Carbohydrate Research, 139 (1985) 1–11
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

FLUORESCENT LABELING OF CARBOHYDRATES AND ANALYSIS BY LIQUID CHROMATOGRAPHY. COMPARISON OF DERIVATIVES USING MANNOSIDOSIS OLIGOSACCHARIDES*

ERIC COLES, VERNON N. REINHOLD**, AND STEVEN A. CARR
Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115 (U.S.A.)
(Received June 25th, 1984; accepted for publication, August 30th, 1984)

ABSTRACT

To enhance resolution and detectability of carbohydrates by liquid chromatography, two fluorescent labels, introduced into oligosaccharides by reductive amination, were compared by use of standard sugars and a complex, biological sample of D-mannose oligomers obtained from the urine of a mannosidosis patient. Both labels, 2-aminopyridine and 7-amino-1-naphthol, improved the chromatographic efficiency and detection sensitivity. However, reductive amination with the pyridinylamine derivative was incomplete. The Schiff-base intermediates left in the mixtures were only partially resolved by chromatography and complicate the patterns. In contrast, the naphtholamine derivatives were completely reduced and, in addition, possess enhanced fluorescence.

INTRODUCTION

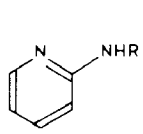
The reducing end of an oligosaccharide molecule provides a site that may be specifically labeled with groups that are both fluorescent and u.v. absorbing. Reductive amination has been used to introduce various residues at this hemiacetal position, and two aromatic amines, 2-aminopyridine[1–7] (**1**) and 7-amino-4-methylcoumarin[8], have been studied for this purpose. The labeled products were purified by t.l.c., electrophoresis[1–8], and l.c.[4,5,7], and were detected with greatly increased sensitivity by fluorescence emission.

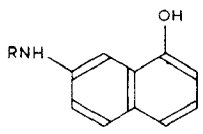
In an effort to introduce better methods for the analysis of glycoconjugates, we have now compared the previously described derivatives[1–7] of **1** with a second, new label possessing improved properties, 7-amino-1-naphthol (**7**). The derivatives of 7-amino-4-methylcoumarin were not considered because of their loss of fluorescence during subsequent permethylation, an important factor for ensuing structural evaluation. Improvements in resolution and completeness of derivatiza-

*This work was supported by a grant from the National Institute of General Medical Sciences (PO1 GM-26625), National Institutes of Health.
**To whom correspondence should be addressed.

0008-6215/85/$ 03.30

tion and the overall enhancement of sensitivity were evaluated by use of ^{14}C-labeled D-glucose, a series of standard mono-, di-, and tri-saccharides, and a biological sample. This sample was difficult to analyse and contained a mixture of oligosaccharides obtained from the urine of patients suffering from mannosidosis, which accumulates in urine as a product of a lysosomal-enzyme deficiency. By use of these samples, compounds derivatized with both **1** and **7** showed improved l.c. resolution and increased detector sensitivity when compared to the starting materials. However, the derivatives of **1** exhibited incomplete reduction of the Schiff-base adduct, which resulted in a more complex chromatogram. In contrast, the derivatives of **2** were completely reduced and had approximately eight times the fluorescence when compared to the derivatives of **1**. Liquid-chromatography and mass-spectral evidence of these findings are presented along with a discussion of the incomplete reduction of derivatives of **1**.

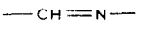




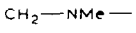
1 R = H
2 R = $(Man)_2$—X
3 R = $(Man)_3$—X
4 R = $(Man)_4$—X
5 R = $(Man)_5$—X
6 R = $(Man)_6$—X

7 R = H
8 R = $(Man)_2$—X
9 R = $(Man)_3$—X
10 R = $(Man)_4$—X
11 R = $(Man)_5$—X
12 R = $(Man)_6$—X

X = 1-acetamido-1,2-dideoxy-D-glucit-1-yl



13



14

EXPERIMENTAL

Methods. — Liquid-chromatography separations were performed with equipment from Waters Associate (Milford, MA 01757) consisting of two M6000A pumps which maintained a column-head pressure between 10–20 MPa, a U6K universal injector, model 660 solvent programmer, R401 differential refractometer, and model 450 variable-wavelength detector. Fluorescence was detected with a model FS970 spectrofluorometer from Kratos Analytical Instruments (Ramsey, NJ 07446). Mass spectra were obtained with a Finnigan-MAT 312 instrument (San Jose, CA 95134), fitted with a combined c.i.–e.i. source. Direct chemical ionization (d.c.i.) was performed as previously described[9]. Fast atom bombardment (f.a.b.) analysis of underivatized materials was performed with the same instrument. Details of sample matrix and instrumental parameters have been previously described[10].

