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Sanfilippo syndrome type C: Deficiency of acetyl-CoA:α-glucosaminide *N*-acetyltransferase in skin fibroblasts

(heparan sulfate/mucopolysaccharidosis/lysosomes)

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ABSTRACT **Removal of *N*-sulfated glucosamine residues during degradation of heparan sulfate is accomplished by the sequential action of three enzymes. Action of sulfamidase results in the formation of α-glucosaminide residues. Removal of these groups requires conversion to α-*N*-acetylglucosaminide by the action of an acetyltransferase in the presence of acetyl-CoA, followed by hydrolysis by α-*N*-acetylglucosaminidase. In fibroblast homogenates from three patients with Sanfilippo syndrome type C (mucopolysaccharidosis III C), a biochemical variant of the Sanfilippo syndrome, complete deficiency of the acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity was detected. Activities of all lysosomal hydrolases known so far to degrade mucopolysaccharides, including those of sulfamidase and α-*N*-acetylglucosaminidase, were in the range of controls. Acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity was normal in fibroblasts of patients with other genetic mucopolysaccharidoses, including Sanfilippo syndrome A and B.**

The Sanfilippo syndrome (mucopolysaccharidosis III) belongs to the group of inborn errors of mucopolysaccharide degradation. The typical clinical features of this syndrome are severe progressive mental retardation and skeletal deformities that are generally less severe than in the Hurler and Hunter syndromes (1). Biochemically the Sanfilippo syndrome is characterized by the intralysosomal storage of heparan sulfate-like mucopolysaccharide fragments in all organs (2, 3) and excessive excretion of these fragments in the urine (4, 5). Two enzyme defects have been known to cause the Sanfilippo syndrome. In the Sanfilippo A form, a sulfamidase (*N*-sulfoglucosaminide sulfamidase) (6, 7) and in B form an α-*N*-acetylglucosaminidase (EC 3.2.1.50) (8, 9) are deficient. Both enzymes split linkages unique to heparan sulfate and heparin. Deficiencies of enzymes that split linkages that heparan sulfate and heparin have in common with other mucopolysaccharides cause the Hurler and Scheie syndromes (α-L-iduronidase, EC 3.2.1.76) (10, 11), the Hunter syndrome (L-iduronide-2-sulfate sulfatase) (12), the β-glucuronidase deficiency (EC 3.2.1.31) (13, 14), and a newly described mucopolysaccharidosis deficient in *N*-acetylglucosamine-6-sulfate sulfatase (15).

Several patients with the typical clinical symptoms of the Sanfilippo syndrome and excessive urinary excretion of exclusively heparan sulfate but normal activities of sulfamidase and α-*N*-acetylglucosaminidase have been reported.§¶ The heparan sulfate stored in fibroblasts of these patients, tentatively designated as having the Sanfilippo syndrome type C, bears terminal glucosamine residues (H. Kresse, K. von Figura, and U. Klein, unpublished data), leading to the erroneous suggestion of an α-glucosaminidase deficiency.§¶

We report here a new enzyme that in the presence of acetyl-CoA acetylates α-glucosaminyl residues, which result during heparan sulfate degradation by the action of sulfamidase on

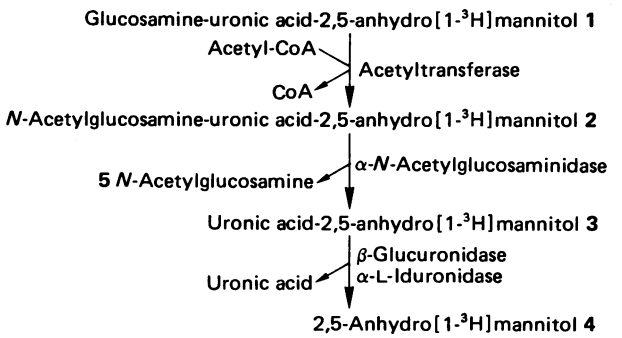

FIG. 1. Degradation of unacetylated trisaccharide 1 by homogenates from normal human skin fibroblasts.

N-sulfated glucosamine residues. In a following step, the *N*-acetylglucosaminyl residues are removed by the α-*N*-acetylglucosaminidase. The acetyl-CoA:α-glucosaminide *N*-acetyltransferase was found to be deficient in fibroblast homogenates from three patients with Sanfilippo syndrome type C.

