Journal of Chromatography, 533 (1990) 11–21
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam

CHROMBIO. 5499

Pulsed amperometric detection of carbohydrates in lysosomal storage disease fibroblasts: a new screening technique for carbohydrate storage diseases

HENK J BLOM, HANS C ANDERSSON, DONNA M KRASNEWICH and WILLIAM A GAHL*

Section on Human Biochemical Genetics, Human Genetics Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892 (U S A)

(First received May 16th, 1990, revised manuscript received July 23rd, 1990)

ABSTRACT

A first step in determining the metabolic defect in patients with an unknown storage disease is to identify the stored material In the case of fibroblasts storing carbohydrates, this can be accomplished by trifluoroacetic acid (TFA) hydrolysis producing monosaccharides which are separated by anion-exchange chromatography and quantitated by pulsed amperometric detection. This technique separates neutral, amino, and acidic monosaccharides in a single run with a detection limit of 50 pmol The method, applied to hydrolyzed 100 000 *g* supernatants of ten normal fibroblast sonicates, revealed a mean ± S.D content of the following monosaccharides (in nmol/mg of protein) fucose, 7 ± 3, galactosamine, 4 ± 2, glucosamine, 20 ± 3, galactose, 11 ± 3. mannose, 27 ± 6, glucuronic acid, 56 ± 28, iduronic acid, 17 ± 11 Six mucopolysaccharidosis fibroblast strains (types I, II, IIIB, IVA, VI and VII) contained 2 to 8 times the normal glucuronic acid levels, and types I and II exhibited 10- to 30-fold normal levels of iduronic acid and 40-fold increases in galactosamine All the mucopolysaccharidoses could be distinguished from normal based upon an increased concentration of some monosaccharide Fibroblasts from patients with mannosidosis and fucosidosis contained 7-fold normal amounts of mannose and 11-fold normal amounts of fucose, respectively The quantitation of monosaccharides in fibroblasts after TFA hydrolysis can identify cells that store excess amounts of a glycosaminoglycan, glycoprotein, oligosaccharide or, presumably, a glycolipid. This may comprise the first step toward identifying novel lysosomal storage disorders and point the way toward new glycoconjugate degradative pathways

INTRODUCTION

The degradation of glycosaminoglycans (GAGs), glycoproteins, glycolipids and oligosaccharides by specific hydrolytic enzymes occurs primarily in cellular lysosomes The monosaccharides produced generally cross the lysosomal membrane by carrier-mediated transport [1–4]. Mutations in lysosomal hydrolytic enzymes can result in storage of macromolecules, *e.g.* GAGs, causing lysosomal storage disorders, *e g.* mucopolysaccharidoses (MPSs) [5]. Similarly, impaired lysosomal monosaccharide transport can result in accumulation of these small molecules and the characteristic clinical manifestations of a storage disorder [3,6].

0378-4347/90/$03.50

To screen for lysosomal carbohydrate storage diseases, thin-layer chromatography of carbohydrates in urine has been employed. This technique is not quantitative, overlapping of spots can interfere with a definitive diagnosis, and patients may fail to excrete increased amounts of the stored material in their urine [5]. Currently, the specific diagnosis of clinically suspected lysosomal storage diseases requires the direct measurement of individual lysosomal enzymes in fibroblasts or leucocytes. Deficiency states have been described for more than twenty lysosomal enzymes and the phenotypes of these diseases overlap, making diagnosis laborious and expensive. In addition, novel disorders will not be recognized by this process

We now describe a convenient screening method for quantitating monosaccharides in human fibroblasts cultured from normal individuals and patients with carbohydrate storage disorders. The technique, which can be performed with or without acid hydrolysis, employs high-performance liquid chromatography (HPLC) for separation and pulsed amperometric detection for quantitation of carbohydrates. In addition to aiding in the diagnosis of known lysosomal storage diseases by identifying elevations of certain monosaccharides, the method can be applied toward identifying the carbohydrate stored in novel or currently unrecognized disorders of carbohydrate metabolism.