The specific conditions for l.c. are shown for each chromatogram in the legend of the corresponding figure. Fluorescence was detected with an excitation wavelength of either 240 nm for the derivatives of **7** or 232 nm for the derivatives of **1**, with a 320 nm cutoff filter on the detector side. An amplification of 1060 V on the photomultiplier tube and a scale of 1.0 μA were used unless otherwise noted.

Materials. — Solvents for l.c. were obtained from Burdick & Jackson Laboratories Inc. (Muskegon, MI 49442); Spherisorb l.c. packing materials from Phase Separations Inc. (Norwalk, CT 06850); and $NaCNBH_3$, methyl iodide, 2-aminopyridine (**1**), and 7-amino-1-naphthol (**7**) from Aldrich Chemical Co. Inc. (Milwaukee, WI 53201). D-[U-^{14}C]-Glucose was obtained from New England Nuclear (Boston, MA 02118). Radioactive-flow detection was performed with a TRACE 7140 instrument manufactured by Packard Instruments Co. (Downers Grove, IL 60515). Mannosidosis oligosaccharides were kindly provided by Dr. C. D. Warren (Massachusetts General Hospital, Boston, MA 02114).

Preparation of fluorescent derivatives. — Preparation of the derivatives[1,3,5] of **1** was performed with several modifications[9]. The analog derivatives of **7** were prepared by dissolving **7** (2 mg) in dimethyl sulfoxide (50 μL). The solution was added to the dried oligosaccharides and the mixture was heated for 30 min at 105°. To this was added a solution (50 μL) made by adding $NaCNBH_3$ (5 mg) to methanol (400 μL) and glacial acetic acid (40 μL). The mixture was heated for 2.5 h at 105°, dried, and subjected to a partition according to Folch *et al.*[11]. The upper layer was removed and dried, and reextraction of the bottom layer insured complete recovery of all derivatized components.

Permethylation. — Permethylation was performed on materials prior to analysis by direct chemical-ionization mass spectrometry (d.c.i.–m.s.). This technique provided both molecular weight and sequence information[9] for a complete characterization of the eluted components. Samples were permethylated according to Hakomori[12], as modified by Sanford and Conrad[13]. The potassium anion was used as described by Valent *et al.*[14]. A single methylation-step for an extended period proved inadequate for complete derivatization. On the other hand, repeating the procedure frequently produced overmethylation, a problem associated with 2-acetamido-2-deoxyhexose-containing oligomers. The maximum yields of the fully permethylated products was obtained with two methylation steps carried out within 15 min each. The same conditions were used for the preparation of the permethylated derivatives of **7**. These conditions were optimal for **2** and may vary for other oligosaccharides.

RESULTS

Group separation of oligosaccharides. — Human urinary oligosaccharides obtained from a mannosidosis patient were chromatographed on an amino-Spherisorb column (250 × 4.6 mm) (Fig. 1) to give five major areas. This provided also some indications of the heterogeneity within several of the peaks, especially in