MATERIALS AND METHODS

[1-^{14}C]Acetyl-CoA (specific activity 60 mCi/mmol) was obtained from Amersham Buchler. α-*N*-Acetylglucosaminidase purified from human urine was that described earlier (16).

Preparation of Radioactive Oligosaccharides. The *N*-acetylated ^{3}H-labeled trisaccharide **2** (see Fig. 1), *O*-α-D-2-acetamido-2-deoxyglucosyl-(1→4)-*O*-uronosyl-(1→4)-2,5-anhydro[1-^{3}H]mannitol (GlcNAc-UA-[1-^{3}H]aMan-ol) was isolated from heparan sulfate and was a gift from R. Basner of this institute (17). Unacetylated ^{3}H-labeled trisaccharide **1** *O*-α-D-2-amino-2-deoxyglucosyl-(1→4)-*O*-uronosyl-(1→4)-2,5-anhydro[1-^{3}H]mannitol (GlcN-UA-[1-^{3}H]aMan-ol) was prepared by hydrolysis of 88 nmol of **2** in 3.5 ml of 2 M trifluoroacetic acid for 23 hr at 70° under N_2. After lyophilization the products were separated by high-voltage electrophoresis at pH 1.7. The unacetylated trisaccharide **1** was the only migrating radioactive compound and contained 46% of the total radioactivity. The unacetylated trisaccharide **1** was eluted from the paper and subjected to chromatography on a Sephadex G-25 (Pharmacia) column, from which it eluted in a symmetric peak with a K_{av} of 0.61 (the disaccharide UA-[1-^{3}H]aMan-ol **3** and the *N*-acetylated trisaccharide **2** had K_{av} values of 0.64 and 0.57, respectively). Analysis of the nonmigrating ^{3}H-labeled products by paper electrophoresis at pH 5.3 showed the presence of *N*-acetylated trisaccharide **2**, UA-[1-^{3}H]aMan-ol **3**, and

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§ Kresse, H. & von Figura, K. (1975) *Third International Symposium on Glycoconjugates*, Brighton, England, July 6–12 (abstr.).

¶ Kresse, H., von Figura, K. & Bartsocas, C. (1976) *Meeting of European Society for Human Genetics*, Athens, May 8–9 (abstr.).

[1-^{3}H]aMan-ol 4 (8, 36, and 10% of the starting radioactivity, respectively). The disaccharide uronosyl-(1→4)-2,5-anhydro[1-^{3}H]mannitol 3 (UA-[1-^{3}H]aMan-ol) was prepared from heparin (18). The uronic acid in the heparan sulfate/heparin-derived oligosaccharides 1–3 could be glucuronic and/or iduronic acid.

Cell Culture. Fibroblasts from the skin of normal individuals or patients affected with mucopolysaccharidoses were maintained in culture as previously described (19). The Sanfilippo C form cell lines were obtained from male patients of German and Greek origin (W. W. and A. V.) and from a female patient of Dutch origin (M. D.). Detailed case reports of these patients will follow elsewhere (C. Bartsocas, H. Groebe, J. J. P. van de Kamp, K. von Figura, H. Kresse, and U. Klein, unpublished).

For enzyme determination, confluent flasks (25 cm^2) were treated with 0.1% trypsin (Flow Laboratories) and the cells were collected by centrifugation and suspended in 0.2–0.5 ml of 0.15 M NaCl. Homogenates were prepared by ten cycles of freezing and thawing and assayed for protein (20) and enzyme activities.

Assay of Acetyl-CoA:α-Glucosaminide *N*-Acetyltransferase Activity. Unless otherwise stated the incubation mixture contained 220 pmol of unacetylated trisaccharide 1 (about 11,000 cpm), 5–20 μg of cell protein, 0.14 M sodium acetate at pH 5.5, 3.8 mM NaN_3, and 2 mM acetyl-CoA in a final volume of 7 μl. Paraffin oil (5 μl) was layered on the incubation mixture. After incubation for 1–48 hr at 37° the incubation mixture was subjected to paper electrophoresis at pH 1.7. The cell homogenate converted substrate 1 into the *N*-acetylated trisaccharide 2, which was further degraded by α-*N*-acetylglucosaminidase to the disaccharide 3. Action of β-glucuronidase and/or α-L-iduronidase led to the liberation of 4 (see Fig. 1). Activity of the *N*-acetyltransferase was expressed as nmol of substrate converted into products 2–4 per mg of cell protein in 24 hr. In control incubations with boiled about enzyme, 2% of the radioactivity remained at the origin.

