Carbohydrate Research, 78 (1980) 249–256

A ^{3}H-LABELLED TRISACCHARIDE FROM HEPARIN AS SUBSTRATE FOR ACETYL-CoA: 2-AMINO-2-DEOXY-α-D-GLUCOSIDE *N*-ACETYLTRANSFERASE

UDO KLEIN* AND KURT VON FIGURA

Institute of Physiological Chemistry, University of Münster, Waldeyerstraße 15, D 4400 Münster (West Germany)

(Received March 15th, 1979; accepted for publication, April 19th, 1979)

ABSTRACT

The tetrasaccharide fraction obtained by gel chromatography after treatment of commercially available heparin with nitrous acid was reduced with NaB^3H_4 and then hydrolysed with 2M trifluoracetic acid at 70° for 3 days. By gel chromatography and electrophoresis, the ^{3}H-labelled trisaccharide **1** bearing an unsubstituted 2-amino-2-deoxy-D-glucosyl group in the non-reducing position was obtained (18% from the ^{3}H-labelled tetrasaccharide). By sequential, enzymic degradation, the structure α-D-GlcN-(1→4)-β-D-GlcA-(1→4)-[1-^{3}H]aManol† was obtained for **1**, which is a substrate for acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase, an enzyme that is deficient in the Sanfilippo C syndrome. In human-skin fibroblasts, the pH optimum of acetyl transfer onto **1** was between pH 5.5 and 7.0, and dependent on the buffer. An apparent K_m for **1** of 0.14mM was found.

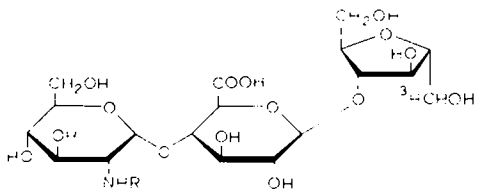
INTRODUCTION

The Sanfilippo C syndrome (mucopolysaccharidosis III C) belongs to the group of heritable disorders of mucopolysaccharide degradation[1–3]. Patients suffering from this disease store, exclusively, heparan sulphate having terminal 2-amino-2-deoxy-D-glucosyl groups[4]. Recently, an acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase was found[5] to be absent in cultivated, skin fibroblasts from patients affected with Sanfilippo C syndrome. A labelled trisaccharide having the structure 2-amino-2-deoxy-α-D-glucosyl-(1→4)-(aldosyluronic acid)-(1→4)-2,5-anhydro-D-[1-^{3}H]mannitol (GlcN-UA-[1-^{3}H]aManol), originally prepared[5,6] from heparan sulphate, was a substrate for this transferase. Heparan sulphate is not a suitable source of a trisaccharide bearing an unsubstituted 2-amino-2-deoxyglucosyl group in the non-reducing position, since treatment of heparan sulphate with nitrous acid yields only a small amount of the tetrasaccharides[6,7] from which the trisaccharide is

*To whom reprint requests should be addressed.
†aManol connotes 2,5-anhydro-D-mannitol.

prepared. Moreover, heparan sulphate is not available commercially. On the other hand, heparin, which is available commercially, yields a higher proportion of tetrasaccharides on treatment with nitrous acid[7].

We now describe the preparation from heparin of the trisaccharide α-D-GlcN-(1→4)-β-D-GlcA-(1→4)-[1-^{3}H]aManol (**1**), which proved to be a substrate suitable for detecting homozygotes of the Sanfilippo C syndrome.



1 R = H
2 R = Ac

EXPERIMENTAL

Materials. — NaB^3H_4 (specific activity, 278 mCi/mmol), ^{14}C-labelled acetyl-CoA (specific activity, 60 mCi/mmol), and heparin were commercial materials. GlcNAc-UA-[1-^{3}H]aManol and the ^{3}H-labelled, unsulphated disaccharide from heparan sulphate were gifts from R. Basner[6] of this Institute; GlcN-UA-[1-^{3}H]-aManol, prepared from heparan sulphate, has been described[5] previously; and IdA-[1-^{3}H]aManol was prepared according to the method of DiNatale *et al.*[8].

