Gargoylism: Hydrolysis of β-Galactosides and Tissue Accumulation of Galactose- and Mannose-Containing Compounds

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ABSTRACT The sugars present in hydrolyzed extracts of human liver and brain were analyzed by gas-liquid chromatography after conversion to their alditol acetates. The samples analyzed were obtained from control subjects, patients with gargoylism, and patients with a few other kinds of storage disorders. Accumulation of galactose was demonstrated in the liver and the brain of two patients with gargoylism, and in the liver samples, high levels of mannose were found too. We also studied the hydrolysis of a number of galactosides by homogenates from different tissues in the control subjects and in the patients. Separation methods and kinetic studies demonstrated the presence in normal human tissues of two different β-galactosidases, which we call enzyme A and enzyme B, respectively. Enzyme A hydrolyzed all the β-galactosides tested. Enzyme B hydrolyzed the synthetic substrates tested (4-methylumbelliferyl-, *p*-nitrophenyl-, *o*-nitrophenyl-, and phenyl-β-galactoside) but not the natural substrates tested (ceramide-β-galactoside, ceramide lactoside, transferrin glycopeptide, and keratan sulfate). Enzyme B also exerted β-glucosidase activity. In various tissues from patients with gargoylism, deficiency of β-galactosidase A could be demonstrated.

It is suggested that the high level of galactose found in the hydrolyzed extracts of tissues from gargoylism patients is due to storage of galactose-rich glycosaminoglycans and glycopeptides, and that this storage is a result of the deficiency of β-galactosidase A.

The high level of mannose in the liver from gargoylism patients seems to indicate storage of glycopeptide, adding a new group of substances to those known to be stored in gargoylism.

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INTRODUCTION

In gargoylism the metabolism of glycosaminoglycans (earlier called acid mucopolysaccharides) and glycolipids (gangliosides) is disturbed (1, 2). The clinical symptoms and signs of the pathological accumulation of different substances involve many organs, are progressive, and finally lead to death.

On the basis of electronmicroscopical studies, gargoylism has been suggested to be a lysosomal disease (3). Recently, markedly decreased activity of MU-β-galactosidase[1] (β-D-galactoside galactohydrolase, EC 3.2.1.23) was demonstrated (4–7). β-galactosidase activity is known to be present in the lysosomes of man (6) as well as in those of the animal (8).

The apparent possibility exists that the demonstrated decrease in β-galactosidase activity in patients with gargoylism may reflect the absence of an enzyme which is necessary for the normal catabolism of the glycosaminoglycans and glycolipids which are known to be stored in pathological amounts in this disease. We do not known whether these glycosaminoglycans and glycolipids contain much galactose, nor do we know which is the natural substrate for the β-galactosidase which is deficient in gargoylism.

In the present study we have, therefore, measured the level of galactose and other sugars in hydrolyzed tissue extracts from patients with gargoylism, patients with other kinds of storage diseases, and normal control subjects. We have also tried to characterize the β-galactosidase deficiency by using a large number of substrates and by performing separation experiments and enzyme kinetic studies with different kinds of tissues. The substrates used include four synthetic β-galactosides (MU-

[1] Abbreviations: MU, 4-methylumbelliferyl; PNP, para-nitrophenyl; ONP, ortho-nitrophenyl; P, phenyl.

β-galactoside, PNP-β-galactoside, ONP-β-galactoside, and P-β-galactoside), two galactolipids (ceramide-β-galactoside and ceramide-β-lactoside), one glycopeptide derived from transferrin, and one glycosaminoglycan (keratan sulfate).

METHODS

Tissue preparations

Normal controls. Tissue specimens were obtained 3 hr postmortem from two children killed in a road accident, and 5–15 hr postmortem from adults who had died of various diseases supposed not to engage liver, spleen, kidney, or brain primarily. Secondary damage to these tissues could not be excluded, but more strictly normal tissues could not be obtained.

Gargoylism patients. Three of the patients with gargoylism have been described earlier (6) and will, in accord with their previous nomenclature, be named *case 1, case 4, and case 7,* respectively. Samples have now been obtained from one further patient, a 7 yr old boy, who will be named *case 8.* These patients had clinical symptoms and signs in all respects compatible with the diagnosis of gargoylism, and large amounts of dermatan sulfate and heparan sulfate were found in the urine. The tissues from cases 4 and 7 were frozen 7 hr and about 15 hr, respectively, postmortem. The tissues from cases 1 and 8 were obtained by surgical biopsy and were frozen immediately.