Analytical Methods. *Determination of Lysosomal Enzymes that Degrade Mucopolysaccharides.* Activities of α-L-iduronidase (21), L-iduronide-2-sulfate sulfatase (according to a modification of the method in ref. 22), sulfamidase (6), α-*N*-acetylglucosaminidase (23), *N*-acetylgalactosamine-6-sulfate sulfatase (24), β-galactosidase (EC 3.2.1.23) (16), arylsulfatase B (25) (EC 3.1.6.1), β-glucuronidase (16), *N*-acetylglucosamine-6-sulfate sulfatase (17) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) (16) were determined as described.

Electrophoresis. High-voltage electrophoresis on Whatman 3 MM paper was carried out either in 1.9 M formic acid, pH 1.7, at 50 V/cm for 45 min or in pyridine/acetic acid, pH 5.3 (0.08 M with regard to pyridine) at 75 V/cm for 45 min. For identification of *N*-acetylglucosamine, electrophoresis was carried out in a borate buffer system as described (26).

RESULTS

Demonstration of acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity in human skin fibroblasts

Unacetylated trisaccharide 1 was incubated with fibroblast homogenates under a variety of conditions, but it was not degraded unless acetyl-CoA was included in the assay mixture (Fig. 1). Variation of conditions included the use of acetate, citrate, and phosphate buffers of pH between 4 and 7, harvesting fibroblasts by trypsin or scraping, preparation of fibroblast homogenates by freezing and thawing or sonication, and use of homogenates before and after dialysis against 0.15 M NaCl. In the presence of acetyl-CoA the unacetylated trisaccharide 1 was converted into the *N*-acetylated trisaccharide 2, the disaccharide 3, and 2,5-anhydro-[1-^{3}H]mannitol 4 (Fig. 2 *A* and *B*). The acetyl transfer appears to be catalyzed enzymatically, as shown by the absence of acetyl transfer when boiled homogenates were used (Fig. 2 *C* and *D*). The appearance of products 2–4 suggests that degradation of the unacetylated trisaccharide 1 starts with the acetylation of the

