Comparative Studies of the Sugar Chains of Aminopeptidase N and Dipeptidylpeptidase IV Purified from Rat Kidney Brush-Border Membrane†

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ABSTRACT: Asparagine-linked sugar chains of rat kidney aminopeptidase N and dipeptidylpeptidase IV were investigated comparatively. Oligosaccharides released from the two enzymes by hydrazinolysis were fractionated by paper electrophoresis, serial chromatographies on columns of immobilized *Aleuria aurantia* lectin, concanavalin A, phytohemagglutinin E_4, and *Datura stramonium* agglutinin, and Bio-Gel P-4 (<400 mesh) column chromatography. Structures of oligosaccharides in each fraction were assumed by their effective molecular sizes and behaviors on the four lectin columns and then confirmed by sequential exoglycosidase digestion and methylation analysis. The sugar chains of aminopeptidase N and dipeptidylpeptidase IV are almost the same as those of rat kidney γ-glutamyltranspeptidase reported previously [Yamashita, K., Hitoi, A., Matsuda, Y., Tsuji, A., Katunuma, N., & Kobata, A. (1983) *J. Biol. Chem. 258*, 1098–1107]. They are a mixture of 5 high mannose type sugar chains and 32 (for aminopeptidase N) or 26 (for dipeptidylpeptidase IV) mono-, bi-, tri-, and tetraantennary complex type sugar chains. The unique feature of the complex-type sugar chains of both enzymes is that they all contain the bisecting *N*-acetylglucosamine residue and are incompletely galactosylated in their outer-chain moieties.

γ-Glutamyltranspeptidase, dipeptidylpeptidase IV, and aminopeptidase N are anchored to the brush-border membrane of the epithelial cells of kidney, intestine, and liver by a small hydrophobic domain located at the N-terminal of the polypeptide chain (Semenza, 1986). These three enzymes are produced by the same cell and transported to the same apical segment of plasma membrane, although they turn over independently. Because hydrophobic signal peptides of these enzymes cannot be removed after transport from the Golgi membrane to the apical side of the plasma membrane, they remain inserted in the membrane (Kreil, 1981). It has been speculated without proof that some carbohydrate linked near the cleavage site of the three enzymes may inhibit the action of signal peptidase. However, the signal peptide theory cannot explain why the three enzymes are exclusively localized at the apical side membrane. Therefore, another recognition mechanism to confine the enzymes to the apical side segment of the membrane may exist. In view of the recent finding that the sugar moiety of subcellular glycoproteins functions as a traffic signal to transport them to a specific subcellular site, it is of interest to elucidate the structures of the sugar chains of apical and basolateral membrane glycoproteins. We have previously found that rat kidney γ-glutamyltranspeptidase contains a rather unusual set of sugar chains with extraordinary complexity (Yamashita et al., 1983a). In order to find out if the presence of unusual sugar chains is a characteristic feature common to the apical side membrane glycoproteins, a comparative study of the sugar-chain structures of aminopeptidase N and dipeptidylpeptidase IV was performed.

Materials and Methods

Materials. Methyl α-D-mannopyranoside, D-galactono-γ-lactone, *Arthrobacter ureafaciens* sialidase (Uchida et al., 1974), and chitin were purchased from Nakarai Chemical Co., Kyoto. Bio-Gel P-4 (<400 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA. BrCN-activated Sepharose 4B and concanavalin A (Con A)–Sepharose were purchased from Pharmacia Fine Chemicals, Uppsala. Bovine epididymal α-fucosidase and jack bean meal were purchased from Sigma Chemical Co., St. Louis, MO. Jack bean β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase were prepared by the method of Li and Li (1972). Snail β-mannosidase (Sugahara et al., 1972) was kindly supplied by Seikagaku Kogyo Co., Tokyo. α-Mannosidase, which cleaves only the Manα1→2Man linkage (Yamashita et al., 1980), was purified from the mycelium of *Aspergillus saitoi* according to the method reported previously (Ichishima et al., 1981). Diplococcal β-galactosidase, diplococcal β-*N*-acetylhexosaminidase, and endo-β-*N*-acetylglucosaminidase D were purified according to the method of Glasgow et al. (1977). *N*-Acetylglucosamine oligomers were prepared according to previous reports (Crowley & Goldstein, 1981; Yamashita et al., 1987). *Aleuria aurantia* lectin (AAL)–Sepharose was prepared as reported previously (Yazawa et al., 1984). *Datura stramonium* agglutinin (DSA)–Sepharose was prepared as reported previously (Yamashita et al. 1987). Phytohemagglutinin E_4 (E-PHA)–agarose was kindly provided from Honen Oil Co., Ltd., Tokyo. NaB^3H_4 (348 mCi/mmol) was purchased from New England Nuclear, Boston, MA. NaB^2H_4 (98%) was obtained from Merck Co., Darmstadt, FRG.