EXPERIMENTAL

Chemicals

Monosaccharides were purchased from Sigma (St. Louis, MO, U.S.A) or Aldrich (Milwaukee, WI, U.S.A), with the exception of iduronic acid (IdUA). IdUA was obtained from Dr. G. Ashwell, who synthesized it according to Shafizadeh and Wolfrom [7]. Heparan sulfate (bovine kidney), dermatan sulfate (bovine mucosa) and keratan sulfate (bovine cornea) were from Sigma, and trifluoroacetic acid (TFA) (spectrophotometric grade) was from Aldrich. Sodium acetate and sodium hydroxide (50%, w/w) were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). A standard amino acid mixture was purchased from Pierce (Rockford, IL, U.S.A.).

Fibroblasts

Normal fibroblast strains GM 1489, 1501, 3440, 3651, 5659 and 5757, as well as mutant strains from patients with MPS I (GM 798), MPS II (GM 1929), MPS IIIB (GM 2552), MPS IVA (GM 593), MPS VI (GM 519), MPS VII (GM 121), mannosidosis (GM 4518) and fucosidosis (GM 292) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.) Other normal cell strains were obtained after informed consent from healthy individuals initially referred to rule out an unrelated diagnosis.

Fibroblasts were cultured in Dulbecco's modified eagle medium (Bioproducts, Walkersville, MD, U S.A.) fortified with 10% heat-inactivated fetal calf serum

(Gibco, Grand Island, NY, U.S.A) and 2 m*M* glutamine and antibiotics as described previously [3,6]. Two to three weeks after reaching confluency, the cells were harvested by trypsinization and washed 4 times with cold phosphate-buffered saline. The cell pellet was sonicated twice for 20 s in 2 ml of cold distilled water. After removing the nuclear and membrane fractions by centrifugation at 100 000 *g* for 60 min, the supernatant, containing soluble carbohydrates, was subjected to TFA hydrolysis. The protein content of the supernatant was determined by the bicinchoninic acid method [8].

TFA hydrolysis

TFA (4 *M*, 0.75 ml) was added to 0.75 ml of a cell culture's 100 000 *g* supernatant, and the mixture was heated at 100°C for 3 h The tubes were cooled and the mixture was lyophilized. The dry residue was taken up in 300 μl of 0.01 *M* sodium hydroxide and filtered into autosampler tubes using 0.45-μm Millipore-type HV filters (Yonezawa, Japan).

For the hydrolysis of heparan sulfate, dermatan sulfate and keratan sulfate, 300 μl of 4 *M* TFA were added to 0.05 mg of each GAG dissolved in 300 μl of water, and treated as above.

Separation and detection of monosaccharides

Monosaccharides were analyzed on a Dionex HPLC apparatus equipped with a gradient pump and an Ionchrom pulsed amperometric detector (E_1 = 0.05 V, T_1 = 480 ms; E_2 = 0.60 V, T_2 = 360 ms; E_3 = −0.60 V, T_3 = 240 ms). A Dionex eluent degas model was used to sparge and pressurize the eluents. Samples (15–150 μl) were injected using a Shimadzu SIL-6A autoinjector connected to a Shimadzu SCL-6A system controller. Monosaccharides were separated on a Dionex Carbopac AS6 column (250 mm × 4 mm) with an AS6 guard column (25 mm × 3 mm) at a flow-rate of 1.0 ml/min. The detector signal was plotted and integrated using a Spectra-Physics 4270 integrator

Neutral monosaccharides [fucose (Fuc), galactose (Gal), glucose (Glc) and mannose (Man)] and amino monosaccharides [galactosamine (GalN) and glucosamine (GlcN)] were separated at a low sodium hydroxide concentration. After increasing the sodium hydroxide concentration to 0.1 *M*, acidic monosaccharides such as sialic acid, glucuronic acid (GlcUA) and IdUA were separated by gradient increases in the sodium acetate concentration (from 0 to 0.7 *M*). Three different eluent reservoirs (0.001 and 0.2 *M* sodium hydroxide and 1 5 *M* sodium acetate) were employed to achieve the gradient as shown in Fig. 1.

