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CHROMATOGRAPHIC SEPARATION OF OLIGOSACCHARIDES FROM MANNOSIDOSIS URINE*

CHRISTOPHER D. WARREN, AGNES S. SCHMIT, AND ROGER W. JEANLOZ†
Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114 (U.S.A.)
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ABSTRACT

A mixture of oligosaccharides was isolated from mannosidosis urine by a rapid and convenient procedure employing adsorption on activated charcoal. The mixture was partially fractionated into a homologous series of compounds by a rapid procedure employing a preparative, liquid chromatograph, and a more complete separation was obtained by a second chromatographic step employing a solid phase having more-powerful resolving properties, or by preparative-layer chromatography. The series of oligosaccharides was completely separated by 7-MPa, liquid chromatography (l.c.) on a Micropak NH_2-10 column; the analysis could be performed with isocratic or gradient solvent-systems, and did not involve derivatization. With the isocratic system, a strict relationship was observed between retention time and the number of D-mannosyl residues. The use of 1,4-diaminobutane as a column "restorer" was evaluated.

INTRODUCTION

The "high-mannose" oligosaccharides constitute one of the principal classes of *N*-glycoprotein glycan chains[1], having various numbers of D-mannosyl residues linked to a "core" of di-*N*-acetylchitobiose, which is, in turn, linked to an L-asparagine residue in the protein chain. Synthesized, or isolated, high-mannose oligosaccharides provide (*1*) exogenous substrates for the study of glycosyltransferases and glycosidases; (*2*) authentic compounds for the development of such physical methods as n.m.r. spectroscopy, mass spectrometry, and circular dichroism; and (*3*) starting materials for the chemical synthesis of biosynthetic intermediates[2]. They are usually prepared by exhaustive digestion of a glycoprotein

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†To whom inquiries should be sent.

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with Pronase, followed by purification of the resulting glycopeptides by ion-exchange and gel-permeation chromatography, and release of the carbohydrate chains by enzymic action or by treatment with hydrazine. These procedures are time-consuming and complicated, and only small amounts of material can be obtained therewith. Therefore, another source of material would be advantageous.

We now describe the use of urine, from patients suffering from mannosidosis, as the source material, and the production of "high-mannose" oligosaccharides in good yields by analytical and preparative liquid-chromatography under pressure, without having to resort to derivatization or to use of column modifiers.

Mannosidosis is a lysosomal-storage disease[3] in which high concentrations of "high-mannose" oligosaccharides are excreted in the urine, because of an α-D-mannosidase deficiency. These oligosaccharides contain between two and nine residues of D-mannose, and a single residue of 2-acetamido-2-deoxy-D-glucose[4]. Previously, mannosidosis oligosaccharides have been isolated by fractionation on a charcoal–Celite column, followed by ion-exchange and preparative paper-chromatography[5], gel-permeation chromatography[6] followed by electrophoresis, or a combination of gel-permeation and ion-exchange chromatography[4]. Glycoprotein-derived oligosaccharides have been separated by l.c. under elevated pressure either in ion-exchange[7] or in "amino-bonded"[8] (NH_2-10) columns. We here report the convenient and rapid separation of milligram quantities of mannosidosis oligosaccharides by a combination of methods.