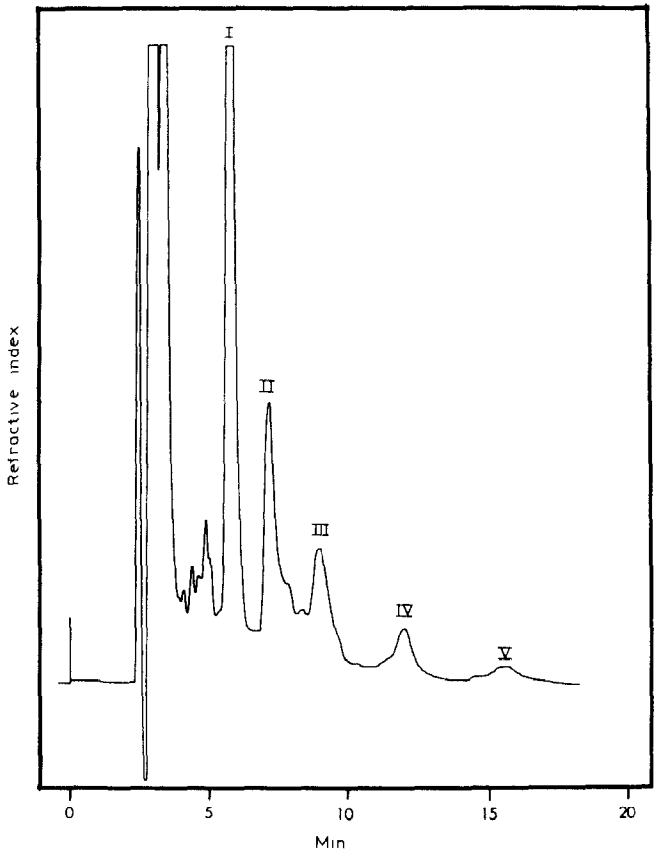


Fig. 1. Liquid chromatography of a mixture (1 mg) of D-mannose oligomers from a mannosidosis urine on a column (250 × 4.6 mm i.d.) of amino-Spherisorb (5μ) in 11:9 acetonitrile–water at 1 mL/min and detection by refractive index (8 ×): I, $(Man)_2$-GlcNAc; II, $(Man)_3$-GlcNAc; III, $(Man)_4$-GlcNAc; IV, $(Man)_5$-GlcNAc; and V, $(Man)_6$-GlcNAc.

the areas labeled $(Man)_3$-GlcNAc and $(Man)_4$-GlcNAc. Because of the great diversity in concentration and the poor dynamic range of the detectors, only the lower oligomers were observed in this single injection. The five areas were collected for the preparation of fluorescent derivatives.

Fluorescent derivatives of **1**. — Each of the five major areas, designated $(Man)_2$-GlcNAc through $(Man)_6$-GlcNAc (Fig. 1), were isolated, dried, and the derivatives of **1** prepared[9]. The published procedure was modified by treating the carbohydrate with the amine previous to adding the reducing agent, in order to prevent direct reduction of the sugar. The products were placed on an amino-Spherisorb column and eluted with a gradient of increasing polarity (Fig. 2A–2E). Each of the resulting chromatograms showed a marked increase in chromatography efficiency and sensitivity. Calculations of the sensitivity with **2** indicated that for each μg of the starting trisaccharide, an area of ~2.5 cm^2 would be obtained for **2** (for instrumental amplification settings, see l.c. conditions). This represents a 1500-fold