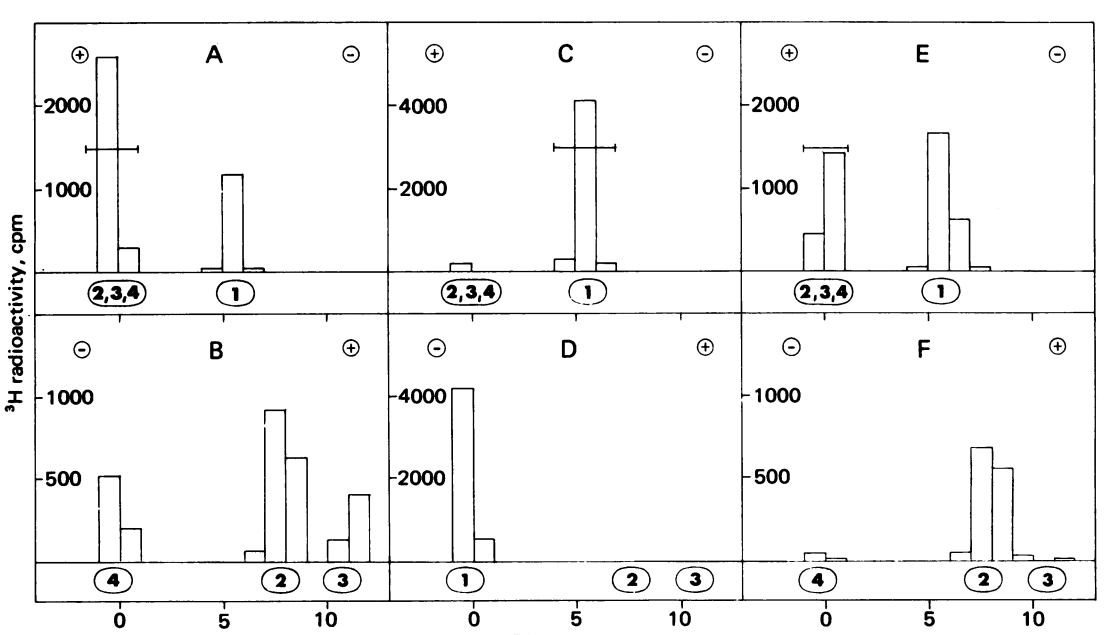

FIG. 2. Paper electrophoresis of acetyl-CoA:α-glucosaminide *N*-acetyltransferase assays. (*A*, *C*, and *E*) Distribution pattern of ^{3}H radioactivity after electrophoresis at pH 1.7. The ^{3}H radioactive material of the areas indicated by the horizontal bars was eluted, brought to dryness, and subjected to electrophoresis at pH 5.3 (*B*, *D*, and *F*). The following enzyme sources were used: (*A* and *B*) homogenate from normal human skin fibroblasts (incubated in the presence of acetyl-CoA); (*C* and *D*) boiled fibroblast homogenate, homogenate from Sanfilippo C fibroblasts, or homogenates from normal fibroblasts incubated in the absence of acetyl-CoA; (*E* and *F*) homogenate from Sanfilippo B fibroblasts (incubated in the presence of acetyl-CoA). The standards 1–4 are those of Fig. 1.