RESULTS

A mixture of authentic monosaccharides representing constituents of common glycoproteins, glycolipids, oligosaccharides and GAGs was injected into the HPLC apparatus (Fig. 2). Separation of the neutral, amino and acidic mono-

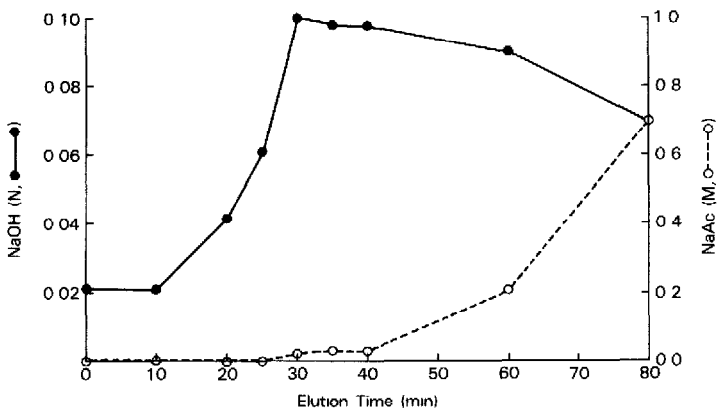

Fig 1 Sodium hydroxide and sodium acetate gradients used for separating monosaccharides

saccharides was achieved, with a detection limit of approximately 50 pmol for Fuc, Man, Glc, Gal, GalN, GlcN, GlcUA and IdUA. In addition, the following compounds were easily detected using 250 pmol: mannosamine, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), N-acetylmannosamine (ManNAC), D-arabinose, D-xylose, D-ribose, adenosine, cytidine, guanosine, uridine, 2-deoxy-D-ribose, 2-deoxy-D-glucose, L-rhamnose, D-fructose, D-gluconic acid, D-galactonic acid, D-galacturonic acid, D-mannuronic acid, N-glycolylneura-

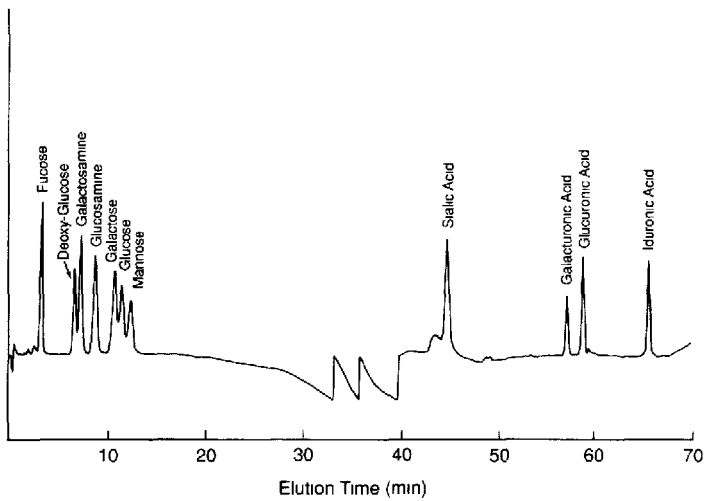

Fig. 2 Separation of standard monosaccharides in a single chromatogram Each of the neutral and charged sugars was present in the mixture in the amount of 625 pmol. The sensitivity can be increased by an order of magnitude Artifacts between 30 and 40 min are due to automatic zeroing for buffer changes Neutral and amino monosaccharides have a retention time of 2–15 min and charged monosaccharides elute between 40 and 70 min

minic acid, glucosamine-2-sulfate, D-mannose-6-phosphate and sialyl-lactose (data not shown). Peak areas correlated linearly with monosaccharide amounts injected, up to 4 nmol. No peaks were detected after injection of a standard amino acid mixture containing 2.5 nmol of 35 different amino acids.

