some movement of the backbone, less than 1 Å in distance, is required). Experiments to distinguish between these two amino acids in KDPG aldolase are now in progress. It is also evident that other amino acids which could serve as bases, such as lysine or arginine, could participate in such a mechanism in other aldolases. The critical requirement is a chain length adequate to allow a carbon-carbon bond rotation which results in proper orientation of the basic function.

We have been able to build models in which either retention or inversion of configuration at C-3 of KDPG would occur. In these two sets of models, completely different locations of the glutamate in the active site and, presumably, in the peptide sequence are required. Thus the two cases in which a single base interacts with both substrates to give either inversion or retention are entirely different and independent on an evolutionary scale. It has been observed with muscle and yeast aldolase (*9*), L-rhamulose-1-phosphate aldolase (*10*) and two other aldolases active on DL-2-keto-4-hydroxyglutarate (*11*), that both the exchanging proton and the aldehyde approach the same face of the enzyme-bound enolate. In addition we have evidence that retention of configuration is also the case for KDPG aldolase. From this evidence, and on the assumption that a two-step rather than a concerted process is occurring, only the case for retention of configuration is shown in Fig. 2. Thus it seems that single base–mediated proton activation in aldolases contributes to an active site model allowing all exchanging reagents to approach a single (solvent) face of the bound enolate in the active site.

From the concept of a single base one might predict observable intramolecular proton transfer among aldolases, as in the case of phosphoglucose isomerase (*12*), which transfers protons between adjacent carbon atoms so that both exchanging protons approach a single face of the enolate intermediat (*13*), and which has in its active site a glutamate residue that reacts with a substrate analog (*14*). Triose phosphate isomerase transfers protons in the same way (*13*) and also has a glutamate residue in its active site (*15*). It now becomes apparent that a carboxylate, in which the oxygen atoms bridge a 2.2-Å distance, might be an ideal reagent for transferring protons between adjacent carbon atoms as catalyzed by isomerases, providing the single base required for *cis* hydrogen transfer generally seen among isomerases (*16*). The distinction between a carboxylate serving as the base for either an aldolase or an isomerase might be viewed as rotation of a C-C bond in the side chain as compared to the rotation of the carboxyl group itself.

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Mucopolysaccharidosis: Secondarily Induced Abnormal Distribution of Lysosomal Isoenzymes

Abstract. *Total activities of acid hydrolases in liver of two patients with mucopolysaccharidosis are decreased for β-galactosidase, α-galactosidase, and arylsulfatase A; total activities of four other hydrolases are normal or increased. The isoenzyme distribution of five hydrolases (β-glucuronidase, α-glucosidase, β-galactosidase, N-acetyl-β-glucosaminidase, and α-galactosidase) is abnormal in that the isoelectric points (by isoelectric focusing) of these enzymes are more acid than in control liver. Along with the isoenzyme abnormalities different kinds of glycolipids were stored in kidney, liver, and brain. The isoenzyme abnormalities can be reproduced in vitro by addition of chondroitin sulfate to a homogenate of normal liver, suggesting that stable binding occurs between mucopolysaccharides and the hydrolase molecules. After the addition of chondroitin sulfate, the total activity of β-galactosidase is inhibited, whereas other hydrolases are affected only slightly or not at all.*

Patients with mucopolysaccharidoses type 1, 2, and 3 show a striking reduction in the β-galactosidase activity in different organs (*1–3*). The diminished value for the β-galactosidase is rather specific as a number of other lysosomal enzymes—especially *N*-acetyl-β-hexosaminidase, β-glucuronidase, and α-fucosidase—show greatly enhanced values (*1*). The reduction of the β-galactosidase activity is correlated to the deficiency of one thermolabile, slow-moving isoenzyme fraction (*4*). Owing to a lack of experimental evidence for a relation between mucopolysaccharide accumulation and this rather specific deficiency of the β-galactosidase, no logical explanation so far was available. It seemed unlikely however that the deficiency of the β-galactosidase activity was the fundamental defect, because (i) it was found in genetically different diseases, and (ii) patients with GM_1 gangliosidosis, a severe disorder in which β-galactosidase is almost totally lacking, do not store or excrete excessive amounts of chondroitin sulfate B or heparan sulfate (*5*).