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Table I: Physicochemical Characteristics of Rat Kidney γ-Glutamyltranspeptidase, Aminopeptidase N, and Dipeptidylpeptidase IV

	γ-glutamyl-trans-peptidase	amino-peptidase N	dipeptidyl-peptidase IV
M_r	70000	260000	220000
subunit M_r			
heavy	46000	homodimer	homodimer
light	23000	(130000)	(110000)
no. of sugar chains/mol	3–5	5.5	5.4

Purification of Rat Kidney Aminopeptidase N and Dipeptidylpeptidase IV. A microsome fraction of rat kidney was prepared according to the method described previously (Yamashita et al. 1983a). Aminopeptidase N and dipeptidylpeptidase IV were solubilized from the microsome fraction by incubation with papain at 37 °C for 60 min. The enzymes were then precipitated by ammonium sulfate fractionation (55–90%), dissolved in 50 mM imidazole buffer, pH 7.2, and dialyzed against the same buffer. The enzyme solution was subjected to gel permeation chromatography with use of an Ultrogel AcA 34 column (2 × 100 cm), and the fractions containing aminopeptidase N and dipeptidylpeptidase IV activities were pooled. The two enzymes were then separated by DEAE-cellulose column chromatography (Matsuda et al., 1983), and the fractions containing each enzyme were pooled and concentrated by ultrafiltration through Amicon YM30. The samples were then applied to a Bio-Gel P-10 column (1 × 45 cm) and eluted with deionized water. The enzyme preparation, thus obtained, gave a single band stained with Coomassie brilliant blue after being subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

Oligosaccharides. NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$[1] and NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ were obtained from Bence Jones protein Wh λ by hydrazinolysis followed by reduction with NaB^3H_4 (Ohkura et al., 1984). Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$(Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$) and Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→$4GlcNAc_{OT}$(Man_3·GlcNAc·$GlcNAc_{OT}$) were obtained as reported by Hitoi et al. (1987). $(Man\alpha1\rightarrow2)_{0-4}$[Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→$4GlcNAc_{OT}$](Man_{5-9}·GlcNAc·$GlcNAc_{OT}$) was obtained from bovine pancreatic ribonuclease (Liang et al., 1980).

Isolation of the Asparagine-Linked Sugar Chains of Rat Kidney Aminopeptidase N and Dipeptidylpeptidase IV as Oligosaccharides. Rat kidney aminopeptidase N (11 mg) and dipeptidylpeptidase IV (15 mg) were subjected to an 8-h hydrazinolysis as reported earlier (Takasaki et al., 1982). After N-acetylation, 30% of the oligosaccharide fractions released from aminopeptidase N and dipeptidylpeptidase IV were reduced with NaB^3H_4. Lactose (10 nmol) was added to each fraction as an internal standard. [^{3}H]Lactitol and the tritium-labeled oligosaccharide fraction were separated by paper chromatography. The total yields of radioactive oligosaccharides obtained from aminopeptidase N and dipeptidylpeptidase IV were 1.38 × 10^6 and 1.29 × 10^6 cpm, respectively. The numbers of sugar chains released from aminopeptidase N and dipeptidylpeptidase IV were calculated as 5.5 and 5.4 (Table I) on the basis of the specific activity of the NaB^3H_4 and the molecular weights of aminopeptidase N and dipeptidylpeptidase IV. The specific activity of the NaB^3H_4 was determined from the radioactivity incorporated into lactitol. The remaining oligosaccharides from aminopeptidase N were reduced with NaB^2H_4 to obtain samples for methylation analysis.

Fractionation of Oligosaccharides by Serial Affinity Chromatographies on Immobilized Lectin Columns. The tritium-labeled oligosaccharide fraction (2 × 10^5 cpm) or deuterium-labeled oligosaccharide fraction, dissolved in 50 μL of 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.4, containing 0.02% NaN_3, 0.15 M NaCl, and 1 mM each of $MnCl_2$, $MgCl_2$, and $CaCl_2$, was applied to a Con A–Sepharose (2-mL), an AAL–Sepharose (5-mL), or a DSA–Sepharose (2-mL) column equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 0.02% NaN_3, and allowed to stand at 4 °C for 30 min. The oligosaccharides bound to the columns were then eluted with 10 mL of the same buffer, followed by 5 mL of 10 mM Tris-HCl buffer, pH 7.4, containing 0.02% NaN_3 and 0.1 M methyl α-D-mannoside (for Con A), 1 mM α-L-fucose (for AAL), and 1% of a mixture of equal amounts of *N*-acetylglucosamine dimer, trimer, and tetramer (for DSA). An E-PHA–agarose column (1 mL) was equilibrated with phosphate-buffered saline (6.7 mM KH_2PO_4/0.15 M NaCl, pH 7.4), and bound oligosaccharides were eluted with the same buffer at room temperature.