RESULTS AND DISCUSSION

The oligosaccharides from mannosidosis urine were separated from salts, urea, and other metabolites by chromatography on a charcoal–Celite column, or, for large-scale preparations, by treatment in 1-l batches with activated charcoal until t.l.c. showed that >90% of the oligosaccharides had been adsorbed. Elution with aqueous ethanol released the oligosaccharides, contaminated with traces of salts, urea, and an orange-colored pigment having an R_1 value similar to that of $(Man)_3GlcNAc$ and very strong, u.v. absorbance. The oligosaccharides could be readily resolved by t.l.c. in a variety of solvent systems; therefore, preparative layer-chromatography (p.l.c.) was performed, and samples of $(Man)_2GlcNAc$ to $(Man)_8GlcNAc$ that each gave a single, major spot in t.l.c. were obtained. For large-scale analysis, rapid separation was obtained by preparative, "medium-pressure" liquid chromatography (~0.2 MPa, m.p.l.c.) using a Chromatospac Prep-10 column in which a high-quality silica gel is the solid phase. Partial resolution of 1–4-g samples of oligosaccharides could be achieved in one day (see Table I). Further resolution was obtained by a second, medium-pressure, liquid chromatography, using a Lichroprep Si-60 column in which l.c.-grade silica gel is the solid phase (see Table II). The fractions from the Lichroprep Si-60 column, when analyzed by t.l.c. (see Fig. 1), showed, in addition to the major oligosaccharide, small amounts of higher and lower members of the series. Final purification of these fractions was

TABLE I

PARTIAL SEPARATION OF OLIGOSACCHARIDES FROM MANNOSIDOSIS URINE BY PREPARATIVE LIQUID-CHROMATOGRAPHY

Components[a]	*Yield*[b] *(mg)*
M_2	574
$(M_2)M_3(M_4)$	300
$(M_3)M_4(M_5)(M_6)$[c]	278
$M_4M_5M_6(M_7)$[c]	187
$M_6M_7(M_8)$	77
$(M_6)M_7M_8$	64
$(M_7)M_8(M_9)$	29
$(M_7)M_8M_9$	68
$>M_9$	92

[a]M_2, M_3, M_4, M_5, M_6, M_7, M_8, and M_9 are abbreviations for $(Man)_2GlcNAc$–$(Man)_9GlcNAc$, identified by t.l.c.; $>M_9$ refers to material with an R_F value lower than that of $(Man)_9GlcNAc$ in t.l.c.; parentheses refer to minor components. [b]This figure is approximate, because the samples contained silica gel. [c]These two fractions were later employed for h.p.l.c. in the presence of 1,4-diaminobutane (see Fig. 3).

TABLE II

YIELDS OF OLIGOSACCHARIDES FROM MANNOSIDOSIS URINE[a]

Component	R_F[b]	*mg*[c]	*pmol*[d]
M_2	0.37	660	774
M_3	0.32	86	240
M_4	0.27	95	97
M_5	0.22	44	40
M_6	0.19	41	20
M_7	0.16	24	11
M_8	0.12	22	4
M_9	0.10	32	3

[a]Abbreviations are the same as for Table I. [b]R_F values were calculated from t.l.c. in solvent C on 20-cm plates (see Fig. 1). [c]Amount isolated, by pooling fractions eluted from m.p.l.c. column that contained mainly one oligosaccharide according to t.l.c., followed by rechromatography (m.p.l.c. or p l.c.), and does not necessarily represent the actual proportion in the urine. [d]Amount determined to be present in a typical sample of urine, calculated from integration data based on the u.v. absorption of benzoyl groups[13] at 230 nm.

achieved by a third liquid chromatography, this time under "high pressure" (~7 MPa, h.p.l.c.), using a Micropak NH_2-10 column.

With 11:9 (v/v) acetonitrile–water as the mobile phase, $(Man)_2GlcNAc$ was eluted with a T_R similar to that of D-glucose, followed closely by $(Man)_3GlcNAc$. When the elution was isocratic (see Table III and Fig. 2), a plot of log T_R against the number of D-mannosyl residues showed a perfectly linear relationship. Faster separation was obtained by gradient elution, but this was impractical for semi-preparative work, because refractometry could not be used for detection. When

