu mol.ml^{-1}.hr^{-1}$ and 1.852 $\mu mol.ml^{-1}.hr^{-1}$ for E 1and E 2, respectively. After 0, 9, 14, 19 and 24 hours cells were harvested, the lipids extracted and separated by HPTLC (ascending technique in system chloroform-methanol-water (70:30:05, v/v/v)).

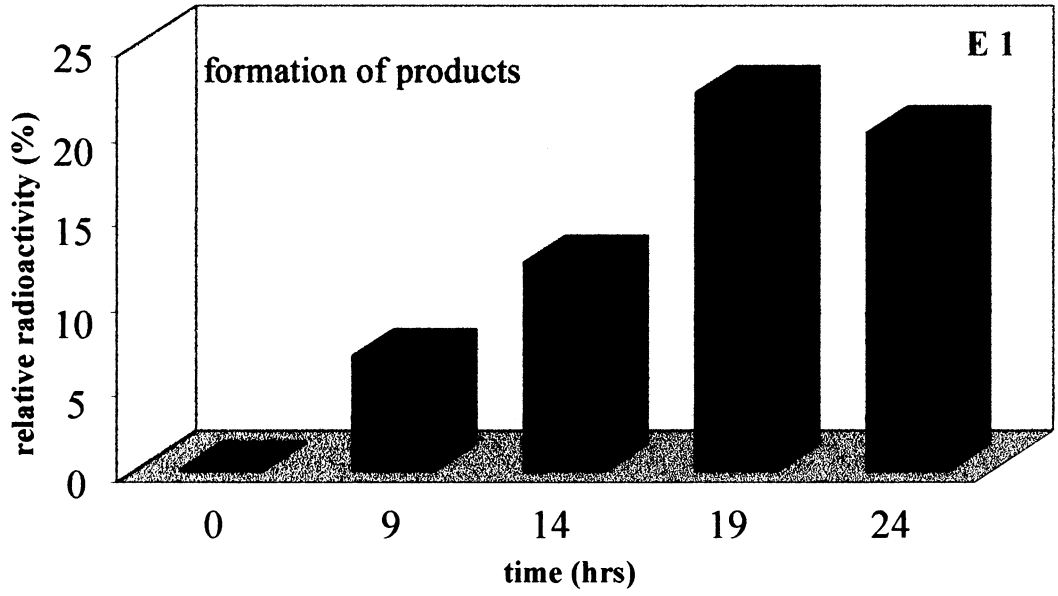
A: Accumulation of Gb_3Cer by α-galactosidase A deficient cells before the application of α-galactosidase A

B: The formation of products after application of recombinant α-galactosidase A (illustration of 24 hrs experiment)

C



D



C: Quantification of products (a sum of all products) formed by α-galactosidase deficient cells after the action of E 2.

D: Quantification of products (a sum of all products) formed by α-galactosidase deficient cells after the action of E 1.

Discussion

In the research of lysosomal storage diseases cell culture feeding experiments with radioactively labelled glycolipids and analysis of their metabolites have been frequently used for various purposes such as investigation of general metabolic pathways, assessment of residual activity in enzyme deficient cells or diagnosis of storage disorders. These experiments were especially useful for identification of defects on catabolic pathways in metachromatic leukodystrophy [54], GM-1 gangliosidosis or SAP deficiencies and another lipidoses [65, 66].

Efficient targeting of radiolabelled glycolipid substrates into the lysosomes and their transport through the plasma membrane may cause some difficulties. The radioactively labelled substrates can be applied in the form of micelles, formed after the addition of minute volume of the substrate ethanol solution into culture medium. The use of higher concentrations of ethanol, however, may be toxic for the cells. Another commonly employed method is the application of the substrates incorporated in phospolipid liposomes.