Other storage disorders. Tissues from three patients with *Gaucher's disease* and one patient with *juvenile amaurotic idiocy* were studied. They had been stored for up to 4 yr at −20°C. Tissues from one patient with *Tay-Sachs' disease* and two patients with a *late-infantile storage disorder* (progressive encephalopathy with storage of lipofuscin-like material in a pair of female twins, 13 yr old) had been stored for between a few weeks and a few months at −20°C.

Determination of sugars by gas-liquid chromatography

The tissue was extracted with lipid solvents. 10–40 mg dry, lipid-free extract was used for hydrolysis. Rhamnose was added and the material was neutralized. All these procedures were as described by Walborg, Christensson, and Gardell (9).

For reduction and acetylation[2] the freeze–vacuum dried hydrolyzed extract was dissolved in 1 ml of water. Then 200 μl of the solution was evaporated to complete dryness, 400 μg sodium borohydride in 200 μl 1 M ammonia was added, and the solution was left at room temperature for 2 hr. The excess of sodium borohydride was removed by adding glacial acetic acid until no further bubbling was observed. The solution was then evaporated to dryness. Methanol was added and the evaporation was repeataed four times. Thereafter, 200 μl of a mixture of equal parts of acetic anhydride and pyridine was added, the tube was closed by melting, and it was heated at 100°C for 1 hr. Then about 1 volume of water and some methanol was added, and evaporation was performed to dryness. The residue was dissolved in a small volume of chloroform.

For gas-liquid chromatography of the alditol acetates we used a slight modification of one of the methods described by Sjöström, Haglund, and Jansson (10).

[2] Lindahl, U. 1969. Personal communication.

A 1–2 μl sample of the chloroform solution was injected into the gas chromatograph (Aerograph, model 1520). The columns were 2 m long, made from ⅛ inch stainless steel, and were filled with a mixture containing 1.5% ethylene glycol succinate and 1.5% silicone oil (XF-1150) on 100–120 mesh Gas-chrom P, purchased from Professor G. Widmark, Department of Analytical Chemistry, University of Stockholm. The standard separation conditions were: injector temperature 260°C, detector cell temperature 217°C, and column temperature 195°C.

When we wanted to obtain a good separation between the sugars appearing early in the chromatography, we used a lower column temperature (temperature programming) during the first 5–10 min of the run.

Commercial monosaccharides of purest grade available were used as reference substances. A small amount of rhamnose was added to each tissue sample before analysis and served as an internal standard. The peak area for each component found was calculated by triangulation. The relative detector response for each monosaccharide was determined.

To test the reliability of the assay with gas-liquid chromatography, the amount of glucose and galactose recovered in tissue extracts was compared with the amounts found on enzymatic analysis (method described in reference 11, also compare below). Similar results were obtained.

Enzyme activity determinations

Substrates. MU-β-galactoside, ONP-β-galactoside, PNP-β-galactoside, and P-β-galactoside were obtained from the same sources as described previously (5, 12).

Ceramide-β-galactoside (*N*-lignoceroyl-DL-sphingosyl-β-D-galactoside, kerasin) and ceramide-β-lactoside (*N*-lignoceroyl-DL-sphingosyl-β-D-lactoside, cytolipin H) were obtained from Seravac Laboratories, Maidenhead, England. Transferrin glycopeptide (sialic acid-free) was prepared from transferrin (a gift from AB KABI, Stockholm) by treatment with pronase and mild acid hydrolysis as described by Jamieson (13). Keratan sulfate was a gift from Dr. L.-Å. Fransson (Department of Physiological Chemistry, University of Lund) (14).

Galactose dehydrogenase. This enzyme was obtained from C. F. Boehringer & Sons, Mannheim, West Germany.

Performance of the assay. MU-β-galactosidase activity was assayed with the same method as that described in earlier publications (5, 6, 15, 16). Acetate buffer pH 4.5 was used and no Triton X-100 was added.