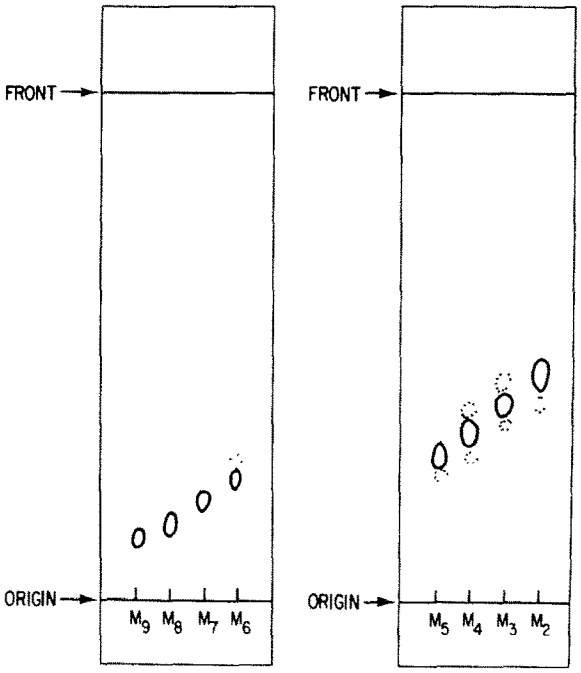

Fig 1 Oligosaccharides, after two stages of preparative liquid-chromatography, chromatographed on Fisher "Rediplates", using solvent *C* and the anisaldehyde reagents [The Fig. shows tracings of the original spots, which are elongated, because each consists of an unresolved, anomeric mixture Abbreviations M_2, M_3, *etc* , are given in footnote to Table I.]

TABLE III

LIQUID-CHROMATOGRAPHIC ANALYSIS OF OLIGOSACCHARIDES OF MANNOSIDOSIS URINE, SEPARATED IN THE ISOCRATIC MODE[a]

Oligosaccharide[b]	*Retention time* (T_R, *min*)[c]
M_2	4 2
M_3	5.2
M_4	6 6
M_5	8 4
M_6	10 5
M_7	13 0
M_8	16 2
M_9	20 0

[a]Conditions: Micropak NH_2-10 column (30 cm × 4 mm) under a pressure of ~7 MPa; mobile phase: 11·9 (v/v) acetonitrile–water; flow rate: 1.5 mL/min; detector: Schoeffel UV, 190 nm (0.08 aufs); and temperature: 25° (see Fig. 2). [b]Abbreviations, see footnote to Table I. [c]Results represent averages of five or more determinations for each oligosaccharide

h.p.l.c. was performed on samples resulting from a single chromatography on the Chromatospac Prep-10 column, the presence of an orange-yellow pigment that

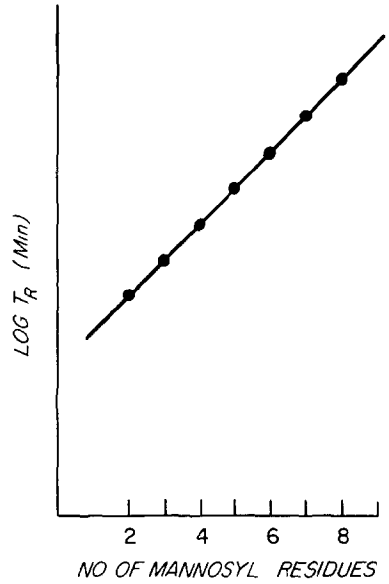

Fig. 2. Linear relationship between logarithm of retention time (min) and the number of D-mannosyl residues in the oligosaccharide (see Table III).