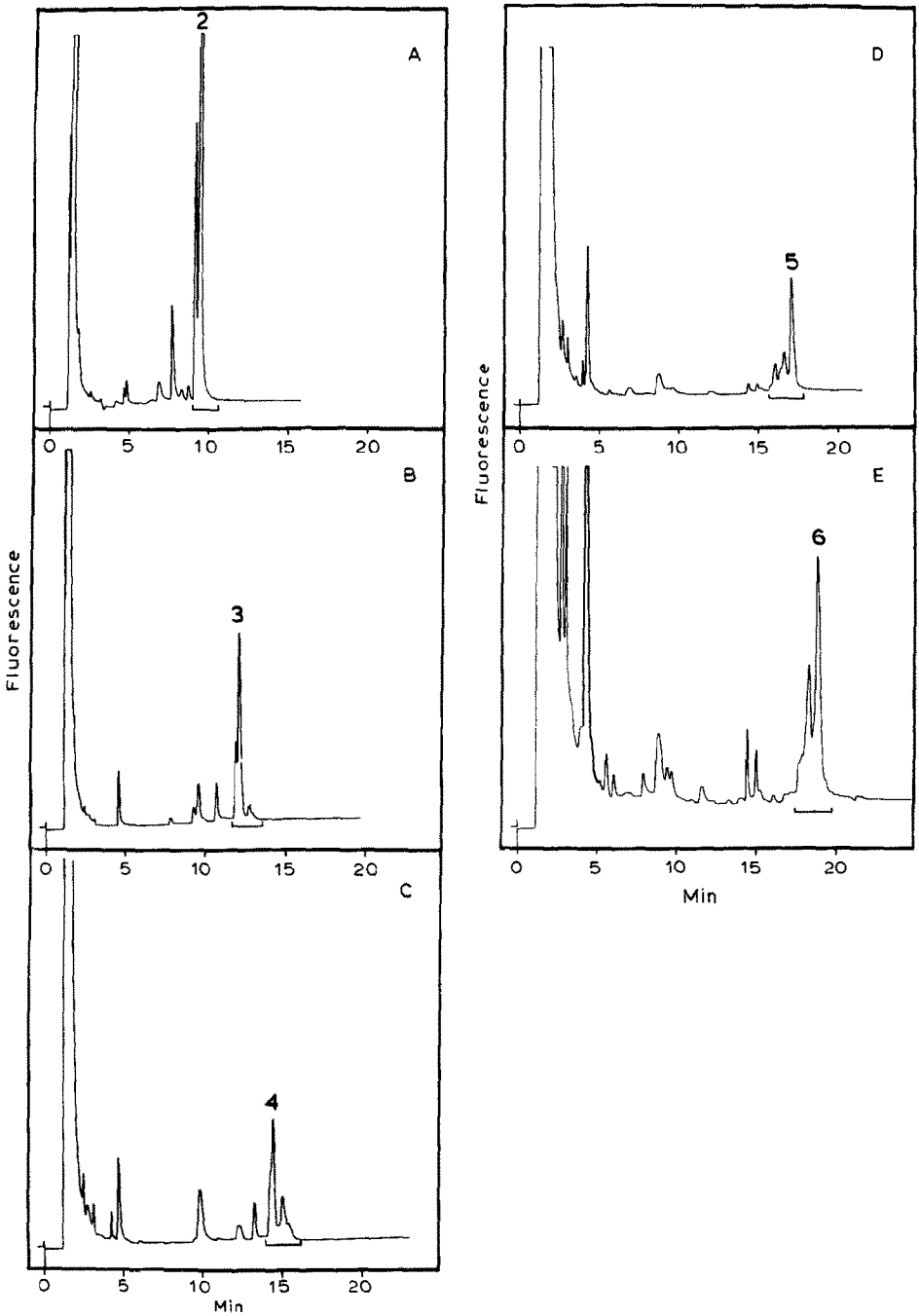


Fig. 2. Rechromatography of fractions described in Fig. 1, after derivatization with **1**, on a column (250 × 4.6 mm i.d.) of amino-Spherisorb, in 20–70% water (0.15M NH_4OH)–acetonitrile, at 2 mL/min with a 20-min program. The fractions were detected >320 nm by fluorescence excitation at 232 nm. The bracketed areas shown in each panel indicate the areas collected for permethylation and d.c.–m.s. analysis: A–E, compounds **2–6**.