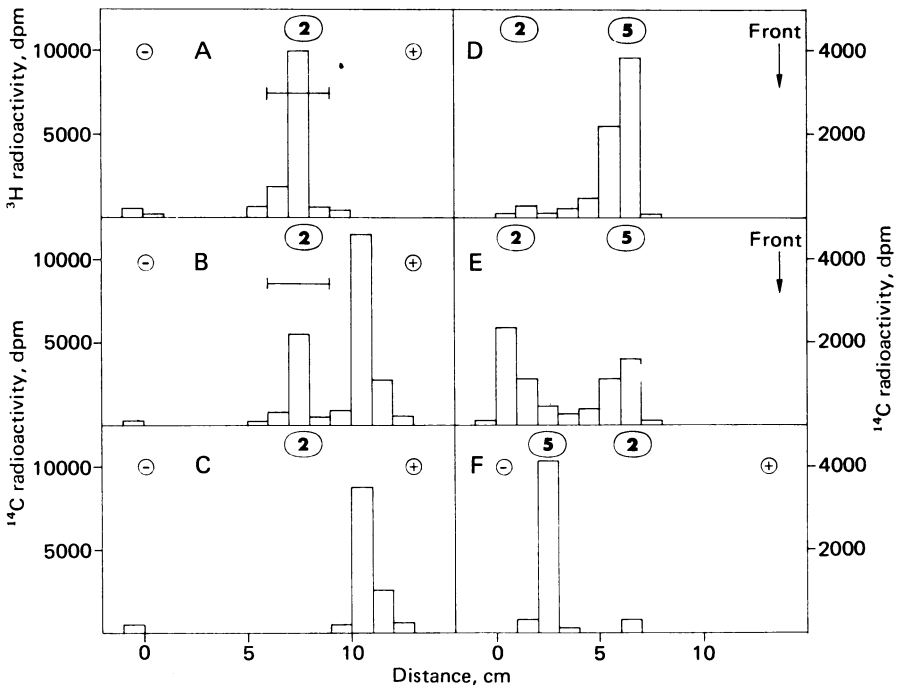

FIG. 3. Demonstration of acetyl transfer from [^{14}C]acetyl-CoA onto unacetylated trisaccharide **1**. Unacetylated trisaccharide **1**, 5 nmol, was incubated in the presence of 0.63 mM [^{14}C]acetyl-CoA (specific activity 60 mCi/mmol) with α-*N*-acetylglucosaminidase-deficient homogenate obtained from Sanfilippo B fibroblasts in 20 μl of 0.14 M sodium acetate, pH 5.5. After 24 hr, 140 nmol of acetyl-CoA in 2 μl of water was added and incubation was continued for another 48 hr. In the blank incubation the trisaccharide **1** was omitted. The incubation mixtures were put on 0.5 × 2 cm columns of an anion-exchange resin (AG 1-X2, Cl^- form, Bio-Rad) in water. Trisaccharide **1** was eluted with water. Anionic material was eluted with 1 M HCl, brought to dryness, and applied to high-voltage electrophoresis at pH 5.3. ^{3}H and ^{14}C radioactivity were measured for the assay (*A* and *B*, respectively) and ^{14}C radioactivity for the blank (*C*). Counting efficiencies were 31% for ^{3}H and 63% for ^{14}C. The radioactive material comigrating with the *N*-acetylated trisaccharide **2** was eluted. This fraction, containing 1.7 nmol of [^{14}C]acetylated trisaccharide **2** with a specific ^{14}C activity of 31.3 mCi/mmol was re-electrophoresed at pH 1.7. [^{14}C]Acetylglucosamine could be liberated from **2** by digestion at pH 4.5 (24) with either purified α-*N*-acetylglucosaminidase (*D* and *F*) or homogenate from Sanfilippo C fibroblasts (*E*). *N*-[^{14}C]Acetylglucosamine **5** was identified by either thin-layer chromatography (*D* and *E*) or high-voltage electrophoresis in a borate buffer system (*F*). *N*-Acetylated trisaccharide **2** and *N*-acetylglucosamine **5** were used as standards.

α-glucosaminyl residue at the nonreducing terminal. The *N*-acetylated trisaccharide **2** is then degraded to the monosaccharides by α-*N*-acetylglucosaminidase, β-glucuronidase, and/or α-L-iduronidase. The removal of the *N*-acetylglucosamine residue by the α-*N*-acetylglucosaminidase is demonstrated by the absence of products **3** and **4** when α-*N*-acetylglucosaminidase-deficient homogenates from Sanfilippo B fibroblasts were used as enzyme source (Fig. 2 *E* and *F*).

Acetyl transfer from acetyl-CoA onto the unacetylated trisaccharide **1** could be directly demonstrated by using [1-^{14}C]acetyl-CoA as cosubstrate and Sanfilippo B fibroblast homogenate (deficient in α-*N*-acetylglucosaminidase) as enzyme source (Fig. 3). As reaction product *N*-[^{14}C]acetylated trisaccharide **2** was isolated by ion-exchange chromatography and high-voltage electrophoresis (Fig. 3 *A–C*). *N*-[^{14}C]Acetylglu-

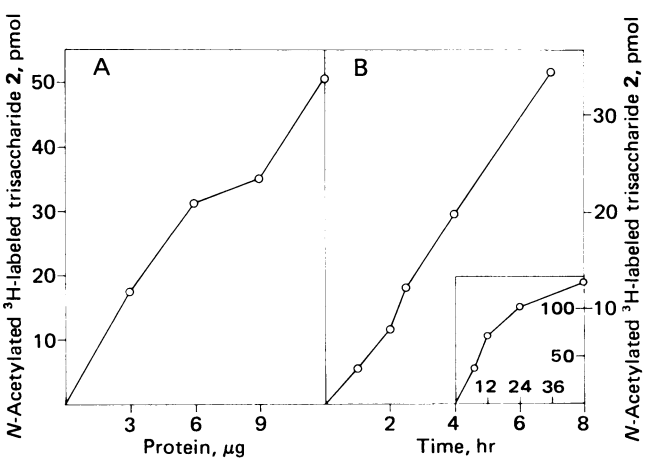

FIG. 4. Dose and time dependence of *N*-acetylation of unacetylated trisaccharide **1**. (*A*) Incubation for 8 hr. (*B*) Incubation with 9 μg of protein.

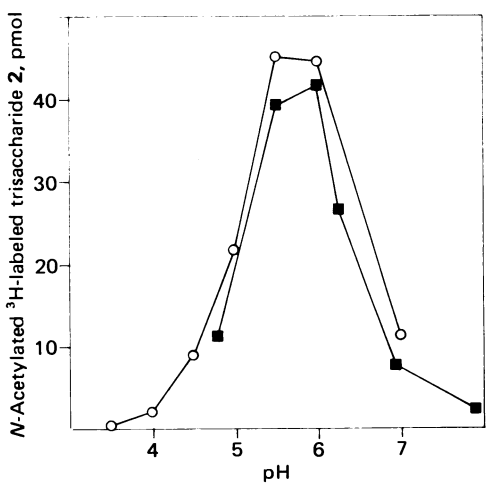

FIG. 5. pH dependence of acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity. Unacetylated trisaccharide **1** was incubated under standard conditions in the presence of 0.14 M sodium acetate buffers, pH 3.5–7.0 (○), and 0.14 M sodium phosphate buffers, pH 4.8–7.9 (■).

cosamine liberated from the doubly labeled trisaccharide by digestion with purified α-*N*-acetylglucosaminidase was identified by thin-layer chromatography (Fig. 3*D*) and high-voltage electrophoresis (Fig. 3*F*).