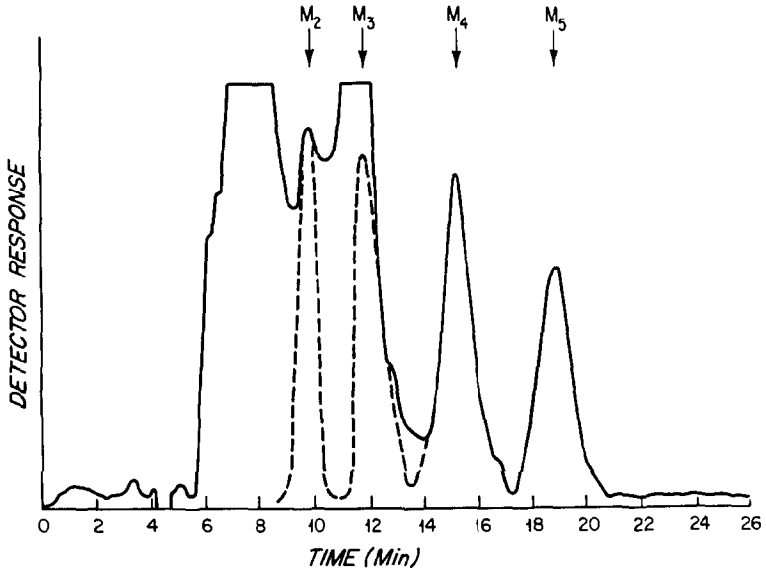

Fig. 3. Liquid chromatogram, under ~7 MPa pressure, of a "crude" fraction of oligosaccharides, showing that peaks for M_2 and M_3 (dotted lines) are completely obscured by the presence of an orange-yellow, urinary pigment. [Chromatographic conditions: Micropak NH_2-10 preparative column (30 cm × 8 mm); isocratic mode; mobile phase, 11:9 (v/v) acetonitrile–water containing 0.01% of 1,4-diaminobutane; flow rate, 2.0 mL/min; temperature, 25°; sample size, 80 μL; refractive index detector, sensitivity 16; and chart speed, 1 cm/min.]

saturated the u.v. and r.i. detectors (see Fig. 3) hindered the detection of $(Man)_2GlcNAc$ and $(Man)_3GlcNAc$. Therefore, for the detection of these lower members of the series of oligosaccharides, an additional, chromatographic step (with m.p.l.c.), or recycling through the h.p.l.c. column, was necessary.

Resolution of oligosaccharides on columns of silica gel modified by treatment[9,10] with 1,4-diaminobutane has been shown to give results similar to those obtained with the NH_2-10 columns. Turco[10], employing either an NH_2-5 column or a column of silica gel modified by treatment with 1,4-diaminobutane[9], achieved very good separations of "high-mannose" chains obtained by hydrolysis of oligosaccharide "lipid intermediates" with dilute acid; preparative separations were not performed by him. However, we observed that, whereas treatment of silica-based columns with 1,4-diaminobutane results in good analytical separations of the high-mannose oligosaccharides, and prolongs the life of aminopropyl-bonded columns, this modifier, even at a very low (0.01%) concentration, produces a highly alkaline, mobile phase (pH 10) that reacts with the oligosaccharides. Even for analytical separations, use of 1,4-diaminobutane is disadvantageous, because its opacity (to u.v. absorbance) limits the detection to determination of refractive index or to radioactive labeling. Therefore, when 1,4-diaminobutane is used to restore the performance of an NH_2-10 column that has begun to deteriorate, each peak fraction should be made neutral with dilute hydrochloric acid immediately after collection, in order to avoid modification of the oligosaccharides.

As a result of the strict relationship between T_R and the number of D-mannosyl residues (see Fig. 2), it is possible to calibrate the NH_2-10 column, so that the composition of a high-mannose oligosaccharide can be predicted from its elution position. Subsequently, the assignment can be confirmed by co-injecting an appropriate oligosaccharide as an internal standard.

EXPERIMENTAL

General methods. — T.l.c. was performed on precoated plates of Silica Gel 60 F254, 0.25-mm thick (E. Merck AG, Darmstadt, Germany), or on "Rediplates" of Silica Gel GF, 0.25-mm thick (Fisher Chemical Co., Pittsburgh, PA 15219). Preparative-layer chromatography (p.c.) was performed on precoated Silica Gel F254 PLC plates, 2-mm thick (Merck) or on precoated plates of Silica Gel F254, 0.5-mm thick (Merck). Solvent systems employed for chromatography were (*A*) 10:10:3 chloroform–methanol–water, (*B*) 3:3:2 1-propanol–acetic acid–water, and (*C*) 3:3:2 2-propanol–ethyl acetate–water; all proportions of solvents were v/v. The spray reagent was 1:1:18 anisaldehyde–conc. sulfuric acid–ethanol[11]. Preparative liquid-chromatography under medium pressure (m.p.l.c.) was performed on Silica gel (40–63 μm, 200–400 mesh, Merck) or on Lichroprep Si-60 (15–25 μm, Merck).