We now describe a generalized abnormality in the isoenzyme distribution of several lysosomal hydrolases, including β-galactosidase, and provide experimental evidence that this abnormality might be due to storage of excessive amounts of mucopolysaccharides. The patients included one (D.J.) with Hunter's disease and one (V.D.K.) with Sanfilippo disease type B (*6*). From patient D.J., autopsy material which had been stored at −20°C for about 2 months was examined; from patient V.D.K., a surgical biopsy piece of liver was studied. Total enzyme activities of

eight hydrolases were measured in the liver homogenates (Table 1). In addition to reduction of α-galactosidase and β-galactosidase and the hyperactivity of *N*-acetyl-β-hexosaminidase, α-fucosidase, and β-glucuronidase, we noted an abnormality of the arylsulfatases. Reduction of α-galactosidase in liver of patients with mucopolysaccharidosis has been described (*1, 2*). Whereas the activity of arylsulfatase A was decreased, the arylsulfatase B was about twice the normal value. Abnormally low activity of arylsulfatase A has been observed in fibroblasts of patients with mucopolysaccharidosis (*7*). The isoenzyme distribution of five hydrolases was then examined by means of isoelectric focusing on polyacrylamide gel (*8*). With the use of a pulsed constant power supply and a vertical cell apparatus (*9*), a high resolution of the isoenzymes was obtained. Because a number of lysosomal enzymes lose their negative charge when treated with neuraminidase (*10*), the separation of the isoenzymes of liver homogenates was performed with and without prior incubation with bacterial neuraminidase (Fig. 1, a to c). In normal liver four enzymes (α-galactosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, and α-mannosidase) could be separated in two main fractions A and B, while β-glucuronidase and α-glucosidase at *p*H 4.5 showed only one isoenzyme. The fraction with the more acid isoelectric point is called A, and the other is referred to as B.

For α-glucosidase (Fig. 1a) a new compound with a more acid isoelectric point is seen, both in Hunter's disease and Sanfilippo's disease. This compound disappears after treatment with neuraminidase. For β-galactosidase (Fig. 1b), an almost complete absence of the fraction B is noted in Hunter's disease. The remaining fraction A is sensitive to neuraminidase. For β-glucuronidase, the patients' isoenzymes again show activity at a more acid isoelectric point than the controls (Fig. 1c); in the Sanfilippo patient this abnormality was far more pronounced than in the Hunter patient. For this enzyme the abnormal pattern did not change after neuraminidase treatment. The distribution of the isoenzymes of α-mannosidase was normal in both patients. With *N*-acetyl-β-glucosaminidase a difference between controls and patients was also noted, although less pronounced than with the first three enzymes as isoenzyme B was moved to a more acid region. Isoenzyme A was at the normal position. There was an abnormal tailing of the isoenzyme A of α-galactosidase, which was removed by treatment with neuraminidase.

These isoenzyme abnormalities were not always present; in cultured fibroblasts and in concentrated, demineralized urine of the Sanfilippo patient the distribution of β-glucuronidase and α-glucosidase was quite normal.

A concurrent examination of stored material in different organs revealed a rather heterogeneous accumulation (Table 2). In kidney of the Hunter disease patient, the amount was about 20 times the normal amount of digalactosylceramide and the ceramide trihexoside increased sevenfold. In the livers of both patients, the ceramide trihexoside was almost doubled while in the brain of the Hunter patient the lactosylceramide content was 20 times the normal. Possibly sulfatide also was increased in brain and kidney.

A mixture of homogenates of normal liver and a liver from a patient with

Table 1. Activity of eight hydrolases in liver. Enzyme activities are expressed as international units per gram of liver tissue. The patient's values are given also as percentage of the mean normal activity. For α-galactosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, and β-glucuronidase, methylumbelliferyl substrates (*17*) were used according to Van Hoof and Hers (*1*). The phosphatase and α-fucosidase were assayed with *p*-nitrophenyl derivatives (*1*). The arylsulfatases A and B were measured according to the method of Baum, Dodgson, and Spencer (*18*).