ONP-β-galactosidase, PNP-β-galactosidase, and P-β-galactosidase activities were also assayed with the same methods as those used earlier (12). Citrate buffer pH 4.5 was used, and no Triton X-100 was added.

The hydrolysis of the natural substrates was measured with galactose dehydrogenase. For the preparation of substrate solutions with ceramide-β-galactoside and ceramide-β-lactoside, 5.0 mg of substance and 40 mg of sodium taurocholate were suspended in 4 ml distilled and deionized water. The tube was heated in boiling water and shaken until all material had dissolved. The addition of taurocholate solubilizes the substrates and activates the enzyme(s) degrading the galactolipids (17)). The solution of ceramide-β-galactoside was 1.48 mmoles/liter and that of ceramide-β-lactoside 1.25 mmoles/liter. Transferrin glycopeptide was used at a concentration of 1 mg/ml in water. For the assay with these three substrates, 25 μl of substrate solution was mixed with 25 μl of acetate buffer (0.2 mole/liter, pH 4.0) and

25 μl tissue homogenate (1:4 in water containing 0.2% Triton X-100).

When keratan sulfate was used as substrate, 100 μl of substrate solution (80 mg/ml) was mixed with 50 μl of acetate buffer and 50 μl of homogenate (prepared without Triton X-100).

After incubation, the samples were centrifuged at +4°C, and free galactose was immediately measured in the supernatant with galactose dehydrogenase in the following way: 50–150 μl of the incubation mixture was mixed with 1.35 ml of 40 mM Tris-HCl buffer pH 8.6, containing 4 mM glutathione and 0.4 mM diphosphopyridine nucleotide (NAD) (18). Fluorescence was measured in an Aminco-Bowman spectrofluorimeter (American Instrument Co., Inc., Silver Spring, Md.) with exciting wavelength 340 nm and emitted wavelength 458 nm. After 6–10 min, a stable reading was reached, and then 5 μl (=25 μg) galactose dehydrogenase was added. After a new plateau had been reached, 1–5 μg of galactose was added in a volume of 10 μl, to serve as internal standard. The amount of galactose liberated was calculated from the difference between the first and the second plateau.

Enzyme kinetics. With all the natural β-galactosides, the substrate concentrations used were close to optimal. A velocity of reaction making 55–65% of V_{max} was obtained, and increasing the substrate concentration did not give any further increase of the reaction velocity, in one case (keratan sulfate) even a decrease was seen by substrate inhibition. The amount of galactose liberated was proportional to the time of incubation up to 8 hr and to the amount of enzyme preparation used in the test, i.e. zero order kinetics were obeyed.

It was checked that spontaneous liberation of galactose did not occur during the incubation conditions with any of the substrates or tissue preparations. Zero time blanks were always prepared, and the readings of these were low in comparison with most incubated samples.

pH dependency. With all the naturally occurring substrates, maximum activity in whole liver homogenates was seen at pH 4.0, both when control and when gargoylism tissues were used. This pH was therefore used in all assays. It is slightly lower than the optimum for the synthetic substrates, which is pH 4.5.

Activators. Triton X-100 was found to activate the enzymatic hydrolysis of the two galactolipids and was, therefore, used in the assay with these two substrates. Triton X-100 did not affect the activities with the other substrates used.

RESULTS

Analysis of sugars in tissues after hydrolysis. The gas-liquid chromatography procedure gave a good separation between different sugars (Fig. 1). The good separation between galactose and glucose was especially valuable.

The results obtained with extracts from brain and liver from control subjects and from patients with different storage disorders are seen in Table I. In patients with gargoylism, high values were obtained for galactose and possibly for mannose in liver, and for galactose in brain. The amount of arabinose found in the