Preparative liquid-chromatography. — This chromatography was performed with a[12] Jobin–Yvon Chromatospac Prep-10 (Instruments S.A., Inc., Metuchen,

NJ 08840), equipped with a hydraulic jack to compress the solid phase, under gaseous N_2 pressure. The column was packed *in situ* just before use, and the sample introduction and elution were conducted under gaseous N_2 pressure (~0.2 MPa). Up to 10 g of sample was loaded onto a column prepared from 200 g of solid phase, and the chromatography was easily completed within one day.

Analytical and semi-preparative liquid-chromatography. — This was performed with a gradient LC Chromatograph, Model 5020 (Varian Associates, Palo Alto, CA 94303) equipped with a microcomputer-based, interactive CRT-keyboard control, solvent and flow programming, and a manual Loop Valve Rheodyne Injector, fitted with a 10-μL loop (or a 100- or 200-μL loop for preparative work). Varian Micropak NH_2-10 columns, analytical (30 cm × 4 mm) or preparative (30 cm × 8 mm), were enclosed in a 30-cm heating-block whose temperature could be programmed to minimize fluctuations in the ambient temperature. A column-inlet filter, Rheodyne Model 7302 (Rainin Instruments, Woburn, MA 01801), and a micro-guard holder containing an H2-GU amino guard column (Rainin), were inserted between the injector and the column. The column led into a UV Spectroflow Monitor, Model SF 770 S (Kratos-Schoeffel, Westwood, NJ 07675) holding a pair of stainess-steel cuvets (8 μL), with the reference cell air-filled. All u.v. analyses reported here were conducted at 190 nm. After passing through the sample cell, the mobile phase either flowed directly into the collector tubes, or, if dual detection was desired, into the refractive-index (r.i.) detector prior to collection. For u.v. detection, either the isocratic or the gradient mode was used, but, for r.i. detection, only the isocratic mode. The Differential Refractive Index Detector, Model 79877A (Hewlett–Packard Co., Palo Alto, CA 94304), was insulated, to help maintain temperature stability. The reference cell was filled with the degassed, reference solution having a composition the same as that in the sample cell. For r.i. detection, the solvents of the mobile phase were degassed by passing helium through it for ~20 min. Prior to sample injection, the degassed, mobile phase of the desired composition was passed through the entire system until equilibrium was reached. A dual-pen, 4-speed, single-span recorder, Model 9176 (Varian), accommodated both the u.v. and r.i. detectors, simultaneously when desired. A variable voltage-divider, inserted between the u.v. detector and the recorder, gave attenuation capabilities between the 10-mV outlet of the detector and the 1-mV recorder. The r.i. detector was equipped with a 1-mV outlet; hence, a voltage divider was unnecessary. The printer-plotter integrator (Hewlett–Packard 3380A) was connected to the 1-V outlet of either the u.v. detector or the r.i. detector, as desired.

Glass-distilled acetonitrile (u.v. cut-off, 190 nm; Burdick–Jackson) was purchased from Rainin Instruments, Inc. To meet u.v. requirements at 190 nm, water was glass-distilled, and subsequently passed through a special ion-exchanger. If necessary, the solvents were filtered through Cellulosic (water) or Zefluor (acetonitrile) filters (0.5 μm pore-size; 47-mm diameter) with a filtration apparatus (Ghia Corporation) from Rainin. Generally, in order to maintain good absorbance

at the 190-nm, u.v. cut-off, filtration was unnecessary, but degassing was always necessary for refractive-index detection. Passing helium of high purity through the solvents for ~20 min removed the air from either solvent.