Analytical Methods. High-voltage paper electrophoresis was performed by using pyridine/acetate buffer, pH 5.4 (pyridine/acetic acid/water 3:1:387), at a potential of 73 V/cm for 60 min. Descending paper chromatography was performed with 1-butanol/ethanol/water (4:1:1) as solvent. Radiochromatoscanning was performed with a Packard radiochromatogram scanner, Model 7201.

Bio-Gel P-4 (<400 mesh) column chromatography (2 cm i.d. × 1.25 m) was performed as reported previously (Yamashita et al., 1982). Radioactivity was determined with a Beckman liquid scintillation spectrometer LS-7000.

Methylation analysis of oligosaccharide was performed as described previously (Endo et al., 1979) except that the time of hydrolysis of permethylated oligosaccharide was prolonged from 2 to 6 h and 0.5 N H_2SO_4 containing 90% acetic acid and NaB^2H_4 was used as a reducing reagent. Analysis of partially O-methylated hexitols and *N*-acetylglucosaminitols was performed with a gas chromatograph–mass spectrometer, Model GC-MS-QP-1000 (Shimadzu Co., Ltd., Kyoto), by using a fused silica capillary column coated with a cross-linked SPB-35 (0.53 mm i.d. × 30 m). The column temperature was programmed from 170 to 230 °C at a rate of 2 °C/min, and the flow rate of carrier gas was 15 mL/min. The molar ratios of partially O-methylated alditol acetates were calculated from the total ion intensities found by GC–MS.

Glycosidase Digestion. Radioactive oligosaccharides [(1–50) × 10^4 cpm, 1–50 nmol] were incubated with one of the following reaction mixtures at 37 °C for 17 h: sialidase digestion, 100 milliunits of enzyme in sodium acetate buffer, pH 5.0 (40 μL); digestion with a mixture of sialidase, diplococcal β-galactosidase, and jack bean β-*N*-acetylhexosaminidase, 50 milliunits of sialidase, 2 milliunits of β-galactosidase, and 3 units of β-*N*-acetylhexosaminidase in 0.2 M citrate/phosphate buffer, pH 5.5 (40 μL); diplococcal β-galactosidase digestion, 5 milliunits of enzyme in 0.2 M citrate/phosphate buffer, pH 6.0 (30 μL); jack bean α-mannosidase digestion, 0.3 unit of enzyme in 0.05 M sodium

[1] Subscript OT is used in this paper to indicate NaB^3H_4-reduced sugars. All sugars mentioned in this paper were of the D configuration, except for fucose, which had an L configuration.

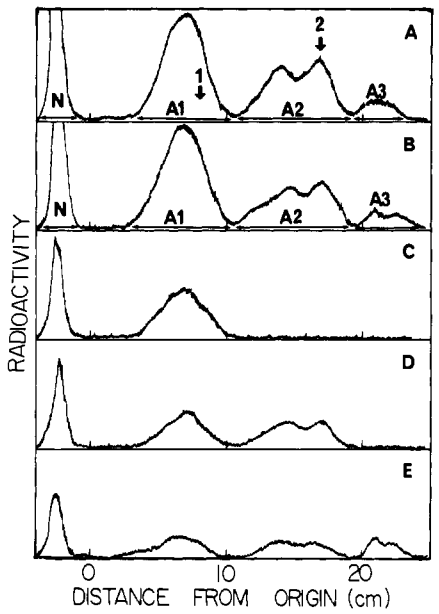


FIGURE 1: Paper electrophoretogram of oligosaccharides released from rat kidney aminopeptidase N (A) and dipeptidylpeptidase IV (B) by hydrazinolysis followed by reduction with NaB^3H_4. Arrows indicate the migration positions of authentic oligosaccharides: (1) NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$; (2) NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$. The radioactive fractions A1, A2, and A3 in (B) were recovered separately by elution with water. An aliquot of each fraction were partially desialylated by sialidase digestion for 3 h. Paper electrophoretograms of the three products, thus obtained, are shown in (C–E), respectively. Partial desialylation of the three acidic fractions in (A) gave results similar to those in (C–E).

acetate buffer, pH 4.5, containing 1 mM $ZnCl_2$ (50 μL); snail β-mannosidase digestion, 5 milliunits of enzyme in 0.05 M sodium citrate buffer, pH 6.0 (40 μL); *Aspergillus* α-mannosidase digestion, enzyme (50 ng) in 0.1 M acetate buffer, pH 5.0 (30 μL). One drop of toluene was added to the reaction mixture to prevent bacterial growth. After incubation, the reaction mixture was heated in boiling water for 2 min to stop the reaction.