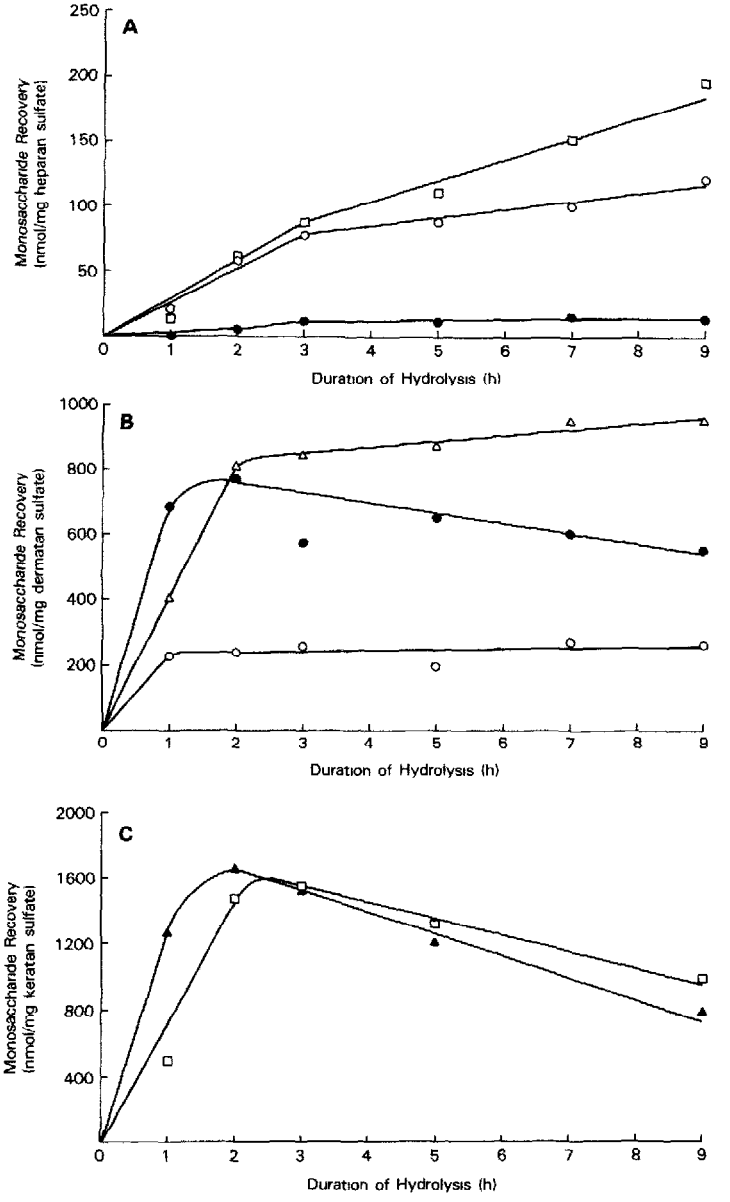

Fig 3 Liberation of monosaccharides from the GAGS heparan sulfate (A), dermatan sulfate (B) and keratan sulfate (C) by TFA hydrolysis △ = GalN, □ = GlcN, ▲ = Gal, ○ = GlcUA; ● = IdUA

Hydrolysis of monosaccharide standards using 2 *M* TFA at 100°C for 3 h gave different yields for each sugar. Results of duplicate determinations were: Fuc, 76%; GalN, 58%; GlcN 70%; Glc, 85%; Man, 98%; sialic acid, 0%, GlcUA, 69%; and IdUA 44%. TFA hydrolysis of N-acetylated monosaccharides yields the free amino monosaccharides, as previously described [9] In addition, TFA hydrolysis of glucosamine-2-sulfate yields free GlcN (data not shown).

GAGs were employed as model carbohydrates to determine the optimal duration of TFA hydrolysis for liberation of single sugars. For heparan sulfate, the yield of GlcN and GlcUA increased steadily for up to 9 h of hydrolysis; small amounts of IdUA were also produced (Fig. 3A) For dermatan sulfate, maximal amounts of GalN, GlcUA and IdUA were formed after 2 h of TFA hydrolysis (Fig. 3B). The hydrolysis of keratan sulfate liberated Gal and GlcN optimally at 2 and 3 h, respectively (Fig. 3C). Based upon these results and similar findings reported for other large glycoconjugates [9], a 3-h period of TFA hydrolysis was chosen for subsequent analyses. Under these conditions, and assuming the average molecular mass of a monosaccharide moiety in GAGs to be 300, the recovery for heparan sulfate was calculated to be only 15%. For dermatan sulfate, the recovery was 50% and for keratan sulfate, 94%.