All glassware used was first cleaned in detergent solution, thoroughly rinsed in water, and cleaned with dichromate–sulfuric acid solution; it was then thoroughly rinsed successively with tap water, distilled water, and the h.p.l.c.-grade water used in the analyses.

Isolation of oligosaccharide mixture from mannosidosis urine. — Urine was collected, treated with phenylmercuric nitrate, and stored at −20°. For examination by t.l.c., a sample (1 mL) was passed through a short column of AG 1-X8 (HCO_3^-) anion-exchange resin, followed by one of AG 50W-X8 (H^+) cation-exchange resin (Bio-Rad Labs., Richmond, CA 94804), and examined on Merck plates with solvent *A* (2 elutions), or on Fisher "Redi-plates" with solvents *B* or *C* (1 elution); for comparison, a sample of normal urine was examined in the same way. $(Man)_2GlcNAc$, $(Man)_3GlcNAc$, and $(Man)_4GlcNAc$ were identified by comparison with authentic compounds; $(Man)_5GlcNAc$ to $(Man)_9GlcNAc$ were identified by their migration pattern on the plates, and by their T_R value in l.c.

For isolation, mannosidosis urine (1 L) was treated with activated charcoal (Darco-G 60, Fisher) until t.l.c. showed that only traces of oligosaccharides remained. The charcoal was filtered off onto a Celite bed, washed with water (1 L), stirred with 50% aqueous ethanol (1 L) for 2 h, filtered off (Celite), and washed with aqueous ethanol (1 L). Evaporation of the extracts gave a brown syrup (2 g) containing oligosaccharides, together with small amounts of low-molecular-weight compounds and traces of urea (t.l.c.)

Partial separation of oligosaccharides by l.c. under medium pressure. — A slurry of silica gel (200 g) in solvent *C* (420 mL) was packed in a column under ~0.2 MPa of nitrogen gas in the Chromatospac Prep-10 preparative liquid-chromatograph. A solution of crude mannosidosis oligosaccharides (2–4 g) in 1:1 (v/v) 2-propanol–water (2 mL) was applied to the column, and the sample vessel was washed with the same solvent mixture (2 mL). The oligosaccharides were eluted with solvent *C* at 10 mL/min at ~0.2 MPa, and, after 450 mL of solvent had passed through, 9-mL fractions were collected. Elution was continued until 6 L of solvent had been used, and 600 tubes of eluate collected. On the basis of analysis of every 5th tube by t.l.c. (solvents *A* and *B*), the oligosaccharide fractions were suitably combined, and the solvent was evaporated. When serious overlap between homologs occurred [as for $(Man)_4GlcNAc$, $(Man)_5GlcNAc$, and $(Man)_6GlcNAc$], a mixed fraction was collected for further separation by rechromatography under medium or high (~7 MPa) pressure. For rechromatography under medium pressure, a column was packed with Lichroprep Si-60, and the partially separated oligosaccharides were applied to the column and eluted as just described. After analysis by t.l.c., only fractions containing mainly one homolog were combined (see Table II and Fig. 1). For purification of oligosaccharides by preparative layer-chromatography, a solution of partially separated oligosaccharides in 1:1 (v/v) 2-

propanol–water (2 mL) was applied to a plate (up to 50 mg for a 0.55-mm plate; up to 200 mg for a 2-mm plate), which was developed in solvent *A*. When the solvent front had reached to within 5 cm of the top, the plate was removed from the tank, dried *in vacuo* overnight, and redeveloped. For the 2-mm plates, a second drying and a third elution were necessary. After being dried in a stream of air, the plates were viewed under u.v. irradiation (254 nm), and the shapes of the bands ascertained from the presence of traces of absorbant and fluorescent pigments. Then, a 0.5-cm strip was cut from each plate and sprayed with the anisaldehyde reagent. Bands corresponding to each oligosaccharide were scraped from the plate, and extracted overnight with solvent *A*. After filtration (Celite), the filtrate was evaporated, to give a residue that was extracted with 1:1 (v/v) ethanol–water. Filtration of the suspension, and evaporation of the filtrate, gave the oligosaccharides, pure according to t.l.c., but containing residual silica gel that could be removed by chromatography on Bio-Gel P-2.