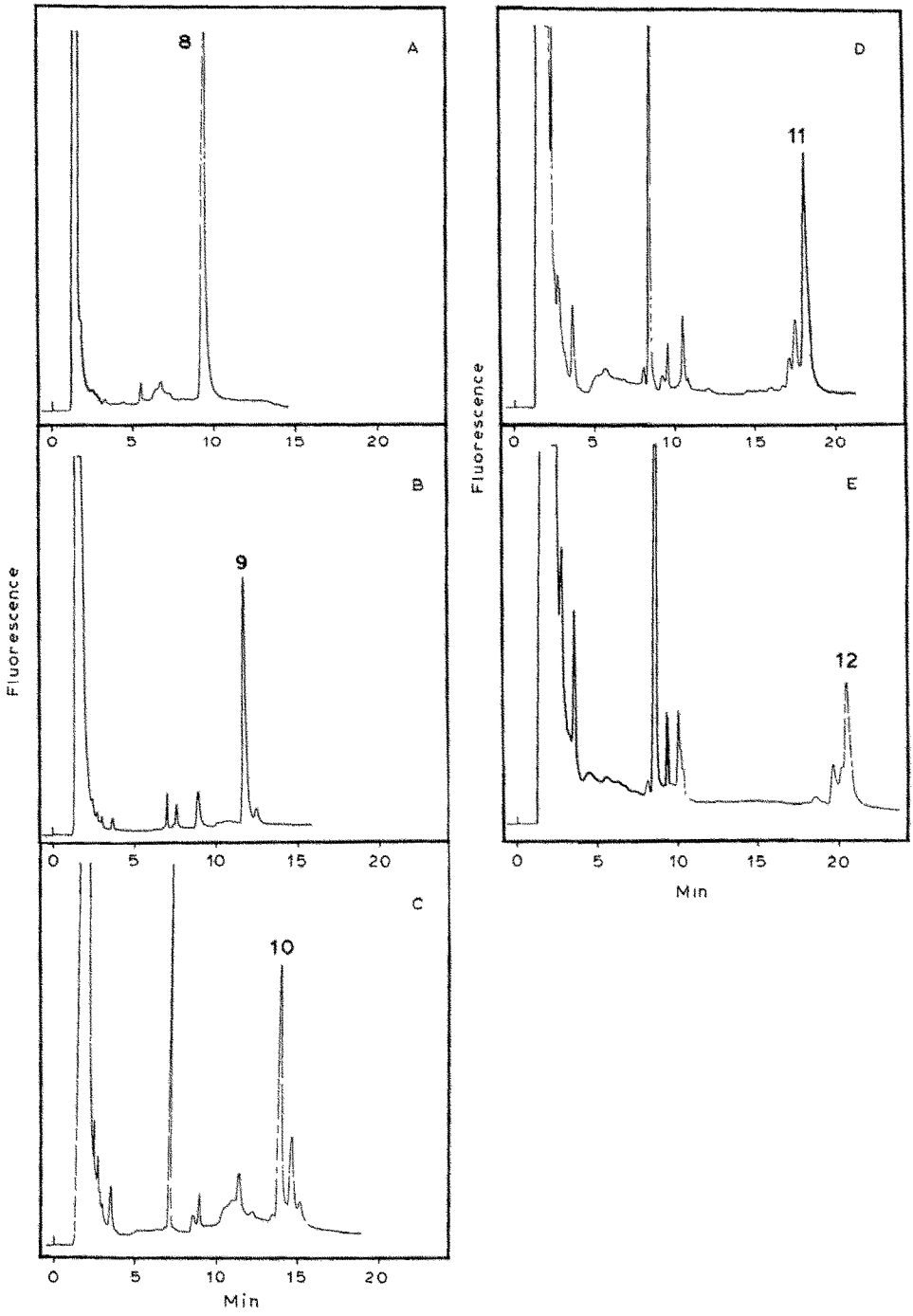


Fig. 3. Rechromatography of fractions described in Fig. 1, after derivatization with **7**, on a column (250 × 4.6 mm i.d.) of amino-Spherisorb, in 20–70% water (0.15M NH_4OH)–acetonitrile, at 2 mL/min with a 20-min program. For detection and treatment, see legend to Fig. 2: A–E, compounds **8–12**.

enhancement in sensitivity, as compared to the direct analysis of $(Man)_2$-GlcNAc by use of refractive-index detection as shown in Fig. 1. The same yield of fluorescence was obtained for each of the higher oligomers based on their molar concentrations. The yield of fluorescence was calculated from the data obtained with the derivative of D-[U-^{14}C]-glucose (see later) and corrected for the yield of derivatives of **1**.

Concomitant with the increase in sensitivity was a dramatic improvement in resolution, which clearly revealed the presence of structural isomers within each oligomer-size class (compare Fig. 1 to Fig. 2). Heterogeneity in the structures of these urinary mannosidosis oligosaccharides has been previously reported[15–19]. The methods of separation described in these earlier reports, however, were inadequate to resolve isomeric components, and the heterogeneity was shown by chemical degradation studies. Liquid chromatography has also been employed in the study of these oligomers for the separation of components into oligomer classes[20,21].

Derivatization with either **1** or **7** introduced several fluorescent compounds that were eluted early and contributed to the chromatographic background. The size of these peaks increased from Fig. 2A to 2E, Fig. 3A to 3E because larger sample aliquots were injected to generate peaks of comparable size. This was necessary because of the diminishing amount of sample in each oligomer class (Fig. 1). We are currently evaluating procedures that completely eliminate this background.