A 3-h TFA hydrolysis at 100°C of the 100 000 *g* supernatant fraction of normal fibroblasts yielded a typical spectrum of monosaccharides (Fig. 4). An initial peak eluting before Fuc was invariantly associated with sample injection and most likely represented unbound material. In addition to the neutral, amino and acidic sugars, other unidentified peaks such as one with an elution time of approximately 25 min (Fig. 4) were prominent in normal fibroblast samples (*n* =

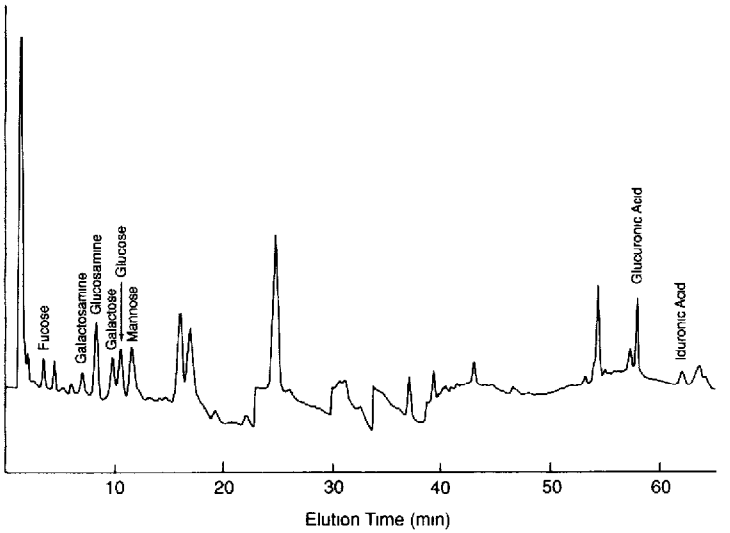

Fig 4 Spectrum of monosaccharides obtained after TFA hydrolysis of the 100 000 *g* supernatant fraction of normal (GM 5757) fibroblasts

TABLE I

CARBOHYDRATE CONTENT OF THE 100 000 *g* SUPERNATANT FRACTION OF SONICATED NORMAL AND MUTANT FIBROBLASTS AFTER TFA HYDROLYSIS

Fibroblast[a]	Carbohydrate content (nmol/mg of protein)						
	Fuc	GalN	GlcN	Gal	Man	GlcUA	IdUA
Mucopolysaccharidoses[b]							
I (Hurler)	14	163	41	22	63	257	166
II (Hunter)	16	179	49	31	79	284	472
IIIB (Sanfilippo)	9	4	28	13	29	220	14
IVA (Morquio)	23	10	40	12	56	103	19
VI (Maroteaux-Lamy)	17	32	52	18	57	477	40
VII (Sly)	26	22	68	21	70	428	32
Glycoproteinoses[b]							
Mannosidosis	6	2	52	8	198	58	11
Fucosidosis	82	5	61	13	54	39	17
Normal (n = 10)							
Mean ± S D	7 ± 3	4 ± 2	20 ± 3	11 ± 3	27 ± 6	56 ± 28	17 ± 11

[a] Fibroblasts were grown for one to three weeks after reaching confluency. A 100 000 *g* supernatant fraction was hydrolyzed at 100°C with 2 *M* TFA for 3 h and subjected to HPLC and pulsed amperometric detection.

[b] Results are means of duplicate determinations.