Phospholipids together with glycolipids form, under certain conditions, small unilamelar hydrophobic particles, which can fuse with plasma membrane, be internalized and transported to the lysosomes in the form of vesicules [58]. Moreover, uptake of the substrates into the cells can be potentiated by receptor-mediated endocytosis. In this approach, molecules, which are recognized by specific receptors on the plasma membrane, are incorporated into the liposomes together with the substrate. Apo-E and -B recognised by LDL receptors on the cell surface are examples of such molecules. Liposome particles with incorporated labelled substrate and coated with Apo-E are recognized by receptors, internalized and transported to the lysosomes. Precultivation of the cells in the lipoprotein-free medium leads to increased expression of appropriate receptor and subsequently to higher uptake of the substrate into the cells [59]. Results of our previous work indicate, that the optimal substrate application is in the form of Apo-E coated liposomes after 48 hour preincubation in the lipoprotein-free medium ("cell starving") [67].

We have also tried to further increase the substrate incorporation by using statins. Statins are normally used for reduction of cholesterol levels by blocking its endogenous synthesis in cells [57]. We hypothesized that addition of statins to the culture medium during preincubation stage could lead to higher LDL receptor expression and subsequently to higher

intake of the liposomes by receptor mediated endocytosis. In our pilot experiments, however, any significant effect of statins was not observed. Therefore these compounds were not included in this study.

[^{3}H]-Sulphatide loading experiments are used for diagnostic purposes, primarily for distinguishing between genuine MLD and arylsulphatase A pseudodeficiency [29]. MLD has several phenotypic variants depending on residual activity of the enzyme. Patients with a late-infantile variant have no or minimal residual activity and are most severely affected. Conversely, the individuals with higher levels of the residual ASA activity typically have milder symptoms and the disease manifests later in the life. We have observed slight substrate degradation (probably contribution of other sulphatases) in late-infantile MLD cells after 5 day incubation of the substrate in the form of liposomes. This rate of degradation was markedly lower than degradation in control cells (Fig. 8A).

Prosaposin deficiency is a very rare metabolic disease, only four cases have been described so far. The only available cultured skin fibroblasts from one of the patients were studied in our Institute. This is the first study of cells with prosaposin defect loaded with sulphatide and globoside.

Sulphatide feeding of prosaposin deficient cells revealed considerable defect in degradation. However, the pattern of accumulated glycolipids in prosaposin deficient cells and MLD cells were different. In the case of prosaposin deficient cells not only sulphatide but also GalCer and Cer were accumulated thus supporting the picture of complex sphingolipidosis (Fig. 8B).

Tritium labelled globoside (Gb_4Cer) isolated from erythrocyte stroma has been employed in metabolic studies of α-galactosidase A deficient cells (Fabry cells) together with control cultures. The advantage of this substrate is that α-linked galactose is in penultimate position in the saccharide chain. The critical substrate, Gb_3Cer, is formed directly in lysosomes by the action of β-N-acetylgalactosaminidase. It ensures that also the next glycohydrolase participating in the Gb_3Cer degradation, i.e. α-galactosidase A is of lysosomal origin. The results of Gb_4Cer loading experiment clearly demonstrate the accumulation of Gb_3Cer in Fabry cells (Fig. 9A) and degradation of this substrate in a control culture. High peak of GlcCer visible in control samples is caused by addition of glucosylceramidase inhibitor conduritol B epoxid. The use of glucosylceramidase inhibitor blocks further degradation of the substrate and helps to evaluate more precisely the TLC radioscans.

Globoside feeding of prosaposin deficient cells also revealed considerable defect in sphingolipid degradation. The similar patterns of degradation products were found in Fabry cells and prosaposin deficient cells, although the underlying reason for the defect of hydrolytic function is different. Accumulation of Gb_3Cer in Fabry disease skin fibroblasts is the result of the defect in α-galactosidase A gene but in the prosaposin deficiency cells the accumulation is due to the lack of SAP proteins, i.e. mutation in a prosaposin gene. SAPs B, -C and -D originate from a proteolytic cleavage of prosaposin protein molecule (see Introduction). The complete absence of prosaposin, in our case due to the mutation in the iniciation codon of PROSAP gene [68], results in generalized accumulation of glycolipids with less then four monosaccharide units. We have observed marked accumulation of Gb_3Cer and also increased amount of LacCer after 5 days of feeding (Fig. 9B). After prolonged incubation of cells with the glycolipids substrate accumulation in prosaposin deficient cells would be even more evident.