RESULTS

Paper Electrophoresis of Oligosaccharide Fractions Obtained from Rat Kidney Aminopeptidase N and Dipeptidylpeptidase IV. Upon paper electrophoresis at pH 5.4, very similar fractionation patterns were obtained from the oligosaccharide fractions of aminopeptidase N and dipeptidylpeptidase IV (Figure 1, parts A and B). The percent molar ratios of N, A1, A2, and A3 calculated on the basis of their radioactivities were 50:29:17:4 for aminopeptidase N and 40:37:19:4 for dipeptidylpeptidase IV. All acidic oligosaccharides in fractions A1, A2, and A3 were converted to neutral oligosaccharides by exhaustive sialidase digestion (data not shown). These three neutral oligosaccharide mixtures from aminopeptidase N and dipeptidylpeptidase IV were designated as AP-A1N, AP-A2N, AP-A3N, DP-A1N, DP-A2N, and DP-A3N, respectively.

It was confirmed by incomplete sialidase digestion that oligosaccharides in fraction A1 were monosialyl derivatives and oligosaccharides in fractions A2 and A3 were disialyl and trisialyl derivatives, respectively (Figure 1, parts C–E). Since the elution profiles of fractions N, A1N, A2N, and A3N derived from aminopeptidase N and dipeptidylpeptidase IV on Bio-Gel P-4 column chromatography and the data of their

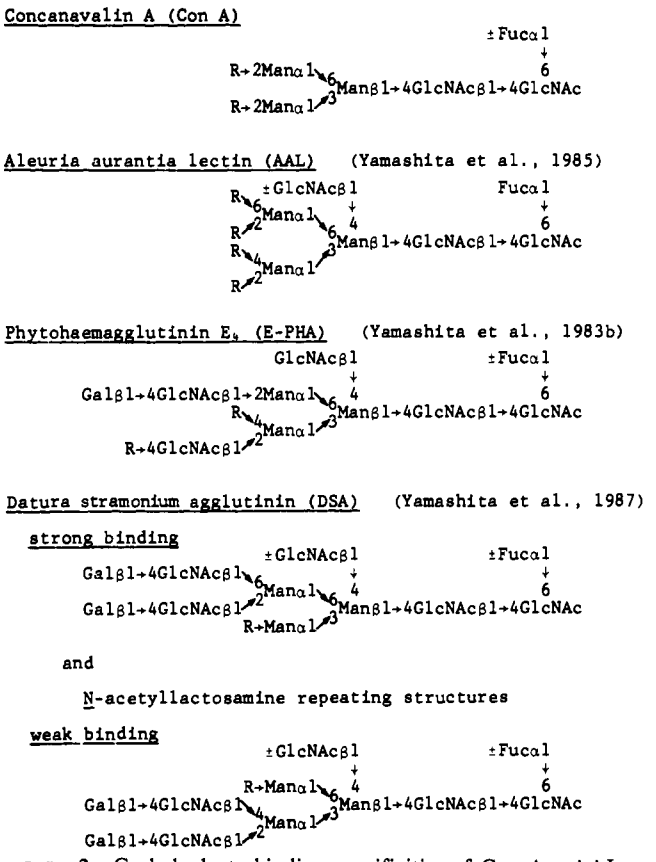


FIGURE 2: Carbohydrate binding specificities of Con A–, AAL–, E-PHA–, and DSA–Sepharose columns elucidated by using complex-type sugar chains. R represents either hydrogen or sugars.

sequential exoglycosidase digestion were too complicated to interpret, they were fractionated further with three different immobilized lectin columns. The carbohydrate binding specificities of these columns are summarized in Figure 2.

Fractionation of the Oligosaccharides Released from Aminopeptidase N and Dipeptidylpeptidase IV by a Con A–Sepharose Column and Preliminary Study of Their Structures. The radioactive oligosaccharide fractions obtained from the two kidney enzymes were exhaustively digested with sialidase. The two radioactive neutral oligosaccharide mixtures, thus obtained, were passed through a Con A–Sepharose column. Ninety-three percent of both oligosaccharide fractions passed through the column without any interaction. The remaining 7% of the two fractions bound to the column and eluted with 0.1 M methyl α-mannopyranoside. The fractions not bound and bound to the column were named Con A(−) and Con A(+), respectively.

Oligosaccharides in the Con A(+) fractions from the two enzymes were separated into five radioactive peaks with mobilities of 12.5, 11.9, 10.9, 9.9, and 9.0 glucose units upon Bio-Gel P-4 column chromatography. By incubation with *Aspergillus* α-mannosidase, which specifically cleaves the Manα1→2Man linkage, all five components were converted to a radioactive oligosaccharide with the same mobility as authentic Man_5·GlcNAc·GlcNAc$_{OT}$ with release of 4, 3, 2, 1, and 0 mol of mannose residues (data not shown). That the heptaitol obtained from these oligosaccharides has the structure Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ was confirmed by a series of experiments as reported previously (Liang et al., 1980). These results indicated that the oligosaccharides in the Con A(+) fractions obtained from aminopeptidase N and dipeptidylpeptidase IV are a series of high mannose type sugar chains,