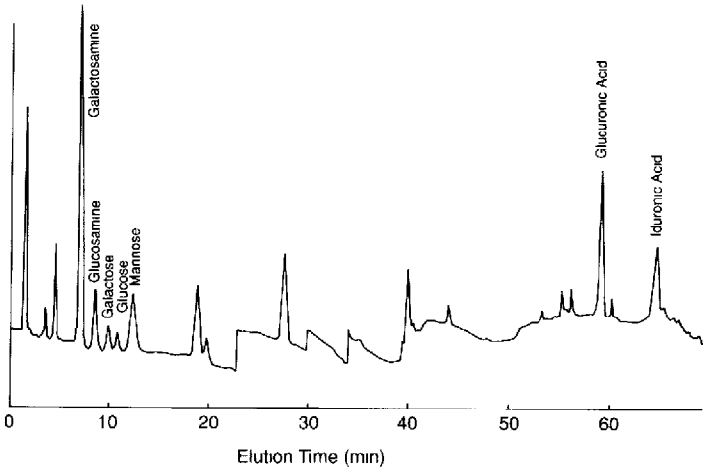

Fig. 5. Spectrum of monosaccharides obtained after TFA hydrolysis of the 100 000 *g* supernatant fraction of MPS I fibroblasts.

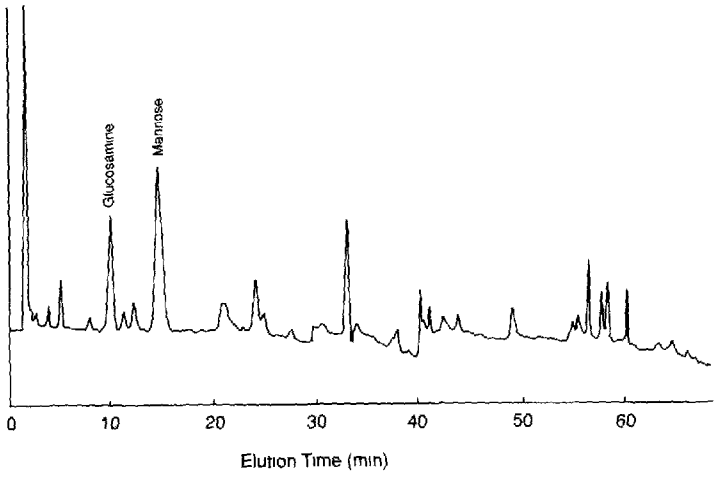

Fig. 6 Spectrum of monosaccharides obtained after TFA hydrolysis of the 100 000 g supernatant fraction of mannosidosis fibroblasts

10). These peaks may represent the products of secondary hydrolysis or oligosaccharides which may have been partially resistant to hydrolysis. For these cell strains, the mean concentrations of seven monosaccharides derived from GAG hydrolysis, expressed in nmol/mg of protein, are given in Table I. GlcUA levels were highest and showed the greatest variation IdUA concentrations also varied somewhat while the other monosaccharide levels were maintained in a close range.

The monosaccharide spectrum of a strain of MPS I fibroblasts was characterized by prominent GalN, GlcUA and IdUA peaks (Fig. 5). Fibroblasts from a patient with mannosidosis displayed an elevated Man peak, apparent on visual inspection (Fig. 6). On quantitation of pertinent monosaccharides, the MPSs (types I–VII) all exhibited markedly elevated levels of GlcUA. MPS types I and II also showed impressive elevations of GalN and IdUA, while MPS VI and VII cells also had moderate increases in GalN and GlcN levels. Man was mildly increased in all the MPSs except type IIIB. Mannosidosis and fucosidosis exhibited remarkably elevated concentrations of Man and Fuc, respectively, along with a mild increase in GlcN (Table I).

DISCUSSION

Several screening techniques, including the use of 1,9-dimethylmethylene blue dye on a paper matrix [10], liquid chromatography [11] and gas chromatography [12], have been directed toward the quantitation of large carbohydrates such as GAGs [10]. When applied to human urine, these methods are non-invasive, rapid and employ a relatively protein-free fluid for analysis. However, urinary excre-

tion of carbohydrates can vary considerably in storage disorders [5], making its use as a screening tool unreliable.