Purification of oligosaccharides by l.c. under "high" pressure. — The partially purified oligosaccharide fractions obtained by medium-pressure liquid chromatography still retained contaminating, yellow-orange pigments that obscured the peaks of the lower-molecular-weight oligosaccharides when samples were injected into the liquid chromatograph (see Fig. 3). Attempts to remove these pigments prior to injection by passing samples through Sep-Pak cartridges (Waters Associates, Milford, MA 01757) were unsuccessful. Hence, it was necessary to collect selected cuts of peak fractions, and repeatedly re-inject these into the chromatograph until all of the pigments had been removed. Eluted fractions of a given oligosaccharide were

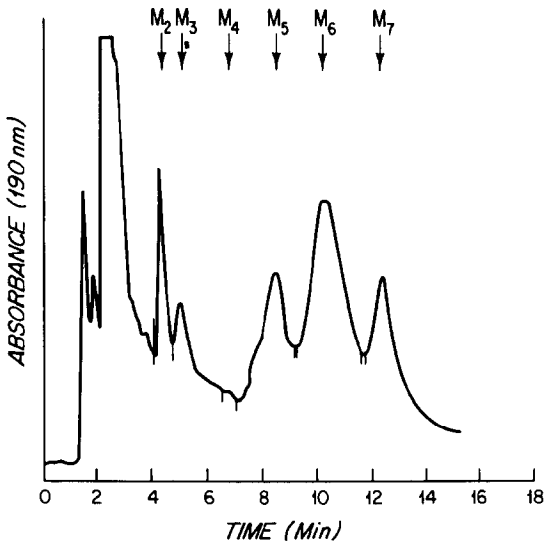

Fig. 4 Liquid chromatogram, under ~9 MPa pressure, of a partially purified oligosaccharide fraction, rich in M_6, in the isocratic mode. [Conditions: Micropak NH_2-10 column (30 cm × 4 mm); mobile phase, 11:9 (v/v) acetonitrile–water; flow rate, 1.5 mL/min; sample size, 10 μL; temperature, 26°; u.v. detector, 190 nm (0.04 aufs); and chart speed, 1 cm/min.]

then pooled, evaporated to dryness under nitrogen (40°), and the residue dissolved in the minimum volume of 1:3 (v/v) acetonitrile–water. If the resulting solution was not completely clear, it was filtered through a stainless-steel, Swinney adapter fitted with a hydrophilic prefilter and a 0.2-nm pore-size filter disc compatible to both acetonitrile and water (Millipore Corporation, Bedford, MA 01730). If traces of silica remained in the sample filtrates, they were removed by passage through Bio-Gel P-2 (Pasteur pipet). The filtrates were evaporated to dryness under nitrogen (40°), the residue dissolved in 1:3 (v/v) acetonitrile–water, and the solution was mixed thoroughly, and analyzed in the Chromatograph (Micropak NH_2-10 column). A typical chromatogram of such a separation is shown in Fig. 4. The pooled, eluted fractions of each sample were repeatedly chromatographed in this way, until a pure oligosaccharide was obtained.

Aliquots of the eight pure oligosaccharides (M_2–M_9) were combined in a given volume of 1:3 (v/v) acetonitrile–water, and a sample (10 μL) was injected. In the gradient mode, this mixture could be separated into the pure components within 16 min (see Table IV and Fig. 5). In the isocratic mode, the last oligosaccharide, M_9, was eluted within 20 min (see Table III).