Table II: Methylation Analysis of Con A(−) Fractions Obtained from Aminopeptidase N (AP) and Dipeptidylpeptidase IV (DP) before and after Sialidase Digestion

partially methylated sugars	Con A(−) fractions[a] (molar ratio)			
	AP	AP + sialidase	DP	DP + sialidase
fucitol				
2,3,4-tri-*O*-methyl-1,5-di-*O*-acetyl	0.5	0.6	0.4	0.5
galactitol				
2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl	0.8	2.5	1.1	2.4
2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl	0.6	tr[b]	0.4	0.0
2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetyl	1.2	0.0	1.0	0.0
mannitol				
3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetyl	1.3	1.2	1.4	1.2
3,6-di-*O*-methyl-1,2,4,5-tetra-*O*-acetyl	0.3	0.3	0.3	0.3
3,4-di-*O*-methyl-1,2,5,6-tetra-*O*-acetyl	0.2	0.2	0.2	0.2
2-mono-*O*-methyl-1,3,4,5,6-penta-*O*-acetyl	*1.0*	*1.0*	*1.0*	*1.0*
(*N*-methylacetamido)-2-deoxyglucitol				
1,3,5,6-tetra-*O*-methyl-4-mono-*O*-acetyl	0.3	0.3	0.3	0.3
1,3,5-tri-*O*-methyl-4,6-di-*O*-acetyl	0.5	0.4	0.4	0.5
3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl	1.7	1.8	1.8	1.7
3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl	2.8	2.6	2.6	2.8

[a] Numbers in the table were calculated by taking the italic value as 1.0. [b] Trace, less than 0.05.

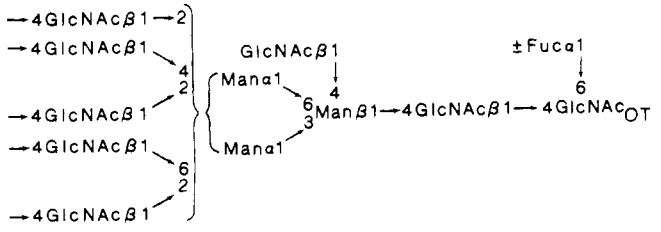
the structures of which are shown in Figure 7.

Upon Bio-Gel P-4 column chromatography, both Con A(−) fractions from the two enzymes gave multiple radioactive peaks distributing from 12.8 to 18.5 glucose units. After digestion with a mixture of diplococcal β-galactosidase and jack bean β-*N*-acetylhexosaminidase, however, only two radioactive components with the same elution positions as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ and Man_3·GlcNAc·$GlcNAc_{OT}$ were detected in both cases. That these two oligosaccharides have the structures Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ and Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4$GlcNAc_{OT}$ was confirmed as described previously (Yamashita et al., 1983a; Hitoi et al., 1987) (data not shown). These results indicated that all oligosaccharides in the Con A(−) fractions are complex-type sugars having either a fucosylated or nonfucosylated trimannosyl core.

Methylation analysis of the Con A(−) fractions obtained from aminopeptidase N and dipeptidylpeptidase IV revealed (Table II) that the mannosyl residues occur as

$$\rightarrow 2Man1\rightarrow,\ \begin{matrix}\rightarrow 4\\ \rightarrow 2\end{matrix}Man1\rightarrow,\ \begin{matrix}\rightarrow 6\\ \rightarrow 2\end{matrix}Man1,\text{ and }\begin{matrix}\rightarrow 6\\ \rightarrow 4\\ \rightarrow 3\end{matrix}Man1$$

The absence of 2,4-di-*O*-methylmannitol indicated that all of the β-mannosyl residues of the trimannosyl cores are substituted with a bisecting *N*-acetylglucosamine residue. Detection of 1,3,5,6-tetra- and 1,3,5-tri-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitols is in accord with the fact that both fucosylated and nonfucosylated trimannosyl cores are included among the oligosaccharides as described already. Detection of only 3,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol as the di-*O*-methyl derivative of *N*-acetylglucosamine indicated that the *N*-acetylglucosamine residues within the sugar chains are all substituted at their C-4 position. Therefore, the complex-type sugar chains of the two enzymes can be written as follows:



Fractionation of the Complex-Type Oligosaccharides by Serial Chromatographies with AAL–Sepharose and E-PHA–Agarose Columns. In order to analyze the structure of each complex-type oligosaccharide, fractions N, A1N, and A2N + A3N from the two kidney enzymes were passed through a Con A–Sepharose column. Among the six neutral fractions, AP-N and DP-N were separated into Con A(−) and Con A(+) fractions. The remaining four, AP-A1N, AP-A2N + AP-A3N, DP-A1N, and DP-A2N + DP-A3N, did not bind to a Con A–Sepharose column. All six Con A(−) fractions were separated into two fractions by affinity chromatography on an AAL–Sepharose column. The fractions that passed through the column without any interaction were named AAL(−). The other fractions that were bound and eluted from the column with a 0.5 mM L-fucose solution were named AAL(+). Each of the eight fractions was further separated into two fractions by affinity chromatography on an E-PHA–agarose column. The fractions that passed through the column were named E-PHA(−), and the fractions retarded on the column were named E-PHA(+). Distribution of the amount of oligosaccharides in each fraction expressed as the percent molar ratio to total oligosaccharides in Con A(−) fractions obtained from the two enzymes is summarized in Table III.