Enzyme	Control ($N = 5$)		Hunter		Sanfilippo type B	
	Mean	Range	Activity	Percent	Activity	Percent
α-Fucosidase	0.28	0.18–0.59	0.65	233	0.48	170
β-Glucuronidase	0.38	0.22–0.98	0.69	182	1.33	350
N-Acetyl-β-glucosaminidase	2.39	2.00–4.30	7.17	300	8.67	363
Acid *p*-nitrophenylphosphatase	2.07	1.61–2.79	1.86	90	3.73	180
α-Galactosidase	0.079	0.042–0.127	0.017	22	0.038	48
β-Galactosidase	0.63	0.58–1.62	0.12	19	0.18	28
Arylsulfatase A	1.07	0.56–2.08	0.18	17	0.15	14
Arylsulfatase B	0.24	0.11–0.44	0.43	180	0.56	235

Table 2. Glycolipids in liver, kidney, and brain expressed in nanomoles per 100 mg (dry weight). Quantitative determination was performed by gas liquid chromatography (*19*) and by densitometry analogous to the method of Sandhoff *et al.* (*20*); CMH, ceramide monohexoside; CDH, ceramide dihexoside; CTH, digalactosylglucosylceramide; CTeT, *N*-aceylgalactosaminyldigalactosylglucosylceramide; Glu, glucosylceramide; Gal, galactosylceramide; Lac, lactosylceramide; Digal, digalactosylceramide; Sul, sulfatides. For the controls the mean value is given with the range of values in parentheses. Figures in italics are significantly different from normal values.

Organ	CMH		CDH		CTH	CTeT	Sul
	Glu	Gal	Lac	Digal			
			Hunter's disease				
Liver	25	< 5	80	< 5	*60*	35	< 5
Kidney	20	15	*135*	*775*	*600*	185	135
Brain, gray	< 10	1,425	*345*	< 10	< 10	< 10	1,125
Brain, white	< 10	7,735	*270*	< 10	< 10	< 10	3,380
			Sanfilippo disease type B				
Liver	25	< 5	100	< 5	*55*	*55*	< 5
			Controls				
Liver ($N=8$)	30 (10–40)	< 5	70 (30–110)	< 5	20 (15–30)	15 (10–35)	< 5
Kidney ($N=6$)	15 (10–25)	15 (5–15)	20 (15–25)	35 (20–50)	100 (60–130)	125 (90–200)	85 (35–120)
Brain ($N=4$)							
Gray	< 10	1,500 (620–1,950)	20 (10–30)	< 10	< 10	< 10	500 (420–620)
White	< 10	12,650 (10,500–15,950)	40 (10–70)	< 10	< 10	< 10	3,100 (2,680–3,850)