Preparation of a ^{3}H-labelled tetrasaccharide. — A solution of sodium heparinate (2.5 g) in water (20 ml) was treated for 15 min at −5° with nitrous acid (80 ml) prepared[9] from H_2SO_4 and $Ba(NO_2)_2$. After addition of 50% aqueous ammonium amidosulphonate (20 ml), the mixture was stored at room temperature for 30 min before loading on a column (5 × 220 cm) of Sephadex G-25 (superfine), equilibrated and eluted with M NaCl. The fraction eluting[6] between 2340 and 2930 ml (35% of the total uronic acid) was desalted on a column (3.6 × 230 cm) of Sephadex G-25 (medium) by elution with water. A solution of the tetrasaccharide in 0.1M sodium borate (pH 8.0, 3.5 ml) was treated with 0.38 mmol of NaB^3H_4 for 4 h, and then with $NaBH_4$ (3 mmol) for 3 h. The pH of the mixture was brought to 5 with acetic acid before loading on a column (3.6 × 240 cm) of Sephadex G-15 equilibrated and eluted with M NaCl. After desalting on Sephadex G-25, as described above, the ^{3}H-labelled tetrasaccharides were twice subjected to chromatography on Sephadex G-15, and desalted when they had a specific activity of 19.8 mCi/mmol and contained 21% of the original uronic acids.

Preparation of ^{3}H-labelled trisaccharide **1**. — A solution of the foregoing ^{3}H-labelled tetrasaccharides in 2M CF_3CO_2H (8 ml) was kept for 72 h at 70° under N_2, and then concentrated *in vacuo*. The residue was subjected to electrophoresis on Whatman 3MM paper in 1.9M formic acid (pH 1.7) at 40 V/cm for 45 min. Radioactive material migrating to the cathode was eluted from the paper, and subjected to paper

electrophoresis in 0.08M pyridine in acetic acid (pH 5.3) at 60 V/cm for 45 min. The non-migrating, ^{3}H-labelled material was eluted from the paper and then eluted from a column (3.6 × 240 cm) of Sephadex G-15 with M NaCl. The fractions containing radioactivity eluting in the position of GlcN-UA-[1-^{3}H]aManol isolated from heparan sulphate[5] were combined, desalted as described above, and freeze-dried to give **1**, which accounted for 18% of the uronic acid present in the ^{3}H-tetrasaccharide. The ^{3}H-labelled trisaccharide **1** was stored in liquid nitrogen, as an aqueous solution containing 2% of ethanol.

Chromatography. — T.l.c. was performed on cellulose plates (Merck) with ethyl acetate–pyridine–acetic acid–water (5:5:2:3), and descending p.c. was performed on water-washed Whatman 3MM paper with ethyl acetate–acetic acid–water[10] (3:1:1).

Enzyme assays. — The standard incubation-mixture contained 0.84 nmol of **1** (~11,000 c.p.m.), 12 nmol of acetyl-CoA, 0.125M sodium chloride in 0.16M buffer, 0.03% of NaN_3, and up to 35 μg of acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase-containing protein in a final volume of 6 μl. After incubation for up to 24 h at 37°, acetylation of the substrate was determined either by paper electrophoresis at pH 1.7 or by the following micro-column procedure. After the addition of 100 μl of H_2O, the mixture was loaded on a column (0.5 × 3 cm) of AG-50W X8 (H^+) resin (200–400 mesh) equilibrated with water. The ^{3}H-labelled products were eluted with 2 ml of H_2O, and the remaining **1** with 2 ml of 0.2M NaCl. Each fraction was assayed for radioactivity in 5 ml of Instagel (Packard, Frankfurt). In control assays incubated either without acetyl-CoA or with boiled enzyme, 5–6% of the total radioactivity was eluted with water.