The investigation of fibroblasts cultured from patients with presumed carbohydrate storage disorders avoids these problems and has several other advantages. Fibroblasts express the defect in most storage disorders, including all the MPSs, and would be expected to express the defect in novel carbohydrate storage diseases. The amount of stored material can be increased by growing the cells long past confluence or by feeding with precursors [6–13]. In this fashion, fibroblasts can be examined for storage of carbohydrates resulting from currently unknown metabolic defects, and precursor studies can be performed once a storage compound is identified. Manipulation of the cell culture environment also opens a broad avenue for pinpointing the defect in an unknown storage disorder

An appropriate technique for quantitating carbohydrates in storage fibroblasts is anion-exchange chromatography at high pH followed by pulsed amperometric detection. This method of analysis has been succesfully applied to the separation of positional isomers of oligosaccharides and glycopeptides [14,15]. For investigating lysosomal storage disorders, fibroblast samples must first be hydrolyzed because heterogeneity of the stored material results in a spectrum of many high-molecular-mass compounds each giving a small detection signal Use of a hydrolysis step makes the pulsed amperometric detection technique applicable to the quantitation of monosaccharide moieties present in glycoproteins, glycolipids, oligosaccharides and GAGS. When employed with a sodium hydroxide gradient followed by a sodium acetate gradient, the method has the added advantage of detecting neutral, amino and charged monosaccharides in a single run The resulting spectrum can reveal a wide variety of disorders of carbohydrate metabolism.

A 3-h hydrolysis in 2 *M* TFA degrades glycoconjugates reasonably well [9] (Fig. 3), but has certain other effects that must be considered. First, TFA splits the N-acetyl group from the amino monosaccharides [9], *e.g.* degrading the GlcNAc of heparan sulfate and keratan sulfate to GlcN and the GalNAc of dermatan sulfate to GalN. Second, N-acetylneuraminic acid (NANA) is completely degraded by TFA hydrolysis, impairing the ability to detect this sugar stored in sialidosis, Salla disease, infantile free sialic acid storage disease (ISSD) sialuria and I-cell disease Fortunately, hydrolysis with 0.1 *M* sulfuric acid not only liberates NANA from oligosaccharides but also preserves NANA [6] and, therefore, can be employed to detect NANA-containing compounds which are stored inside fibroblasts. Finally, the extent of TFA hydrolysis differs for certain model GAGs, such as heparan sulfate, dermatan sulfate and keratan sulfate, and the recovery of monosaccharides from heparan sulfate is quite low. However, this will be true for normal as well as storage disease fibroblasts, and the TFA hydrolysis-pulsed amperometric detection method should still detect the 10-to-100-fold increased concentrations of sugars typically found in storage disease cells.

MPSs and glycoproteinoses were chosen as model carbohydrate storage dis-

orders with which to test this proposal. Using our detection protocol, MPS I (Hurler disease, α-L-iduronidase deficiency) and MPS II (Hunter disease, iduronate sulfatase deficiency) cells were found to contain 100-fold increases of IdUA, 40-fold increases of GalN and 5-fold increases of GlcUA, all reflecting the storage of dermatan sulfate [5]. The modest increase of GlcN most likely occurred because of poor hydrolysis by TFA of heparan sulfate, the other GAG stored in these two MPSs. MPS IIIB (Sanfilippo disease, α-N-acetylglucosaminidase deficiency) stores only heparan sulfate and manifested a 4-fold increase in GlcUA concentration, with normal IdUA and GlcN levels. MPS VI (Maroteaux-Lamy, N-acetylgalactosamine-4-sulfatase deficiency) stores only dermatan sulfate and exhibited a mild increase in IdUA and 8-fold increases in GalN and GlcUA The monosaccharide levels of MPS VII (Sly disease, β-glucuronidase deficiency) resembled those of MPS VI, although MPS VII stores heparan sulfate as well as dermatan sulfate. The impressive storage of IdUA in MPS I and II contrasts markedly with the minimal accumulation of IdUA in MPS types IIIB, VI and VII, despite the fact that the IdUA-containing GAGs dermatan sulfate and heparan sulfate are considered stored in all these diseases. This suggests that a lysosomal endoglycosidase may exist to hydrolyze sugar linkages at specific IdUA residues within a GAG. While this technique does not offer diagnostic specificity, it may act as a guide in selecting appropriate lysosomal enzyme analysis.