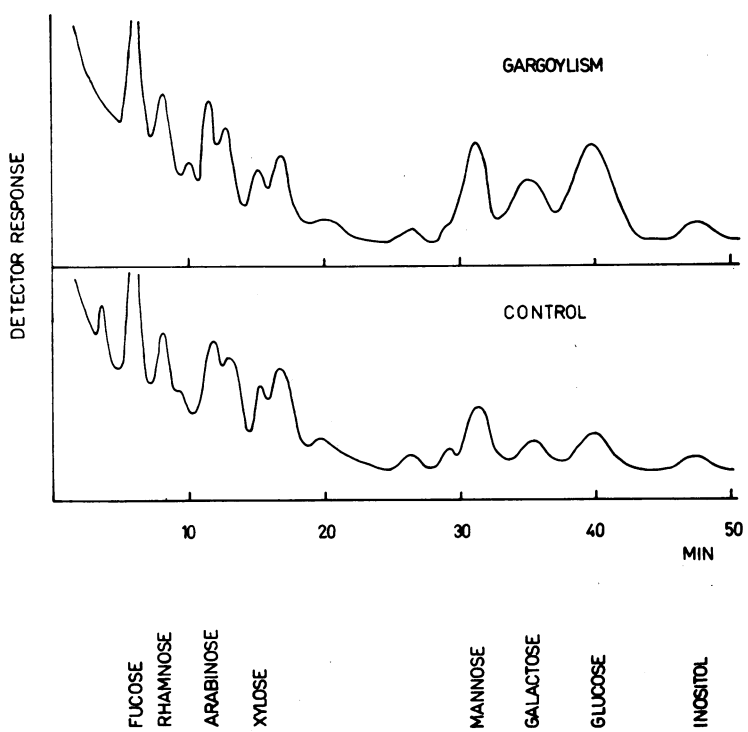

FIGURE 1 Separation of monosaccharides as alditol acetates. Hydrolysates from liver tissue from gargoylism case 4 and one control are shown.

brain from patients with gargoylism was somewhat lower than in the control subjects. In the other kinds of storage diseases, the amounts of different sugars found were not different from those found in the control subjects with the exception of inositol, which showed a high level in liver from patients with Gaucher's disease and from one of the patients with an unclassified storage disorder.

Heat sensitivity of β-galactosidase activities. A purified enzyme preparation (peak II) was inactivated by heating, and the effect was studied with three of the natural substrates (the two galactolipids and transferrin glycopeptide). These activities were inactivated in parallel with the MU-β-galactosidase activity, the heat sensitivity of which has ben described in a previous paper (19).

Subcellular localization of β-galactosidase activities. The subcellular particles in a rat liver homogenate were fractionated as described in reference 6. The activity, with all the natural substrates tested (ceramide galactoside, ceramide lactoside, and keratan sulfate), was located in the lysosomes as earlier described with MU-β-galactoside (6).

TABLE I

Total Level of Sugars in Hydrolysates from Liver and Brain

	Fat free dry weight				
	Arabinose	Xylose	Mannose	Galactose	Inositol
			%		
Liver					
Controls, n = 5	0.231	0.148	0.561	0.174	0.214
Mean and range	(0.109–0.520)	(0.079–0.181)	(0.340–0.735)	(0.075–0.317)	(0.144–0.258)
Gargoylism, case 4	0.350	0.135	0.790	0.580^{+}	0.131
Gargoylism, case 7	0.221	0.035	0.960	0.910^{++}	0.415
Mean value in gargoylism	0.286	0.085	$0.875^{(+)}$	0.745^{++}	0.273
Gauchers disease, n = 3	0.195	0.120	0.513	0.296	0.746^{+}
Mean and range	(0.156–0.235)	(0.112–0.129)	(0.405–0.665)	(0.189–0.373)	(0.500^{+}–1.175^{+})
Juvenile amaurotic idiocy	0.232	0.032	0.509	0.100	0.302
Tay-Sachs disease	0.255	0.170	0.615	0.309	0.149
Late-infantile storage disorder, case 1	0.257	0.125	0.712	0.338	0.548^{++}
case 2	0.319	0.058	0.605	0.369	0.342
Brain					
Controls, n = 5	0.186	0.167	0.595	0.411	1.566
Mean and range	(0.125–0.279)	(0.094–0.330)	(0.349–0.960)	(0.243–0.615)	(1.135–2.930)
Gargoylism, case 4	0.094	0.097	0.440	0.618	0.408
Gargoylism, case 7	0.072	0.046	0.590	$0.845^{(+)}$	1.710
Mean value in gargoylism	$0.083^{(+)}$	0.072	0.515	$0.732^{(+)}$	1.059
Gauchers disease, n = 2, mean value	0.160	0.153	0.550	0.382	1.251
Juvenile amaurotic idiocy	0.148	0.092	0.815	0.800	0.920
Tay-Sachs disease	0.065	0.035	0.463	0.298	1.030
Late-infantile storage disorder, case 1	0.063	0.080	0.485	0.252	2.543
case 2	0.164	0.148	0.545	0.455	1.790