Enzymic characterization of **1**. — Trisaccharide **1** (300 nmol) was incubated in the presence of 0.6mM [^{14}C]acetyl-CoA (60 mCi/mmol) with a homogenate from Sanfilippo B fibroblasts deficient in 2-acetamido-2-deoxy-α-D-glucosidase in 300 μl of 0.1M sodium acetate (pH 5.5). After 24 h, a solution (1.4 μmol) of acetyl-CoA in 20 μl of water was added and incubation was continued for 72 h. The mixture was then loaded on a column (0.5 × 3 cm) of AG-1 X8 (Cl^-) resin, which was equilibrated and eluted with water. Anionic material, including, *inter alia*, the ^{3}H,^{14}C-labelled trisaccharide **2**, was eluted with M HCl, the eluate was concentrated to dryness, and the residue was subjected to paper electrophoresis at pH 5.3 (see above). The radioactivity co-migrating with an *N*-acetylated, ^{3}H-labelled trisaccharide[5] was eluted, subjected to paper electrophoresis at pH 1.7 (see above), and then digested[5] at pH 4.5 with purified, human-urinary 2-acetamido-2-deoxy-α-D-glucosidase. After paper electrophoresis of the digest at pH 5.3, liberated [^{14}C]-2-acetamido-2-deoxy-glucose was eluted from the paper, and identified by t.l.c., or paper electrophoresis in a borate buffer[5]. The resulting ^{3}H-labelled disaccharides were characterized, after elution from the paper, by p.c., which separated glucuronic and iduronic acid-containing disaccharides[10]. 2-Acetamido-2-deoxy-α-D-glucosidase, β-D-glucuronidase, and α-L-iduronidase activities were assayed as previously described[11,12].

Human 2-acetamido-2-deoxy-α-D-glucosidase was purified[11] from fresh, human urine.

Human-skin fibroblasts from mucopolysaccharidosis and control patients were maintained in culture[13], and harvested by trypsinization[13]. Suspensions of fibroblasts were obtained by 10 cycles of freezing and thawing in 0.15M sodium chloride at concentrations of 1–7 mg of protein/ml.

RESULTS

Heparin was treated with nitrous acid under conditions that release all *N*-sulphate groups and convert the resulting 2-amino-2-deoxy-D-glucose residues into 2,5-anhydro-D-mannose[9]. After chromatography on Sephadex G-25, 35% of the total uronic acid was recovered in the position of tetrasaccharides (Fig. 1). After reduction with NaB^3H_4 and chromatography on Sephadex G-15, the ^3H-labelled tetrasaccharides were hydrolysed, to give a trisaccharide having a 2-amino-2-deoxy-D-glucosyl group at the non-reducing terminal. The hydrolysis was monitored by paper electrophoresis at pH 1.7, which separated the cationic, ^3H-labelled oligosaccharides, including **1**, from non-migrating and anionic, ^3H-labelled oligosaccharides (Fig. 2*A*). Cationic, ^3H-labelled material was separated by paper electrophoresis at pH 5.3. Under these conditions, **1** remained and was separated from material having greater negative charge, presumably UA-GlcN-UA-[1-^3H]aManol (Fig. 2*B*). Fig. 3 shows that, after 72 h at 70°, hydrolysis of the ^3H-labelled tetrasaccharides gave an optimum yield of **1**, which was isolated by paper electrophoresis at pH 1.7 and 5.3 followed by gel electrophoresis on Sephadex G-15.

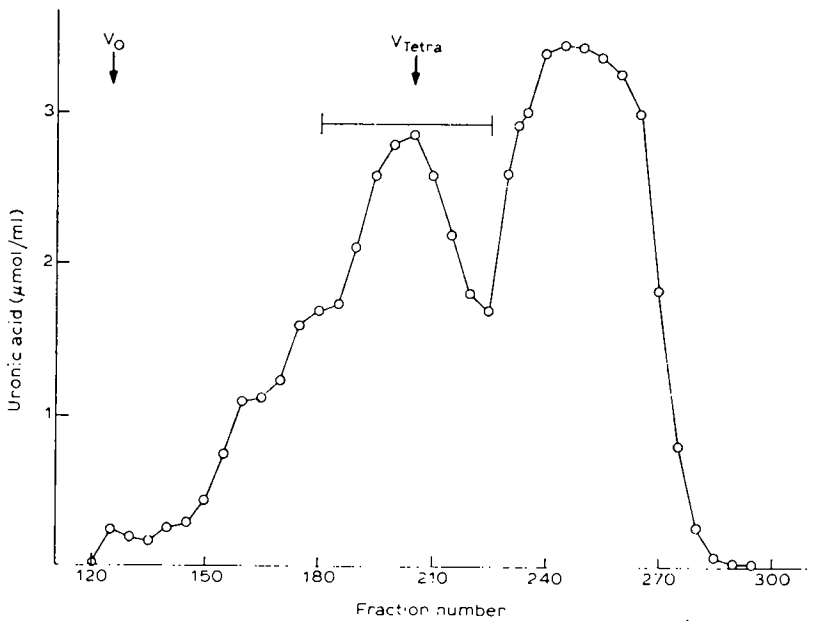


Fig. 1. Gel chromatography of heparin, after treatment with nitrous acid, on Sephadex G-25 (uronic acid assay). The fractions containing tetrasaccharide are indicated by the horizontal bar; V_0 and V_{Tetra} are the elution positions of Dextran Blue and of a standard tetrasaccharide obtained from heparan sulphate[6].