Although TFA degraded standard keratan sulfate extremely well (Fig. 3C), the monosaccharides expected from its hydrolysis, Gal and GlcN, were not prominent in MPS IVA (Morquio disease, galactose-6-sulfatase deficiency), which stores keratan sulfate. This may be because MPS IVA fibroblasts store minimal amounts of keratan sulfate, since the tissue distribution of this GAG is restricted to cornea and cartilage. Alternatively, keratan sulfate may be present in human fibroblasts but may resist TFA hydrolysis, while commercially available keratan sulfate from bovine cornea is very susceptible to TFA hydrolysis

The glycoproteinoses fucosidosis and mannosidosis manifested the anticipated increases in Fuc and Man, along with mild elevations of GlcN, which reflect the fact that GlcNAc-containing oligosaccharides are stored in these diseases [16].

In general, the TFA hydrolysis-pulsed amperometric detection protocol yielded the expected elevations of monosaccharides for MPS and glycoproteinosis fibroblasts, and demonstrated its possible application as a screening method for this group of disorders. This technique can now be adapted to the study of carbohydrate storage diseases, either lysosomal or cytosolic, in which the stored material, whether a GAG, glycoprotein, glycolipid or oligosaccharide, has not yet been identified. Defects of the lysosomal carbohydrate transport system [3,4] would be expected to be identified by expanding the present protocol to include sulfuric acid hydrolysis, which leaves N-acetylated sugars intact. In time, lysosome-rich granular fractions might be studied as an embellishment of this technique for improving the diagnosis and definition of carbohydrate storage disorders.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the "Ter Meulen Fonds" (Amsterdam, The Netherlands). The authors appreciate the helpful comments of Dr. Frank Tietze of NIDDK (Bethesda, MD, U.S.A.) and Dr. George Thomas of the Kennedy Institute and John Hopkins Medical School (Baltimore, MD, U.S.A.). The excellent secretarial work of Carol Becker and Jacqueline Sharkey is gratefully acknowledged.

REFERENCES

1 K Docherty, G V Brenchley and C N Hales, *Biochem J*, 178 (1979) 362
2 G A Maguire, K Docherty and C N Hales, *Biochem J*, 212 (1983) 211
3 M. Renlund, F. Tietze and W. A Gahl, *Science*, 232 (1986) 759
4 A J Jonas, R J Speller, P B Conrad and W P Dubinsky, *J Biol Chem*, 264 (1989) 4953
5 E F. Neufeld and J Muenzer, in C. R. Scriver, A L Beaudet, W S Sly and D Valle (Editors), *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 1989, p 1565.
6 F Tietze, R Seppala, M Renlund, J. J Hopwood, G S Harper, G H Thomas and W A Gahl, *J Biol Chem*, 264 (1989) 15 316
7 F. Shafizadeh and M L Wolfrom, *J Am Chem Soc.*, 77 (1959) 2568
8 P K Smith, R I Krohn, G. T Hermanson, A. K Mallia, F H Gartner, M D Provenzano, E K Fujimoto, N M Goeke, B J Olson and D. C. Klenk, *Anal Biochem*, 150 (1985) 76
9 M R Hardy, R R Townsend and Y C Lee, *Anal Biochem*, 170 (1988) 54.
10 C B Whitley, K A Draper, C. M. Dutton, P A Brown, S L Severson and L. A France, *Clin Chem*, 35 (1989) 2074
11 C Kodama, N Ototani, M Isemura, J Aikawa and Z Yosizawa, *Clin Chem*, 32 (1986) 30
12 P W. Larking, *J. Chromatogr*, 420 (1987) 231
13 P Weiss, F Tietze, W. A Gahl, R Seppala and G Ashwell, *J. Biol Chem*, 264 (1989) 17 635
14 M R Hardy and R. R. Townsend, *Proc Natl Acad Sci U S A*, 85 (1988) 3289.
15 R R Townsend, M R Hardy, D A Cumming, J P Carver and B Bendiak, *Anal Biochem*, 182 (1989) 1
16 A L Beaudet and G H Thomas, in C R. Scriver, A. L. Beaudet, W S Sly and D Valle (Editors), *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 1989, p 1603.