Recently, two new drugs for causal cure of Fabry disease, based on recombinant α-galactosidase A, have been developed [69, 70]. One of the goals of this diploma thesis was to investigate the efficiency of the recombinant enzyme transport from medium into the lysosomal compartment as well as the degradation of critical substrate Gb_3Cer in Fabry cells cured with the recombinant α-galactosidases A.

Enzymes E 1 and E 2 were dissolved according to the manufacturers' guidelines. However, for *in vitro* determination of enzyme activity enormous dilution of the stock solution (80 000 fold) with 0.1 % BSA in water was inevitable.

The activity of both enzymes measured with artificial substrate did not differ significantly. However, under experimental conditions the activity decreased markedly in time. The residual activity was approximately 15% of initial activity after 48 hour in culture medium (Fig .10).

After addition of recombinant α-galactosidase A into the culture of Fabry skin fibroblasts with preloaded tritium labelled Gb_4Cer, the formation of degradation products was observed in case of both of enzymes. Nevertheless, current results show faster *in situ* degradation in cells supplemented with E 1 in comparison with E 2 (Fig. 12). We tried to find out the reason for these differences. E 1 and E 2 were applied into the cell cultures under

identical conditions and their incorporation into the cells was monitored. We observed much more efficient transport of E 1 into the cells than in case of E 2. We believe that this is the underlying reason for differences in degradation rates in both enzymes. Lysosomal enzymes in culture medium are transported to lysosomes by manose-6-phosphate receptor mediated endocytosis [71]. The differences in glycosylation may play vital role in efficiency of enzyme internalization into the cell. Both enzymes are recombinant human α-galactosidase A, but they are produced in different expression systems [69,70] in human fibroblast cell lines or in genetically engineered CHO cells. Due to the differences in expression systems and methods utilized during the manufacturers' process, the enzymes may differ in their glycosylation patterns. These differences may be the reason for higher levels of incorporated enzyme and subsequently faster substrate degradation with E 1 observed in Fabry cell cultures.

However, we have to bear in mind, that these experiments represent results performed only on one cell type and can not be applied to the whole organism. Also the experimental conditions differ from *in vivo* situation. Despite the differences described above, both recombinant α-galactosidases A are efficient in cleaving accumulated Gb_3Cer as it was clearly demonstrated by this study.

Conclusion

I. Degradation studies of glycolipid substrates in cell cultures with specific defects of catabolism lead to the following findings:

- in dynamic experiment with [^{3}H]-sulphatide, block in degradation was proved in ASA-deficient fibroblasts due to inherited defect of ASA gene. Similar block in sulphatide degradation was found in prosaposin deficient cells –in this case caused by the absence of corresponding activator SAP B due to the mutation in prosaposin gene.

- in another series of loading experiments with [^{3}H]-globoside, defect in degradation of Gb_3Cer was determined on α-galactosidase A deficient cells. Using this test, classical and cardiac variants of Fabry disease were clearly distinquished. Defect in Gb_3Cer degradation was also observed in prosaposin deficient cells due to the absence of SAP B, similarly as for sulphatide.

- results on prosaposin deficient cells are unique in that
  - complex defect has been demonstrated for the first time by dynamic experiments with globoside and sulphatide on primary cells
  - it has been proved that not only enzyme defect but also its activator absence can cause fatal block of catabolism

II. Study of efficiency of two recombinant α-galactosidases A (E 1 and E 2) to degrade Gb_3Cer in Fabry fibroblasts. Conclusions are following:

- procedure of *in vitro* measurement of α-galactosidase A activity has been adjusted for highly concentrated enzyme preparations

- both enzymes were internalized by cultured cells but in different rates

- both enzymes were efficient in degradation of preloaded Gb_3Cer in α-galactosidase A deficient cells. It indicates their effectivness in treatment of this defect