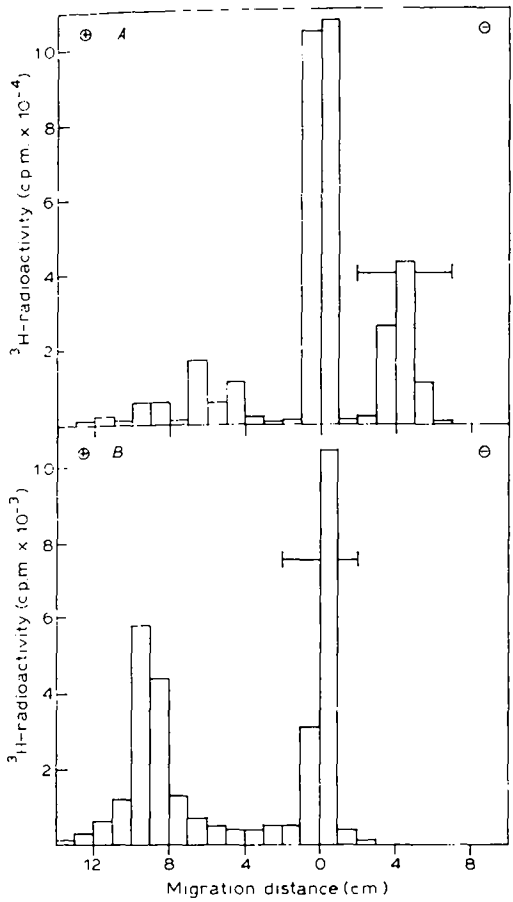


Fig. 2. Paper electrophoresis of the products of hydrolysis of the ^{3}H-labelled tetrasaccharides with 2M CF_3CO_2H at 70° for 24 h under N_2: *A*, at pH 1.7; *B*, at pH 5.3, of the material indicated by the horizontal bar in *A*.

The ^{3}H-labelled trisaccharide **1** proved to be a suitable substrate for determination of acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase activity. The activities determined with **1** in fibroblast suspensions obtained from controls and Sanfilippo C patients were in excellent agreement with those obtained with the previously used, ^{3}H-labelled trisaccharide GlcN-UA-[1-^{3}H]aManol from heparan sulphate[5]. The enzyme activity appeared to be dose-dependent and proceeded linearly with time, as previously shown[5]. Upon prolonged incubation, >90% of **1** was acetylated. The pH-dependence of acetyltransferase activity shows a single optimum at pH 5.5 in phosphate buffers, whereas two optima (pH 6.0 and 7.0) were observated in citrate buffers (Fig. 4). An apparent K_m of 0.14mM was found for **1** (Fig. 5). Under the standard conditions of incubation, increase of acetyl-CoA concentration above 2mM did not affect the rate of acetyl transfer.