The abbreviations used in Tables I–IV: MU-GAL, 4-methylumbelliferyl-β-galactosidase; ONP, ortho-nitrophenyl; PNP, para-nitrophenyl; P, phenyl. Statistical significances in relation to controls: (+) $P < 0.1$; + $P < 0.05$; ++ $P < 0.01$; +++ $P < 0.001$. In gargoylism, case 4, using an ion exchange chromatographic method (9), high values were found for mannose (0.709^{+}) and galactose (0.834^{++}). Mean values in controls with this method were 0.398 and 0.294, respectively (20).

Fractionation of the β-galactosidases by gel filtration. Gel filtration of the supernatant from normal human liver homogenates separates the MU-β-galactosidase activity into three fractions which have been named peaks I, II, and IIII (16) (Fig. 2). It has been shown that these three peaks represent only two different enzymes (19); one of these, which has been named enzyme A, is present in peaks I and II, and the other one, enzyme B, is present in peaks I and III. Enzyme B also exerts β-glucosidase activity (19). When the β-galactosidase activity in such chromatograms was measured with ONP-β-galactoside or P-β-galactoside as substrates, the same three peaks of β-galactosidase activity were found, and the proportions between the peaks were roughly similar to those found with MU-β-galactoside (Fig. 2). When the naturally occurring substrates were tested (ceramide-β-galactoside, ceramide-β-lactoside, keratan sulfate, transferrin glycopeptide), activity was only found in peaks I and II (Fig. 2). This apparently indicates that only enzyme A can hydrolyze the natural substrates.

Fractionation by isoelectric focusing. When liver extracts from control subjects were subjected to isoelectric focusing, and when the synthetic substrates were used, three activity peaks were obtained (Fig. 3). As in the gel filtration experiments, only two of these peaks hydrolyzed the natural substrates (one peak recovered at pH 4.2–4.3 and the other at pH 4.9–5.0). The third β-galactosidase peak (recovered at pH 4.5–4.6) did not hydrolyze the natural substrates. The properties of this peak were studied in detail, by use of the same methods as described previously (19). All results agreed in demonstrating that this peak was essentially identical to β-galactosidase B.

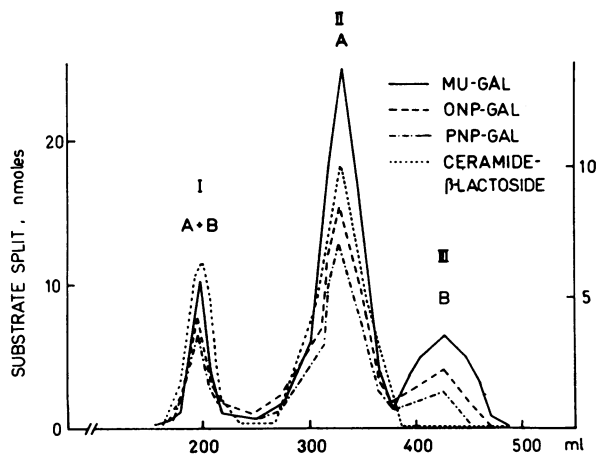

FIGURE 2 Gel filtration on Sephadex G-150 of supernatant from 10 g of control human liver. All enzyme assays performed at pH 4.5. Incubation time 2 hr with all substrates. Abbreviations used: MU-GAL, 4-methylumbelliferyl-β-galactosidase; ONP-GAL, ortho-nitrophenyl-β-galactosidase; and PNP, para-nitrophenyl-β-galactosidase. The left scale is for MU-GAL, the right scale for ceramide-β-lactoside. A pattern, very similar to that of ceramide-β-lactoside, was found also with ceramide-β-galactoside, transferrin glycopeptide, and keratan sulfate.