The substances corresponding to the peaks in Figs. 2A–2E were collected, dried, and subjected to mol. wt. analysis by d.c.i.–m.s. following permethylation (see Table I). Each of the fractions gave the expected protonated molecular ions consistent with the assigned structure. The last eluted material, per-*O*-methylated **6**, was not available in sufficient quantity to obtain mol. wt. information by mass spectrometry. The fraction containing **2** had ~12% of an additional component having a mol. wt. equal to that of the expected product minus 16. This contaminant was eluted slightly ahead of the major peak. To clarify this anomaly, the two peaks were isolated (Fig. 2A), and analyzed directly by f.a.b.–m.s., thereby avoiding any possible complications introduced by permethylation. The major peak showed a single ion at *m/z* 624, which is consistent with a protonated molecular ion for the

TABLE I

ANALYSIS BY DIRECT CHEMICAL IONIZATION–MASS SPECTROMETRY OF PERMETHYLATED DERIVATIVES OF OLIGOSACCHARIDES WITH 2-AMINOPYRIDINE (**1**)

Per-O-methyl derivative of	m/z (MH^+)
2	792
3	996
4	1200
5	1404
6	"1608"[a]

[a]Concentration insufficient to be observed.

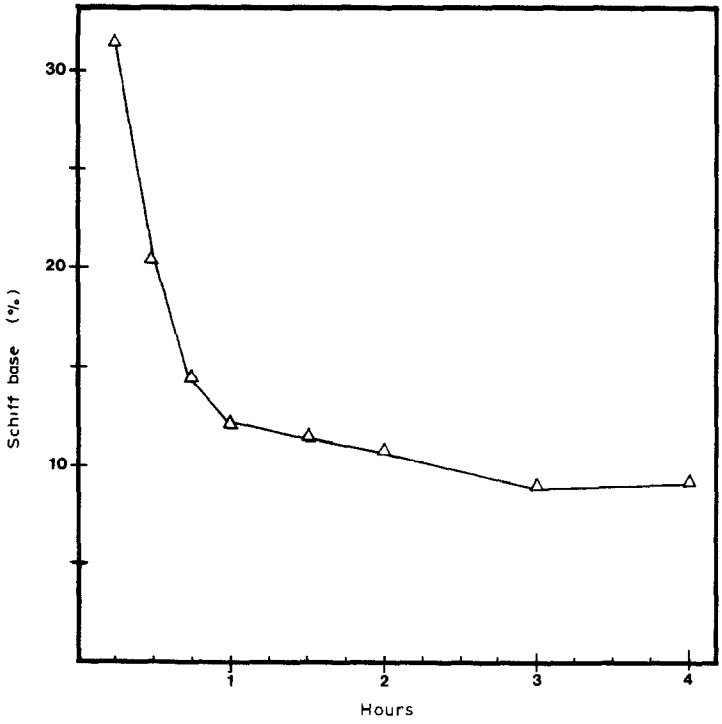


Fig. 4. Time study of reductive amination of $(Man)_2$-GlcNAc. Decrease in Schiff base with **1** in time, as monitored by l.c., to give **2**.

expected product (**2**). The minor component gave a protonated molecular ion at *m/z* 622, suggesting incomplete reduction of the Schiff base intermediate. The resulting aldimine structure **13** cannot be *N*-methylated, which accounts for the 16-dalton decrease in mass observed for this permethylated product, as compared to the expected structure **14**. No evidence for any other material was found in this trisaccharide fraction.

Schiff-base reduction with sodium cyanoborohydride. — The time course of the reductive amination of $(Man)_2$-GlcNAc was studied by l.c. for which aliquots were removed during the reduction (see Fig. 4). The l.c. conditions were changed in order to achieve better resolution of the Schiff-base adduct and its reduced product (see Fig. 5 for l.c. of the products following a reaction of 15 min). Complete reduction of the aldimine adduct could not be achieved under these conditions (see Fig. 4), although addition of acid after 4 h still caused evolution of hydrogen, indicating the presence of an excess of reducing agent. Extending the time of reduction beyond 4 h or adding fresh reducing agent did not affect the product distribution. Chromatographic isolation of this total fraction, followed by repetition of the reduction step converted 50% of the remaining intermediate. These results suggest that a by-product that inhibits further reduction is accumulated during derivatization. One anticipated by-product would be sodium borate (which may chelate with