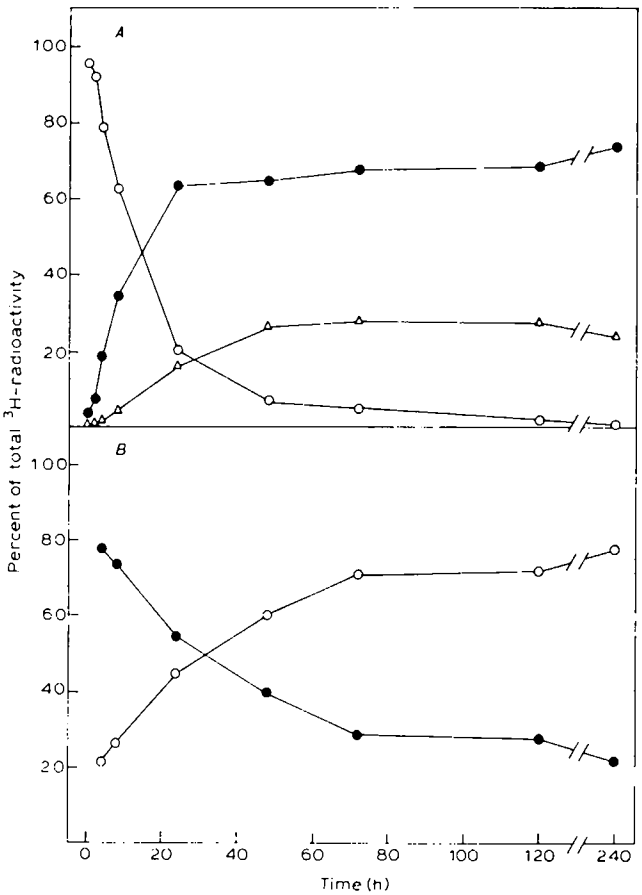


Fig. 3. Hydrolysis of ^{3}H-labelled tetrasaccharides: *A*, analysis by paper electrophoresis at pH 1.7 for percent of radioactivity in neutral (●), cationic (△), and anionic material (○); *B*, paper electrophoresis at pH 5.3 of cationic material from *A*, namely, **1** (○) and ^{3}H-labelled oligosaccharides (●).

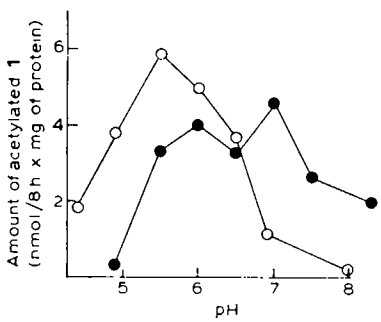


Fig. 4. pH-Dependence of acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase activity: incubation of **1** in 0.16M sodium phosphate (○) and sodium citrate buffers (●) for 8 h.

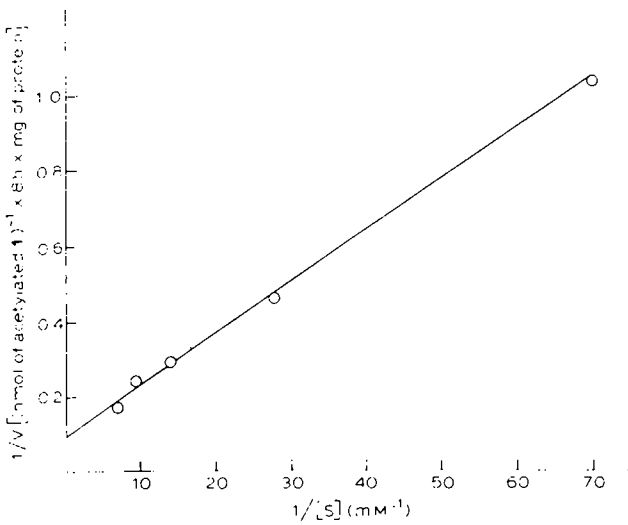


Fig. 5. Acetyl-transfer onto **1** as a function of concentration. The acetyl-CoA concentration was 2mM and the incubation time 8 h.

The structure of **1** was analysed by stepwise, enzymic degradation. After treatment of **1** with a homogenate from Sanfilippo B fibroblasts deficient in 2-acetamido-2-deoxy-α-D-glucosidase in the presence of ^{14}C-labelled acetyl-CoA, the ^{3}H,^{14}C-labelled trisaccharide **2** was obtained. [^{14}C]-2-Acetamido-2-deoxy-D-glucose was liberated by purified, human-urinary 2-acetamido-2-deoxy-α-D-glucosidase from **2** and was identified by t.l.c. The ^{3}H-labelled disaccharide-fraction was identified by p.c. as β-D-GlcA-(1→4)-[1-^{3}H]aManol.

DISCUSSION

Acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase participates in the degradation of *N*-sulphated 2-amino-2-deoxy-D-glucose residues in heparan sulphate and heparin. After removal of the *N*-sulphate groups by sulphamidase, the 2-amino-2-deoxy-D-glucose residues are *N*-acetylated and then cleaved by 2-acetamido-2-deoxy-α-D-glucosidase[5]. Since a deficiency of acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase causes mucopolysaccharidosis III C (Sanfilippo syndrome type C)[5], a suitable substrate for assaying acetyl-CoA: 2-amino-2-deoxy-α-D-glucoside *N*-acetyltransferase was needed.