References

1. UPAC (1999) IUPAC-IUB joint commission on biochemical nomenclature. In *J Mol Biol*, Vol. 286, pp. 963-70.
2. Schwarzmann, G. and Sandhoff, K. (1990) *Biochemistry,* **29,** 10865-10871.
3. Hakomori, S. (1986) *Sci Am,* **254,** 44-53.
4. Kolter, T. and Sandhoff, K. (1998) *Brain Pathology,* **8,** 79-100.
5. Sullards, M.C. (2000) *Methods Enzymol,* **312,** 32-45.
6. Yamashita, T., Wada, R., Sasaki, T., Deng, C., Bierfreund, U., Sandhoff, K. and Proia, R.L. (1999) *Proc Natl Acad Sci U S A,* **96,** 9142-7.
7. Hakomori, S. (1981) *Annu Rev Biochem,* **50,** 733-64.
8. Holmgren, J., Lonnroth, I., Mansson, J. and Svennerholm, L. (1975) *Proc Natl Acad Sci U S A,* **72,** 2520-4.
9. Markwell, M.A., Svennerholm, L. and Paulson, J.C. (1981) *Proc Natl Acad Sci U S A,* **78,** 5406-10.
10. Karlsson, K.A. (1989) *Annu Rev Biochem,* **58,** 309-50.
11. Sandhoff, K. and Kolter, T. (1997) *Clin Chim Acta,* **266,** 51-61.
12. Suzuki, K. (1987) Enzymatic diagnosis of sphingolipidoses. In *Methods in Enzymology*, Vol. 138.
13. Harzer, K., Paton, B.C., Poulos, A., Kustermann-Kuhn, B., Roggendorf, W., Grisar, T. and Popp, M. (1989) *Eur J Pediatr,* **149,** 31-9.
14. Ogawa, K., Sugamata, K., Funamoto, N., Abe, T., Sato, T., Nagashima, K. and Ohkawa, S. (1990) *Hum Pathol,* **21,** 1067-73.
15. Ioannou, Y.A., Zeidner, K.M., Grace, M.E. and Desnick, R.J. (1998) *Biochem J,* **332,** 789-97.
16. Schiffmann, R., Murray, G.J., Treco, D., Daniel, P., Sellos-Moura, M., Myers, M., Quirk, J.M., Zirzow, G.C., Borowski, M., Loveday, K. *et al.* (2000) *Proc Natl Acad Sci U S A,* **97,** 365-70.
17. Kornfeld, S. and Mellman, I. (1989) *Annu Rev Cell Biol,* **5,** 483-525.
18. Desnick, R.J., Ioannou, Y.A. and Eng, C.M. (2001) Fabry Disease. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds.), *The metabolic and molecular bases of inherited disease.* 8th ed. McGrew-Hill, New York, Vol. 3, pp. 3733-3774.

19. Davies, J., Christomanou, H., Winchester, B. and Malcolm, S. (1994) *Hum Mol Genet,* **3,** 667-9.

20. Ashley, G.A., Shabbeer, J., Yasuda, M., Eng, C.M. and Desnick, R.J. (2001) *J Hum Genet,* **46,** 192-6.

21. Branton, M.H., Schiffmann, R., Sabnis, S.G., Murray, G.J., Quirk, J.M., Altarescu, G., Goldfarb, L., Brady, R.O., Balow, J.E., Austin Iii, H.A. *et al.* (2002) *Medicine (Baltimore),* **81,** 122-38.

22. Ioannou, Y.A., Zeidner, K.M., Gordon, R.E. and Desnick, R.J. (2001) *Am. J. Hum. Genet.,* **68,** 14-25.

23. Kleijer, W.J., Hussaarts-Odijk, L.M., Sachs, E.S., Jahoda, M.G. and Niermeijer, M.F. (1987) *Prenat Diagn,* **7,** 283-7.