Table III: Fractionation of Con A(−) Fractions by Serial Affinity Chromatography on an AAL–Sepharose Column and an E-PHA–Agarose Column[a]

Con A(−) fractions from	AAL(−)		AAL(+)	
	E-PHA-(−)	E-PHA-(+)	E-PHA-(−)	E-PHA-(+)
AP-N	18.9 (A)	5.6 (B)	13.2 (G)	5.4 (H
AP-A1N	7.0 (C)	6.1 (D)	8.0 (I)	7.9 (J)
AP-A2N + AP-A3N	5.2 (E)	3.8 (F)	7.0 (K)	5.2 (L)
DP-N	9.6 (A)	4.3 (B)	9.2 (G)	9.2 (H)
DP-A1N	3.3 (C)	6.5 (D)	6.6 (I)	20.6 (J)
DP-A2N + DP-A3N	1.9 (E)	2.0 (F)	9.7 (K)	9.7 (L)

[a] The numbers in the table represent the percent molar ratio of each fraction to the total Con A(−) fraction of oligosaccharides released from either aminopeptidase N or dipeptidylpeptidase IV. The capital letters in parentheses are used in this paper to indicate the fractions.

When the 12 fractions, A–L in Table III, obtained from aminopeptidase N were subjected to Bio-Gel P-4 column chromatography, they were separated into 38 radioactive peaks as shown in Figure 3. The twelve fractions from dipeptidylpeptidase IV were separated into 32 radioactive peaks as shown in Figure 4. These radioactive peaks were named from a to z as shown in Figures 3 and 4. As will be described

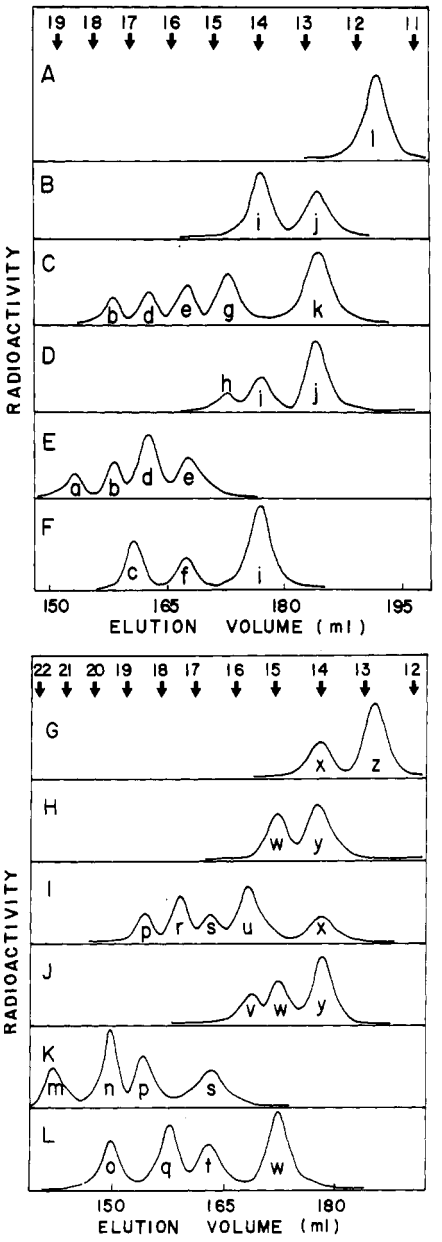


FIGURE 3: Bio-Gel P-4 column chromatograph of oligosaccharide fractions A–L from aminopeptidase N in Table III. Black arrows indicate the elution positions of glucose oligomers, and the numbers indicate those of glucose units.

later, the components with the same mobility but named differently in these figures were confirmed to have isomeric structures.

Structures of Oligosaccharides Released from Components a–z by β-Galactosidase Digestion. The results of diplococcal β-galactosidase digestion of radioactive components a–z are summarized in Figure 5. The elution position of each oligosaccharide before the enzymatic digestion is indicated by a white arrow with the name of the component. Components l and z were not degraded by the β-galactosidase digestion, indicating that they do not contain any Galβ1→4GlcNAc residues as their nonreducing termini.