For the preparation of a substrate closely resembling heparin (heparan sulphate) and having a 2-amino-2-deoxy-D-glucosyl group in the non-reducing position, advantage was taken of the degradation of heparin with nitrous acid, which yields, *inter alia*, tetrasaccharides of the structure UA-GlcN-UA-aMan having various degrees of sulphation. The tetrasaccharides were radiolabelled by reduction with NaB^{3}H$_4$, and the maximum yield of the trisaccharide GlcN-GlcA-[1-^{3}H]aManol (**1**)

was obtained by hydrolysis at 70° for 72 h. Under these conditions, deacetylation is favored, relative to the cleavage of the 2-amino-2-deoxy-D-glucosidic linkage[14]. The internal uronic acid residue of the trisaccharide was glucuronic acid, reflecting the resistance of glucuronosyl-anhydromannitol, but not of iduronosyl-anhydromannitol, towards acid hydrolysis[10,15].

More than 90% of **1** was acetylated by control fibroblasts, whereas the acetyltransferase activity in Sanfilippo C-fibroblasts is zero (within experimental error). The acetyltransferase active towards the 2-amino-2-deoxy-D-glucosyl residues is a lysosomal enzyme[16] and is genetically distinct from the acetyl-CoA: 2-amino-2-deoxy-D-glucose 6-phosphate acetyltransferase (EC 2.3.1.4), as shown by the unaltered acetyltransfer on to 2-amino-2-deoxy-D-glucose 6-phosphate by Sanfilippo C-fibroblasts[17].

REFERENCES

1 H. KRESSE AND K. VON FIGURA, *Abstr. Int. Symp. Glycoconjugates, 3rd., Brighton, England*, 1978, p. 12.
2 H. KRESSE, K. VON FIGURA, AND C. BARTSOCAS, *Abstr. Clin. Genet.*, 10 (1976) 367.
3 C. BARTSOCAS, H. GROEBE, J. J. P. VAN DE KAMP, K. VON FIGURA, H. KRESSE, U. KLEIN, AND M. A. H. GIESBERTS, *Eur. J. Pediatr.*, 130 (1979) 251–258.
4 H. KRESSE, K. VON FIGURA, AND U. KLEIN, *Eur. J. Biochem.*, 92 (1978) 333–339.
5 U. KLEIN, H. KRESSE, AND K. VON FIGURA, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 5185–5189.
6 R. BASNER, H. KRESSE, AND K. VON FIGURA, *J. Biol. Chem.*, 254 (1979) 1151–1158.
7 M. HÖÖK, U. LINDAHL, AND P. H. IVERIUS, *Biochem. J.*, 137 (1974) 33–43.
8 P. DINATALE, I. G. LEDER, AND E. F. NEUFELD, *Clin. Chim. Acta*, 77 (1977) 211–218.
9 J. E. SHIVELY AND H. E. CONRAD, *Biochemistry*, 15 (1976) 3932–3942.
10 I. JACCOBSSON, M. HÖÖK, I. PETTERSSON, U. LINDAHL, O. LARM, E. WIRÉN, AND K. VON FIGURA, *Biochem. J.*, 179 (1979) 77–87.
11 K. VON FIGURA, *Eur. J. Biochem.*, 80 (1977) 525–533.
12 R. BASNER, K. VON FIGURA, J. GLÖSSL, U. KLEIN, H. KRESSE, AND W. MLEKUSCH, *Pediatr. Res.*, (1979) in press.
13 M. CANTZ, H. KRESSE, R. W. BARTSOCAS, AND E. F. NEUFELD, *Methods Enzymol.*, 28 (1972) 884–897.
14 A. NEUBERGER AND R. D. MARSHALL, in A. GOTTSCHALK (Ed.), *Glycoproteins*, Elsevier, Amsterdam, 1966, pp. 190–234.
15 M. HÖÖK, U. LINDAHL, G. BÄCKSTRÖM, A. MALMSTRÖM, AND L. Å. FRANSSON, *J. Biol. Chem.*, 249 (1974) 3908–3915.
16 R. POHLMANN, U. KLEIN, AND K. VON FIGURA, unpublished results.
17 U. KLEIN, unpublished results.