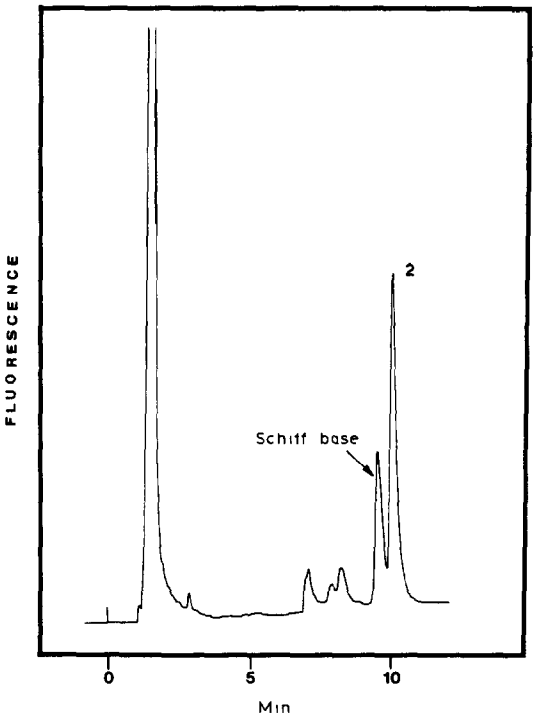


Fig. 5. Reductive amination of $(Man)_2$-GlcNAc to give **2** after 15 min, as monitored by l.c. in 1:3 water–acetonitrile at 2 mL/min. For other conditions, see legend to Fig. 2.

the pyridinyl nitrogen atom and the aldimine group); however, the addition of this salt in an equivalent concentration failed to inhibit the reductive step.

The Schiff-base components were also observed in the chromatograms of **3** and **4** where they appeared as shoulders on the major peak. The aldimine component could not be detected in the chromatograms of **5** (Fig. 2D) and **6** (Fig. 2E), although the limits in sensitivity and the decreasing chromatographic efficiency may have prevented detection by m.s. and l.c. respectively.

Fluorescent derivatives of 7-amino-1-naphthol. — By use of the same general approach as that applied to derivatives of **1**, other fluorescent labels were investigated with regard to preventing the formation of potentially misleading artifacts and improving the sensitivity of the detection. Fractions from each of the five major areas, $(Man)_2$-GlcNAc to $(Man)_6$-GlcNAc (Fig. 1), were collected, dried, and prepared as derivatives of **7**, and the products rechromatographed on an aminopropyl column (Fig. 3A–3E). Derivative **8** gave a single peak (Fig. 3A) in the trisaccharide region, in contrast to the doublet obtained for the derivative of **1** (**2**) (Fig. 2A), indicating that the intermediate Schiff-base is more readily reduced than that of the corresponding analog of **1**. To corroborate this observation and insure that the unreduced material was not coeluted with the expected derivative, the fraction containing **8** was per-*O*-methylated and analyzed by d.c.i.–m.s. In contrast

to the results obtained for **2**, the mass spectra of the corresponding derivative **8** indicated a single component having a mol. wt. consistent with the assigned structure. Lack of heterogeneity in the $(Man)_2$-GlcNAc fraction of the mannosidosis urinary extracts is consistent with results obtained earlier[18,19]. The unreduced aldimine component was also no longer apparent in the fractions containing **9** and **10** (comp. Fig. 3B and 3C with 2B and 2C, respectively). The multiplicity of peaks in the new chromatograms reflects only the contributions of structural isomers having the same degree of polymerization. Although specific structures cannot be assigned to peaks by chromatography alone, the observed multiplicity is consistent with prior work describing multiple isomeric structures when analyzed by the alditol acetate procedure[18,19].

DISCUSSION

The derivatives of **7** have several advantages over the derivatives of **1** and, although it would be incorrect to generalize for a broad range of oligosaccharides, certain major features make this fluorescent-labeling procedure very attractive. First, for the materials studied and the threshold of detectability, the reaction goes to completion and does not leave unreduced intermediates. The fluorescence of **7** is approximately eight fold greater than that of **1**, and the reaction appears to proceed in high yield. A more precise estimate of the yield of the formation of derivatives of **7** was obtained by use of D-[U-^{14}C]glucose. After formation of the derivative with **7**, the mixture was separated by l.c. under conditions suitable for the separations of D-glucose, D-glucitol, and 1-deoxy-1-[7-(1-naphthyl)amino]-D-[U-^{14}C]-glucitol, and the products were determined with a radioactive-flow monitor. The derivative of D-glucose was obtained in >90% yield, which agrees closely with estimates based solely on the fluorescence of pure **2** and the sugar derivatives of **2**. Possible loss of sugar due to reduction prior to amination was avoided in the present procedure as the carbohydrate and amine (in large excess) were condensed separately in order to maximize aldimine formation before addition of the reducing agent.