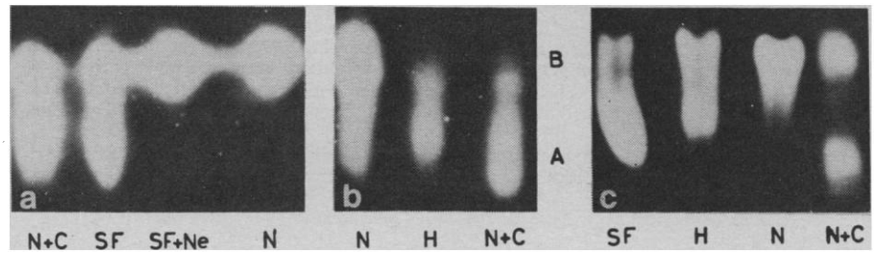

Fig. 1. Isoelectric focusing of (a) α-glucosidase, (b) β-galactosidase, and (c) β-glucuronidase of the liver. Supernatants of homogenates, centrifuged at high speed, of normal and pathological liver were focused along a *p*H gradient in polyacrylamide gel (*8*). Homogenates of normal liver (2 percent) were compared to a mixture (1 : 1) of homogenate of normal liver (2 percent) and chondroitin sulfate (2 mg/ml) and to homogenates treated with bacterial neuraminidase (*8*). (a and b) The *p*H was 3 to 5; (c) the *p*H was 3 to 10; the cathode is at the top; *A* and *B* are fractions; *N*, normal liver; *H*, Hunter liver; *SF*, Sanfilippo liver; *N+C*, mixture of homogenate of normal liver and chondroitin sulfate B; *SF + Ne*, treated with neuraminidase.

Hunter's disease gave us an image for β-glucuronidase, which was the sum of both distributions. The normal fractions were not influenced by the pathological, nor did the abnormal β-glucuronidase isoenzymes disappear. One can conclude from such experiment that no free inhibitor (or activator) is responsible for the abnormalities. However, such mixing experiments give no conclusive answer if a stable complex is formed between the isoenzyme and the inhibitor (or the activator). In order to see whether stable complexes can be made between mucopolysaccharides and lysosomal enzymes we performed the following experiments.

First, a mixture of equal volumes of 2 percent (volume) human liver homogenate and different kinds of chondroitin sulfate (*11*) was subjected to electrofocusing, and the isoenzymes of several hydrolases were localized. The pattern for β-galactosidase (Fig. 1b) was suggestive of the Hunter distribution since the B isoenzyme decreased and the A isoenzyme was normal. The pattern for α-glucosidase (Fig. 1a) showed the same abnormality as in Hunter's and Sanfilippo's diseases. For β-glucuronidase the mixing of chondroitin sulfate and a liver homogenate produced a change of the isoenzyme distribution analogous, but not quite identical, to the Hunter or Sanfilippo distribution in that a new fraction with a more acid isoelectric point appeared at about the same localization as the main fraction of the Sanfilippo distribution (Fig. 1c). In the distribution of the other hydrolases (α-galactosidase, *N*-acetyl-β-glucosaminidase) anomalies analogous to those seen in the patients also appear. The effect of different mucopolysaccharides on the distribution of the isoenzymes of α-mannosidase was negligible. It seems obvious from the experiments that strong binding occurs between some fractions of the hydrolases and the acid mucopolysaccharides. This binding results in new molecules with a more acid isoelectric point. Total activity of the hydrolases was almost unaffected by preliminary incubation of a liver homogenate with five different mucopolysaccharides (0.5 mg/ml), except for β-galactosidase (50 to 60 percent inhibition), α-galactosidase (15 percent inhibition), and β-glucuronidase (25 percent inhibition). Chondroitin sulfate B was the most effective inhibitor in each case. We believe that the biochemical abnormalities in these diseases are the result of a complex mechanism. The primary abnormality resides in the accumulation of one or several types of mucopolysaccharides and is due to one single enzyme deficiency [α-iduronidase (*12*), α-glucosaminidase (*13*), heparan sulfate sulfatase (*14*)]. The accumulated mucopolysaccharides are then partly bound to the hydrolases, which may change some of their properties (such as their isoelectric point). For β-galactosidase this complex of mucopolysaccharide and enzyme results in a reduction in enzymic activity, possibly because of competitive binding of the two galactose molecules at the reducing end of the carbohydrate chain. For other hydrolases such as β-glucuronidase and *N*-acetyl-β-hexosaminidase, the binding may result in a protection of the enzyme against the destructive action of the lysosomal proteinase and cause increased activities, as was first suggested by Van Hoof (*2*). However, stimulated synthesis of some hydrolases may also occur. The accumulation of glycolipids is secondary and may be due to the inability of one or several isoenzymes of a hydrolase to degrade its substrate after it has been bound to a mucopolysaccharide. Indeed, while enzyme activity with methylumbelliferyl substrates are apparently not inhibited, the enzyme activity with macromolecular substrates may be substantially decreased by the binding of mucopolysaccharide (*15*).

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