By comparing the mobilities of each radioactive component before and after the β-galactosidase digestion, the number of galactose residues released from each component was calculated and summarized in Table IV. Since diplococcal β-galactosidase cleaves the Galβ1→4GlcNAc linkage only, these galactose residues should be linked at the C-4 position of the β-*N*-acetylglucosamine residues of components I–VIII. That

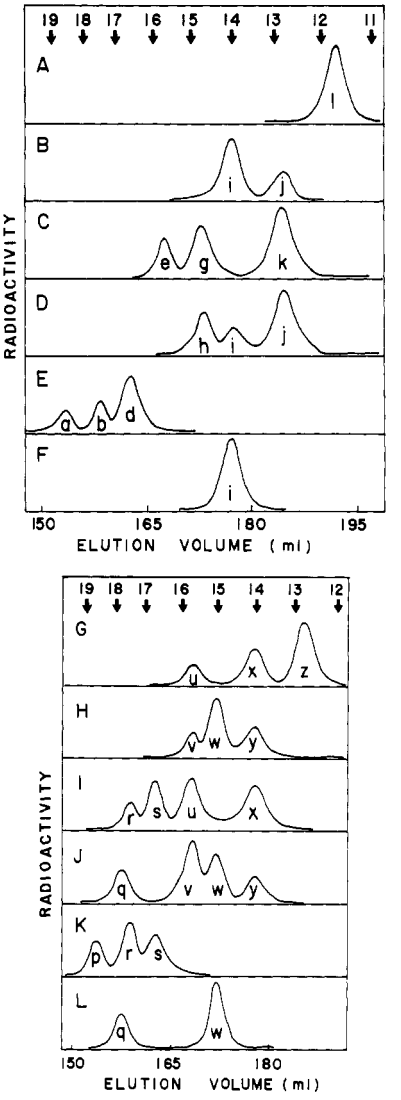


FIGURE 4: Bio-Gel P-4 column chromatograph of oligosaccharide fractions A–L from dipeptidylpeptidase IV in Table III. Black arrows are the same as in Figure 3.

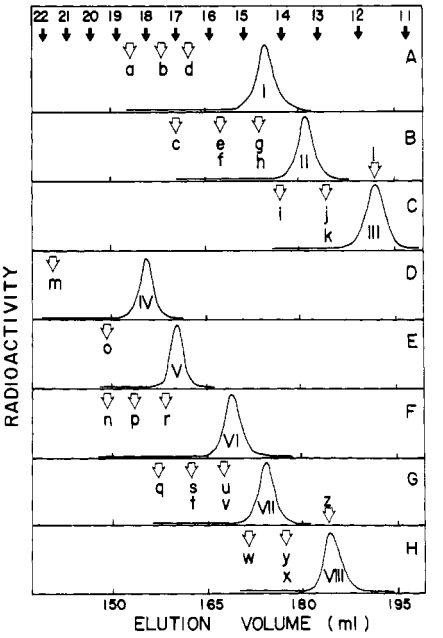


FIGURE 5: Diplococcal β-galactosidase digestion of oligosaccharides a–z in Figures 3 and 4. Black arrows are the same as in Figure 3. White arrows indicate the elution positions of oligosaccharides before the β-galactosidase treatment.

Table IV: Number of Galactose Residues Released by Diplococcal β-Galactosidase Digestion of Oligosaccharides a–z in Figures 3 and 4 and Their Radioactive Products and Behavior of Oligosaccharides a–z on a DSA–Sepharose Column

components	galactose released	behavior on DSA–Sepharose[a]	radioact. product	components	galactose released	behavior on DSA–Sepharose[a]	radioact. product
a	4	+	I	n	4	+	VI
b	3	R, +	I	o	2	–	V
c	3	R	II	p	3	R, +	VI
d	2	–, R, +	I	q	3	R	VII
e	2	R	II	r	2	–, R, +	VI
f	2	–	II	s	2	R	VII
g	1	–	II	t	2	–	VII
h	1	–	II	u	1	–	VII
i	2	–	III	v	1	–	VII
j	1	–	III	w	2	–	VIII
k	1	–	III	x	1	–	VIII
l	0	–	III	y	1	–	VIII
m	3	+	IV	z	0	–	VIII

[a] +, eluted with 1% *N*-acetylglucosamine oligomers; R, retarded in a DSA–Sepharose column; –, passed through a DSA–Sepharose column.

only 3,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol was detected as the di-*O*-methyl derivative of *N*-acetylglucosamine in the methylation data of the Con A(–) fragment (Table II) also supported this assumption.