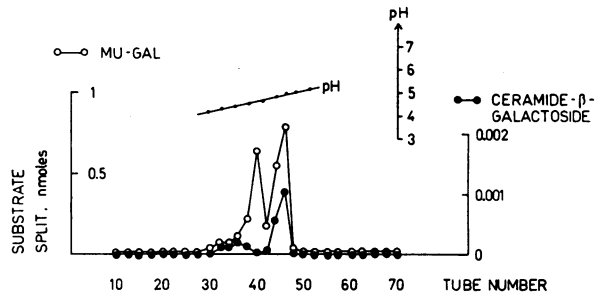

FIGURE 3 Isoelectric focusing of galactosidase activities in supernatant from 1 g of control human liver. With ceramide-β-lactoside and transferrin glycopeptide a pattern was found similar to that for the ceramide-β-galactoside.

β-galactosidase activities in tissues from patients with gargoylism. We measured the activity of β-galactosidase in liver from patients with gargoylism with all the eight β-galactoside substrates used in the present investigation (Table II). It was found that in patients with gargoylism, the hydrolytic activity was lower than in the normal controls, whichever substrate was used.

For all the other tissues tested, the β-galactosidase activity in gargoylism patients tended to be lower than in normal controls.

Table II also shows the tissue distribution of the β-galactosidase activity measured with all the substrates in normal controls and in patients with gargoylism. The relative activity with the different substrates used was approximately the same in all tissues, most probably indicating that they all contained enzyme A and enzyme B in about the same proportion. An exception was urine, which hydrolyzed only MU-β-galactoside. With the natural substrates, however, we were not able to demonstrate any galactosidase activity, even though we used several different methods for concentration of the proteins.

Since the decreased activity of β-galactosidase in gargoylism need not necessarily be the result of decreased enzyme formation, but also could be caused by inhibitory action of some substance accumulated in the tissues of such patients, we mixed the liver extracts from a control subject with that from a patient with gargoylism (Table III). Three of the natural substrates were used for this experiment. No inhibition of the β-galactosidase activity could be demonstrated.

DISCUSSION

Sugars. The gas-liquid chromatography method for assay of sugar has several advantages over the ion-exchange chromatography methods (9); it is technically

TABLE II

β-Galactosidase Activities in Human Tissues

	MU-GAL	ONP-GAL	PNP-GAL	P-GAL	Ceramide galactoside	Ceramide lactoside	Transferrin glyco-peptide	Keratan sulfate
Liver								
Controls, n = 8–13	646* (272–1172)	231 (83–302)	418 (196–542)	324 (230–476)	3.82 (1.93–6.30)	6.96 (3.56–9.44)	50.2 (37.4–76.7)	54.7 (15.6–117)
Gargoylism, case 1	67*	—	—	—	0.31	0.95	5.59	1.4
Gargoylism, case 4	26*	40	47	27	0.89	0.28	6.65	3.5
Gargoylism, case 8	40	—	—	—	0.77	1.79	6.60	2.8
Mean value in gargoylism	44++	—	—	—	0.65+	1.00++	6.27+++	2.5+
Kidney								
Controls, *n* = 1–4	654 ±234 (n = 10)‡				3.20–6.78	8.60	12.3–38.4	52.3–516
Gargoylism, case 4	69++‡				1.61	1.76	2.56	28.1
Spleen								
Controls, n = 1–5	285 ±78 (n = 12)‡				2.74–4.80	5.19	3.84–8.09	16.1–39.5
Gargoylism, case 4	32++‡				0.43	0.55	0.96	8.3
Brain cortex								
Controls, n = 1–6	68 ±17 (n = 11)‡				1.16–2.74	2.80	1.92–4.80	4.3–10.6
Gargoylism, case 4	10++‡				2.09	2.10	1.92	2.2
Urine								
Controls, n = 6	0.63–4.94 (n = 17)‡§	—	—	—	0	0	0	0

All values for tissues in milliunits per gram wet weight at 37°C = nanomoles substrate split (first six substrates) or product formed (transferrin glycopeptide and keratan sulfate) per minute per gram. In the controls most values are given as mean (observed range).
(+)-+++, values differing significantly from control values. (+) $P < 0.1$; + $P < 0.05$; ++ $P < 0.01$; +++ $P < 0.001$.
* From Öckerman, 1968 (6).
‡ From Öckerman, Hultberg, and Eriksson, 1969 (32).
§ U/g creatinine.

easier, more rapid, and more sensitive. The resolution power is also greater. In the present study, analysis for galactose in the tissue extracts was performed not only with the gas-liquid chromatography method, but also enzymatically with galactose dehydrogenase. The two methods gave similar results. The other sugars present were, however, only assayed with gas-liquid chromatography, and their identity is thus not equally well-documented. The values obtained for extracts from liver and brain of normal control subjects can be compared with values obtained in similar controls using ion-exchange chromatography (20). At large, the two methods give similar results, but a few more peaks were obtained with the gas-liquid chromatography method.