The acetyl transfer is dose dependent and proceeds linearly with time for up to 8 hr under the conditions used (Fig. 4). The pH optimum of the acetyl transfer is between 5.5 and 6.0 (Fig. 5).

Deficiency of acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity in fibroblasts from patients with Sanfilippo C syndrome

Fibroblast homogenates from the three patients affected with Sanfilippo C syndrome were unable to acetylate trisaccharide **1**. They had a complete deficiency of acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity (Table 1, Fig. 2 *C* and *D*). All the lysosomal enzymes known to be involved in the degradation of heparan sulfate (α-L-iduronidase, β-glucuronidase, α-*N*-acetylglucosaminidase, iduronide 2-sulfate sulfatase, sulfamidase, and *N*-acetylglucosamine 6-sulfate sulfatase) or in the degradation of other mucopolysaccharides (β-*N*-acetylglucosaminidase, β-galactosidase, arylsulfatase B, *N*-acetylgalactosamine 6-sulfate sulfatase) were present at normal levels. Accordingly the degradation of the *N*-acetylated trisaccharide **2** by homogenates from Sanfilippo C cells was not impaired (Fig. 3*E*).

The deficiency of acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity in Sanfilippo C fibroblasts is not due to the presence of an inhibitor. A mixture of equal portions of homogenates of Sanfilippo C and of normal fibroblasts exhibited 58% of the activity of the unmixed control homogenate activity. Normal acetyl-CoA:α-glucosaminide *N*-acetyltransferase activities were found in homogenates from Sanfilippo A and B fibroblasts and from other mucopolysaccharidoses (Table 1). Mucolipidosis II fibroblasts (I-cell disease) have markedly reduced intracellular activities of a variety of lysosomal hydrolases (27–29). Intracellular acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity from a mucolipidosis II cell line was found to be in the range of controls, as has previously been reported for intracellular activities of acid phosphatase and β-glucosidase in mucolipidosis II fibroblasts (for review see ref. 30).

Table 1. Acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity in homogenates of cultured skin fibroblasts

Subjects	Mucopoly-saccharidosis	Enzyme activity
Controls (n = 5)		9.9 (4.0–18.4)
Sanfilippo C	III C	
W.W.		<0.3
M.D.		<0.3
A.V.		<0.3
Sanfilippo A	III A	7.3
Sanfilippo B	III B	8.4
Hurler	I H	12.3
Scheie	I S	3.5
Hunter	II	7.5
Morquio	IV	3.6
Maroteaux–Lamy	VI	5.5
β-Glucuronidase deficiency	VII	7.8
I-cell	Mucolipidosis II	5.5
Sandhoff	GM_2-gangliosidosis II	5.4

Enzyme activity is expressed as nmol of products 2–4 formed per mg cell protein per 24 hr at 37°. During the incubation 15–50% of the added substrate became *N*-acetylated.