After this work had been completed, an update of the earlier procedures for the preparation of derivatives of **1** was published[22]. This modified procedure utilizes the hydrochloride salt of the pyridinylamine, an aqueous medium, and high concentrations of **1**. Comparison of this procedure for the preparation of **2** with the previous ones indicated the formation of only 2.7% of the aldimine intermediate.

The two very promising methods of fluorescent labeling before separation by l.c. described herein not only enhance the sensitivity of the l.c. detection, but also improve the chromatographic resolution of the products. Similar results have also been obtained with more complex, branched oligosaccharides and will be reported elsewhere.

ACKNOWLEDGMENTS

The authors express their appreciation to Ms. Jeanne Breen for her technical support and aid with the preparation of the manuscript.

REFERENCES

1 S. HASE, T. IKENAKA, AND Y. MATSUSHIMA, *Biochem. Biophys. Res. Commun.*, 85 (1978) 257–263.
2 S. HASE, S. HARA, AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 85 (1979) 217–220.
3 S. HASE, T. IKENAKA, AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 85 (1979) 989–994.
4 S. HASE, T. IKENAKA, AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 85 (1979) 995–1002.
5 S. HASE, T. IKENAKA, AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 90 (1981) 407–414.
6 S. HASE, T. IKENAKA, AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 90 (1981) 1275–1279.
7 M. KOSAKAI AND Z. YOSIZAWA, *J. Biochem. (Tokyo)*, 92 (1982) 292–303.
8 C. PRAKASH AND I. K. VIJAY, *Anal. Biochem.*, 128 (1983) 41–46.
9 V. N. REINHOLD, E. COLES, AND S. A. CARR, *J. Carbohydr. Chem.*, 2 (1983) 1–18.
10 S. A. CARR AND V. N. REINHOLD, *J. Carbohydr. Chem.*, 3 (1984) 381–401.
11 J. FOLCH, M. LEES, AND G. H. S. STANLEY, *J. Biol. Chem.*, 226 (1957) 497–503.
12 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
13 P. A. SANFORD AND H. E. CONRAD, *Biochemistry*, 5 (1966) 1508–1517.
14 B. S. VALENT, A. G. DARVILLE, M. MCNEIL, B. K. ROBERTSON, AND P. ALBERSHEIM, *Carbohydr. Res.*, 79 (1980) 165–198.
15 P. A. OCKERMAN, *Lancet*, 2 (1967) 239–241.
16 N. E. NORDEN, A. LUNDBLAD, S. SVENSSON, P. A. OCKERMAN, AND S. AUTIO, *J. Biol. Chem.*, 248 (1973) 6210–6215.
17 N. E. NORDEN, A. LUNDBLAD, S. SVENSSON, P. A. OCKERMAN, AND S. AUTIO, *Biochemistry*, 13 (1974) 871–874.
18 K. YAMSHITA, Y. TOCHIBANA, K. MIHARA, S. OKADA, H. YABUUCHI, AND A. KOBATA, *J. Biol. Chem.*, 255 (1979) 5126–5133.
19 F. MATSUURA, H. A. NUNEZ, G. A. GRABOWSKI, AND C. C. SWEELEY, *Arch. Biochem. Biophys.*, 207 (1981) 337–352.
20 P. F. DANIEL, D. F. DE FEUDIS, I. T. LOTT, AND R. H. MCCLEUR, *Carbohydr. Res.*, 97 (1981) 161–180.
21 C. D. WARREN, A. S. SCHMITT, AND R. W. JEANLOZ, *Carbohydr. Res.*, 116 (1983) 171–182.
22 S. HASE, T. IBUKI, AND T. IKENAKA, *J. Biochem. (Tokyo)*, 95 (1984) 197–203.