In addition to the sugars listed in Table I, The gas-liquid chromatography also revealed a large peak of glucose plus a few minor, yet unidentified peaks.

The evaluation of the high values for galactose and mannose found in gargoylism patients must be made with care. The number of gargoylism patients is low as is that of control subjects, and there is a marked age difference between the two groups. To obtain control tissue from healthy children is for obvious reasons very difficult. A fact which strongly supports the idea that galactose- and mannose-containing substances are deposited in the tissues of patients with gargoylism is, however, that the values were high also when compared with samples from children with other kinds of storage disorders. Such patients probably are a better control than normal adults, and maybe even better than healthy children would have been.

TABLE III

β-Galactosidase Activities in a Mixture of Control and Gargoylism Liver

	Ceramide galactoside	Ceramide lactoside	Keratan sulfate
	mU/g wet weight		
1. Control*	2.48	2.12	16.8
2. Gargoylism*	0.88	0.18	1.50
3. Mixture of 1 + 2‡	2.82	3.00	13.2
4. Theoretical value for 3	3.36	2.30	18.3
5. Recovery in 3, %.....	84	130	72

* Analysis made on homogenates $\frac{1}{8}$.
‡ Analysis made on a mixture of equal volumes of homogenates $\frac{1}{4}$.

The significance of the high levels of inositol found in the patients with Gaucher's disease and one patient with an unclassified storage disorder is at present unknown.

Enzymes. Several reports have been published in which β-galactosidase activity in various human and animal tissues has been studied. Different substrates have been used such as lactose, synthetic heterogalactosides (MU-β-galactoside, ONP-β-galactoside, PNP-β-galactoside) (2, 21–23), and naturally occurring β-galactosides, ceramide β-lactoside (17, 24–27) and sialic acid–free α_1-acid glycoprotein (28, 29). Keratan sulfate does not seem to have been studied earlier in this respect. In only a few of these investigations has any comparison been made between the β-galactosidase activity measured with synthetic substrates and that measured with naturally occurring substrates. Langley and Jevons (28) recently indicated that the β-galactosidase in animal tissues hydrolyzing MU-β-galactoside might not be identical with that hydrolyzing sialic acid–free α_1-acid glycoprotein. Hajra, Bowen, Kishimoto, and Radin (25) and Bowen and Radin (17, 26) also made some experiments which indicated that the β-galactosidase activity with ceramide-β-galactoside as substrate was partly separable from that found with ONP-β-galactoside as substrate, when the tissue extracts were subjected to gel filtration, lipase digestion, or acid precipitation.

Austin, McAfee, Armstrong, O'Rourke, Shearer, and Bachhawat (30) and Austin, Armstrong, and Shearer (31) found normal (although possibly in the low normal range) β-galactosidase activity in liver, kidney, and brain from patients with gargoylism, using ONP-β-galactoside as substrate. Our experiences thus disagree with that of these authors. The cause of this discrepancy may be that fact that Austin et al. (31) used a substrate concentration which was only 0.23 mmole/liter which is far below optimal substrate concentration. We have used a 12 mM substrate concentration when measuring ONP-β-galactosidase activity. When suboptimal substrate concentrations are used for enzyme activity assay, values that are too low will be obtained, and furthermore, zero order kinetics will not be obeyed. Consequently, varying the amount of enzyme will not affect the results proportionally, which would tend to make all samples look more alike than they really are. In Table IV are shown results which we obtained by using the same technique as that of Austin et al. (31). It is apparent that all the enzyme activity values, as would be expected, are much lower than in Table II. The patient with gargoylism still has lower activity than the controls, but the difference is smaller than when our standard technique is used. The values in Table IV are very similar to those obtained by Austin et al. (31); their values are recalculated to internal units of enzyme activity. The difference in technique thus seems fully to explain the discrepancy.