TABLE IV

CHARACTERISTIC PEAK-AREAS FROM A MIXTURE OF PURE OLIGOSACCHARIDES IN ACETONITRILE–WATER SEPARATED BY LIQUID CHROMATOGRAPHY UNDER THE GRADIENT MODE[a]

Oligosaccharide[b]	T_R *(min)*	*Peak area (%)*	*Amount injected (μg)*
M_2	3.89	18.3	4
M_3	4.60	13.4	6
M_4	5.57	14.5	6
M_5	7.06	14.6	6
M_6	8.65	10.9	9
M_7	10.77	15.0	8
M_8	13.40	6.1	10
M_9	16.25	5.8	10

[a]Conditions: injection of 10 μL; pressure, ~7 MPa; Micropak NH_2-10 column (30 cm × 4 mm); gradient program as in Fig. 5; flow rate, 1.5 mL/min; temperature, 26°; and u.v. detector, 190 nm (0.08 aufs.). [b]Abbreviations, see footnote to Table I.

To investigate the effect of restoration of a Micropak, preparative NH_2-10 column with 0.1% 1,4-diaminobutane[10], two of the crude fractions (see Table Ic) were chromatographed with 11:9 (v/v) acetonitrile–water containing 0.01% (v/v) of 1,4-diaminobutane (see Fig. 3). The eluted fractions, when evaporated in the presence of 1,4-diaminobutane, gave modified oligosaccharides (t.l.c.). Formation of these modifications was prevented by treatment of the eluted fractions with 0.1M hydrochloric acid prior to evaporation under nitrogen, followed by passage through a short column (Pasteur pipet) of AG 50W-X8 (pyridinium$^+$) cation-exchange resin, and washing with 2 column-volumes of water. The combined solu-

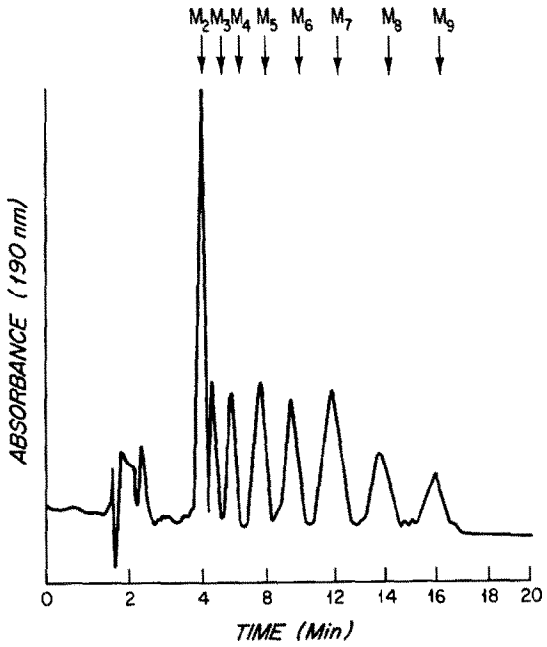

Fig. 5. Liquid chromatogram, under ~9 MPa pressure, of a mixture of pure oligosaccharides, M_2–M_9, in 1:3 (v/v) acetonitrile–water, in the gradient mode (see Table IV). [Conditions: Micropak NH_2-10 column (30 cm × 40 mm); flow rate, 1.5 mL/min; temperature, 26°; sample size, 10 μL; detector: Schoeffel u.v., 190 nm (0.08 aufs); integrator attenuation, 64; chart speed, 0.5 cm/min; and mobile phase: acetonitrile–water (v/v), 55:45 (0 min), 54:46 (7 min), 52:48 (13 min), and 51:49 (16 min).]

tions were concentrated to 1 mL, and the concentrate was applied to a column (1 × 15 cm) of Bio-Gel P-2 (200–400 mesh). Fractions (0.5 mL) were collected, and analyzed by t.l.c., and those containing the desired oligosaccharide were combined, and evaporated. The resulting product was re-examined in a new Micropak NH_2-10, analytical column.

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