24. Hřebíček, M. Grant project IGAMZ (1999).

25. Schiffmann, R., Kopp, J.B., Austin, H.A., Sabnis, S., Moore, D.F., Weibel, T., Balow, J.E. and Brady, R.O. (2001) *Jama,* **285,** 2743-9.

26. Desnick, R.J., Banikazemi, M. and Wasserstein, M. (2002) *Clinical Nephrology,* **1,** 1-8.

27. Matsuura, F., Ohta, M., Ioannou, Y.A. and Desnick, R.J. (1998) *Glycobiology,* **8,** 329-339.

28. Lemansky, P., Bishop, D.F., Desnick, R.J., Hasilik, A. and von Figura, K. (1987) *The Journal of Biol. Chem.,* **262,** 2062-2065.

29. von Figura, K., Gieselmann, V. and Jaeken, J. (2001) Metachromatic leukodystrophy. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds.), *The metabolic and molecular bases of inherited disease.* 8th ed. McGrew-Hill, New York, Vol. 3, pp. 3695-3724.

30. Sandhoff, K., Kolter, T. and Harzer, K. (2001) Sphingolipid Activator Proteins. In Scriver, C.R. (ed.), *The metabolic and molecular bases of inherited disease*, New York, Vol. 3, pp. 3371-3388.

31. James, G.T., Thach, A.B., Klassen, L. and Austin, J.H. (1985) *Life Sci,* **37,** 2365-71.

32. Farooqui, A.A. (1976) *Arch Int Physiol Biochim,* **84,** 479-92.

33. Laidler, P.M., Waheed, A. and Van Etten, R.L. (1985) *Biochim Biophys Acta,* **827,** 73-83.

34. Lukatela, G., Krauss, N., Theis, K., Selmer, T., Gieselmann, V., von Figura, K. and Saenger, W. (1998) *Biochemistry,* **37,** 3654-64.

35. Poretz, R.D., Yang, R.S., Canas, B., Lackland, H., Stein, S. and Manowitz, P. (1992) *Biochem J,* **287 (Pt 3),** 979-83.

36. Kreysing, J., von Figura, K. and Gieselmann, V. (1990) *Eur J Biochem,* **191,** 627-31.

37. Gieselmann, V., Matzner, U., Hess, B., Lullmann-Rauch, R., Coenen, R., Hartmann, D., D'Hooge, R., DeDeyn, P. and Nagels, G. (1998) *J Inherit Metab Dis,* **21,** 564-74.

38. Polten, A., Fluharty, A.L., Fluharty, C.B., Kappler, J., von Figura, K. and Gieselmann, V. (1991) *N Engl J Med,* **324,** 18-22.

39. Barth, M.L., Fensom, A. and Harris, A. (1993) *Hum Genet,* **91,** 73-7.

40. Lee-Vaupel, M. and Conzelmann, E. (1987) *Clin Chim Acta,* **164,** 171-80.

41. Wenger, D.A., Williams, C. (1991) *Techniques in Diagnostic Human Biochemical Genetics: A Laboratory Manual.* Willey-Liss, Inc.

42. Berna, L., Asfaw, B., Conzelmann, E., Cerny, B. and Ledvinova, J. (1999) *Anal Biochem,* **269,** 304-11.

43. Herz, B. and Bach, G. (1984) *Hum Genet,* **66,** 147-50.

44. Gieselmann, V., Polten, A., Kreysing, J. and von Figura, K. (1989) *Proc Natl Acad Sci U S A,* **86,** 9436-40.

45. Barth, M.L., Ward, C., Harris, A., Saad, A. and Fensom, A. (1994) *J Med Genet,* **31,** 667-71.

46. Kihara, H., Fluharty, A.L., Tsay, K.K., Bachman, R.P., Stephens, J.D. and Ng, W.G. (1983) *Prenat Diagn,* **3,** 29-34.

47. Desnick, R.J., Schuette, J.L., Golbus, M.S., Jackson, L., Lubs, H.A., Ledbetter, D.H., Mahoney, M.J., Pergament, E., Simpson, J.L., Zachary, J.M. *et al.* (1992) *Prenat Diagn,* **12,** 357-72.