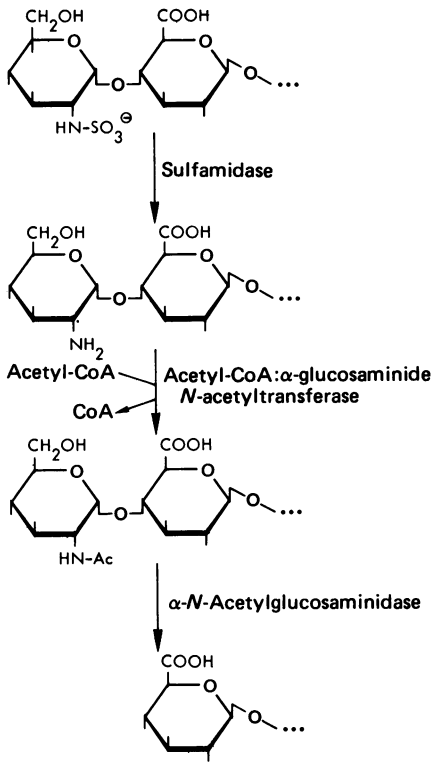

FIG. 6. Pathway of enzymatic degradation of sulfoglucosaminyl residues in heparan sulfate. Sulfamidase is deficient in Sanfilippo syndrome A form (6, 7), and α-*N*-acetylglucosaminidase in Sanfilippo syndrome B form (8, 9). As shown in this paper, an acetyl-CoA:α-glucosaminide *N*-acetyltransferase is deficient in Sanfilippo syndrome C form.

DISCUSSION

Heparan sulfate chains are degraded within the lysosomes by the action of endoglycosidases (31–34), exoglycosidases, and sulfatases (for reviews see ref. 30, 35, 36). The endoglycosidases and sulfatases act on the nonreducing terminal of the polysaccharide chains. *N*- and/or *O*-sulfate groups on the sugar residues at the nonreducing terminal have to be removed by specific sulfatases prior to the action of the exoglycosidases. Removal of the *N*-sulfate group from a terminal *N*-sulfoglucosaminyl residue by the sulfamidase results in formation of an α-glucosaminyl residue, which cannot be removed by the α-*N*-acetylglucosaminidase (23). The present study demonstrates that the terminal α-glucosaminyl residue of a trisaccharide derived from heparan sulfate is *N*-acetylated prior to the removal as *N*-acetylglucosamine by the α-*N*-acetylglucosaminidase (Fig. 6). The sequence by which sulfamidase, acetyl-CoA:α-glucosaminide *N*-acetyltransferases, and α-*N*-acetylglucosaminidase remove *N*-sulfoglucosaminyl residues bears similarity to the sequence of enzymatic steps involved in the formation of these residues. Biosynthesis of *N*-sulfoglucosaminyl residues involves deacetylation of *N*-acetylglucosaminyl residues within precursor polysaccharide chains. The *N*-deacetylated α-glucosaminyl residues are then *N*-sulfated by a specific sulfotransferase (37).

The intralysosomal storage of mucopolysaccharides accompanying the deficiency of lysosomal enzymes in mucopolysaccharidoses has led to the view that degradation of mucopolysaccharides takes place in the lysosomes. Participation of acetyl-CoA:α-glucosaminide *N*-acetyltransferase in the

degradation of α-glucosaminyl residues would therefore suggest its intralysosomal localization. Thus we have found a nonhydrolytic reaction occurring in lysosomes. Intralysosomal occurrence of acetyl-CoA has so far not been described. Whether acetyl-CoA is the donor for the acetylation reaction under *in vivo* conditions remains to be investigated. The acid pH optimum and the absence of acetyltransfer to free glucosamine (not shown) distinguish the acetyl-CoA:α-glucosaminide *N*-acetyltransferase from the previously described acetyl-CoA:glucosamine *N*-acetyltransferase (EC 2.3.1.3) (38) and the acetyl-CoA:glucosamine-6-phosphate *N*-acetyltransferase (EC 2.3.1.4) (39).

The present study clearly demonstrates that homogenates of fibroblasts derived from patients with the clinical diagnosis of Sanfilippo syndrome type C are deficient in acetyl-CoA:α-glucosaminide transferase activity. Biochemically the type C of Sanfilippo syndrome differs from type A and B by normal activities of both sulfamidase and α-*N*-acetylglucosaminidase and the storage of heparan sulfate-like fragments characterized by low anionic charge and content of *N*-unsubstituted glucosamine residues[§¶]; H. Kresse, K. von Figura, and U. Klein, unpublished data). These findings are in accordance with the deficiency of an acetyl-CoA:α-glucosaminide *N*-acetyltransferase activity that is expected to result in the storage of heparan sulfate-like fragments with α-glucosaminyl residues at the nonreducing terminal.

Note Added in Proof. Since submission of this manuscript, deficiency of acetyl-CoA:α-glucosaminide *N*-acetyltransferase was detected in the fibroblast homogenates of 11 patients with Sanfilippo syndrome.

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