TABLE IV

ONP-GAL-β-Galactosidase Activity in Supernatant from Liver Homogenates, Assayed with the Method of Austin et al. (31)

	ONP-GAL at 37°C
	mU/g wet weight
Controls, n = 4	
Mean (observed range)	26 (21–37)
Gargoylism, case 4	10

In extracts of normal human tissues, we recently separated two different enzymes with MU-β-galactosidase activity (19). These enzymes were named A and B. The separation experiments performed in the present study indicate that the naturally occurring substrates are only hydrolyzed by enzyme A, although all the synthetic substrates were hydrolyzed by both enzymes. This seems to be at variance with the findings for animal tissues by other authors (17, 25, 26, 28). However, the methods used by these authors to prepare the enzyme solution studies involve several precipitation steps. If a separation between β-galactosidase A and B occurs during these steps, this may be sufficient to explain the differences in results.

The finding that the natural substrates are hydrolyzed only by enzyme A is especially interesting since earlier we found that enzyme A, but not B, is markedly deficient in gargoylism tissues (32). This strengthens the suspicion that gargoylism is primarily caused by β-galactosidase A deficiency, and that consequently one or more β-galactosides which are the physiological substrate (s) for this enzyme are accumulated. This fits well with the finding of high galactose levels in the tissues.

Relation between enzyme defect and sugar accumulation. Pathological storage of galactose-containing glycolipids, e.g. ceramide β-galactoside and ceramide-β-lactoside, has earlier been shown to occur in gargoylism (33). This fits well with our finding of a decreased hydrolysis of ceramide-β-galactoside and ceramide-β-lactoside. The galactose we found by gas-liquid chromatography could not, however, originally have been present in lipids, since we extracted the lipids before hydrolysis.

It has been shown that glycosaminoglycans (acid mucopolysaccharides) contribute the largest fraction of the material stored in gargoylism (2). Many glycosaminoglycans contain two molecules of galactose in the linkage region between the polysaccharide and the peptide chains (34), and these galactose molecules may contribute at least part of the galactose found in the hydrolysates.

Although β-galactosidase A hydrolyzes keratan sulfate also the deficiency of β-galactosidase A does not

seem to cause accumulation of this substrate in patients with gargoylism, since no increase of keratan sulfate has been demonstrataed. It must be remembered, however, that although enzyme A is low in activity in these patients, it is not completely absent. The activity remaining may be sufficient for the normal need of hydrolysis of some substrates.

We do not know whether the partial deficiency of enzyme A in gargoylism indicates that enzyme A in normal subjects in fact should contain two components, and that only one of them is absent in the gargoyles, or whether it is a single enzyme which in these patients is formed either in smaller amounts than normal, or is formed in normal amounts but is pathologically altered and has lower activity than the normal enzyme.

Although it does seem possible to explain the storage of at least a few of the substances known to be accumulated in gargoylism by the decreased β-galactosidase activity, it is not yet clear why dermatan sulfate and heparan sulfate, the two main storage substances, are accumulated. All attempts to demonstrate galactose liberation from these substances with extracts from normal tissues have so far failed.[8] This does not, of course, exclude the possibility that β-galactosidase A normally catalyzes a slow degradation of dermatan sulfate and heparan sulfate and that the deficiency of β-galactosidase A may be the cause of the storage of these substances.

Finally, which substances are responsible for the increased concentration of mannose in gargoylism patients? Mannose is known to be an important component of several glycoproteins. It has also been suggested that glycosaminoglycans may contain a very small amount of mannose (35), although nothing is known about the exact position of mannose in this molecule, and these findings so far have not been verified. The high level of mannose found, therefore, may indicate pathological storage of glycoproteins or glycopeptides containing this sugar. Hypothetically, mannose in this substances may be located at a more central part of the molecule than in galactose, since this is known to be the case in many normal glycopeptides, and in that case, inability to liberate galactose may cause a storage of mannose also.

Our results support the idea that gargoylism is a true multicomponent storage disorder, with storage not only of glycosaminoglycans and glycolipids, which has already long been known, but also of glycopeptides (glycoproteins).

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[8] Öckerman. Unpublished experiments.

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