48. Wrobe, D., Henseler, M., Huettler, S., Pascual Pascual, S.I., Chabas, A. and Sandhoff, K. (2000) *J Inherit Metab Dis,* **23,** 63-76.

49. Bierfreund, U., Kolter, T. and Sandhoff, K. (2000) *Methods Enzymol,* **311,** 255-76.

50. Wilkening, G., Linke, T., Uhlhorn-Dierks, G. and Sandhoff, K. (2000) *J Biol Chem,* **275,** 35814-9.

51. Schuette, C.G., Pierstorff, B., Huettler, S. and Sandhoff, K. (2001) *Glycobiology,* **11,** 81R-90R.

52. Matsuda, J., Vanier, M.T., Saito, Y., Tohyama, J. and Suzuki, K. (2001) *Hum Mol Genet,* **10,** 1191-9.

53. Booth, C.W., Chen, K.K. and Nadler, H.L. (1975) *J Pediatr,* **86,** 560-4.

54. Kudoh, T. and Wenger, D.A. (1982) *J Clin Invest,* **70,** 89-97.

55. Wigley, C.B., Roberts, P.L., Cartwright, T., Shah, G.P., Dobbie, I., Zicha, D., McAteer, J.A., Davis, J.M. and MacDonald, J.M.e.a. (2002) *Basic Cell Culture.* 2nd ed. Davis. J.M., Oxford.

56. Mahley, R.W. and Innerarity, T.L. (1983) *Biochim Biophys Acta,* **737,** 197-222.

57. Istvan, E.S., Palnitkar, M., Buchanan, S.K. and Deisenhofer, J. (2000) *Embo J,* **19,** 819-30.

58. Asfaw, B., Schindler, D., Ledvinova, J., Cerny, B., Smid, F. and Conzelmann, E. (1998) *J Lipid Res,* **39,** 1768-80.

59. Asfaw, B., Ledvinova, J., Dobrovolny, R., Bakker, H.D., Desnick, R.J., van Diggelen, O.P., de Jong, J.G., Kanzaki, T., Chabas, A., Maire, I. *et al.* (2002) *J Lipid Res,* **43,** 1096-104.

60. Suzuki, K. (1965) *J Neurochem,* **12,** 629-38.

61. Schnaar, R.L. (1994) Method in Enzymology. In *Method in Enzymology.* Academic Press, Vol. 230, pp. 348-370.

62. Berná, L. (1996) *Diploma thesis, Department of biochemistry and microbiology.,* Institute of chemical technology, Prague, pp. 20-24.

63. Hartree, E.F. (1972) *Anal Biochem,* **48,** 422-7.

64. Young, E.P. (1992) *J Inherit Metab Dis,* **15,** 224-30.

65. Schmid, B., Paton, B.C., Sandhoff, K. and Harzer, K. (1992) *Hum Genet,* **89,** 513-8.

66. Sonderfeld, S., Conzelmann, E., Schwarzmann, G., Burg, J., Hinrichs, U. and Sandhoff, K. (1985) *Eur J Biochem,* **149,** 247-55.

67. Dobrovolný, R. (1999) *Diploma thesis, Department of Biochemistry.,* The Charles University, Prague, pp. 9-11.

68. Hulkova, H., Cervenkova, M., Ledvinova, J., Tochackova, M., Hrebicek, M., Poupetova, H., Befekadu, A., Berna, L., Paton, B.C., Harzer, K. *et al.* (2001) *Hum Mol Genet,* **10,** 927-40.

69. TKT Europe (Information Brochure, Enzyme replacement therapies for Fabry disease).

70. Genzyme Corporation (Product Monograph, Enzyme replacement therapy for Fabry disease).

71. Hille-Rehfeld, A. (1995) *Biochim Biophys Acta,* **1241,** 177-94.

Souhlasím s vypůjčením diplomové práce ke studijním účelům:

Jméno a příjmení	Číslo OP	Datum vypůjčení	Poznámka