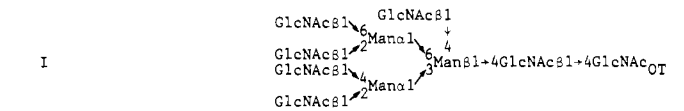
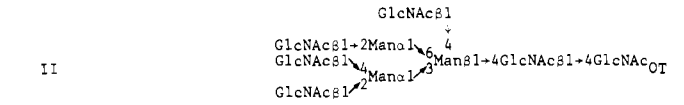
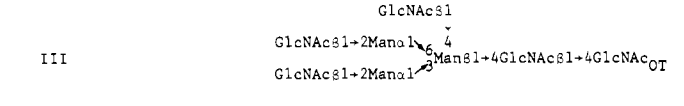
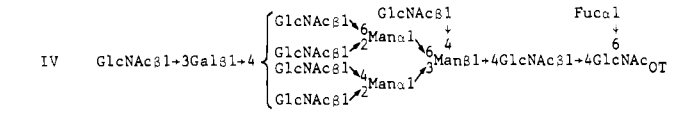
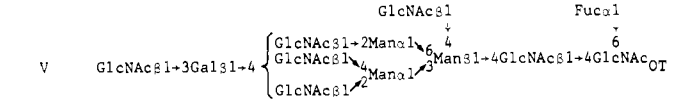
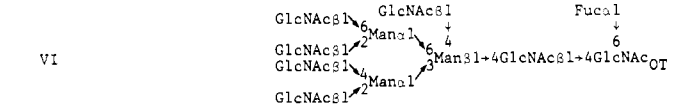
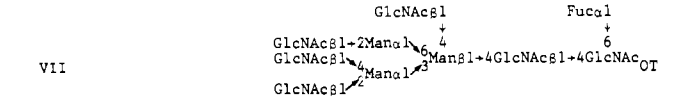
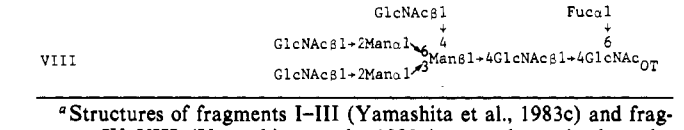
The structures of components I–VIII were determined as shown in Table V by use of the substrate specificities of diplococcal β-*N*-acetylhexosaminidase and periodate oxidation. Because the structural analyses to reach these structural assignments were the same as reported in previous papers (Yamashita et al., 1983a,c), they will not be described here.

The data in Table IV indicated that oligosaccharides a, c, i, n, q, and w are fully galactosylated forms of components I, II, III, VI, VII, and VIII, respectively. The structures of oligosaccharides l and z should be the same as components III and VIII, respectively. Therefore, their structures can be written as shown in Figure 6.

The data in Table IV indicated that oligosaccharides b and p are the trigalactosyl derivatives of components I and VI, respectively. Therefore, four structures are possible for both of them, galactosylated (1) at the 4:2,6 arm,[2] (2) at the 2:2,6 arm, (3) at the 2,4:6 arm, and (4) at the 2,4:2 arm. Among them, oligosaccharides with structure 4 should not be included in b and p because they were not retarded in an E-PHA–agarose column (Table III). When radioactive oligosaccharides b and p were subjected to affinity chromatography on a DSA–Sepharose column, approximately 60% was retarded and eluted with buffer only, while the remaining 40% bound firmly to the column and eluted with *N*-acetylglucosamine oligomer solution in both cases (Table IV). The binding specificity of a DSA–Sepharose column (Figure 2) indicated that oligosaccharides with either structure 1 or 2 should be included in the bound fraction and oligosaccharides with structure 3 in the retarded fraction. Therefore, b and p should be mixtures of the three oligosaccharides shown in Figure 6.

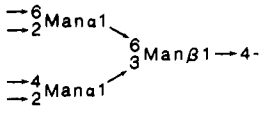
The data in Table IV indicated that oligosaccharides d and r are the digalactosyl derivatives of components I and VI, respectively. Therefore, six possible structures could be included, galactosylated (1) at the :2,6 arm, (2) at the 4:6 arm, (3) at the 2:6 arm, (4) at the 4:2 arm, (5) at the 2:2 arm, and (6) at the 2,4: arm. Because oligosaccharides d and r did not bind to an E-PHA–agarose column, oligosaccharides with structures 4 and 5 should be excluded. When radioactive oligosaccharides d and r were subjected to DSA–Sepharose column chromatography, both were separated into three fractions: a pass through, a retarded, and a bound (Table IV). The bound fractions should contain oligosaccharides with structure 1, and the retarded fractions, those with structure 6. The pass through should contain oligosaccharides with

Table V: Structures of Fragments I–VIII in Figure 5

fragment	structures[a]
I	
II	
III	
IV	
V	
VI	
VII	
VIII	

[a] Structures of fragments I–III (Yamashita et al., 1983c) and fragments IV–VIII (Yamashita et al., 1983a) were determined as described in the cited references.

[2] In order to facilitate expression of the locations of outer chains, a new way of description was adopted. The four possible locations will be described as 2,4:2,6.



Accordingly, the 4:2,6 arm will indicate the $\rightarrow^{6}_{2}$Manα1→6(→4Manα1→3)Manβ1→4- group and the 2,4: arm will indicate the Manα1→6($\rightarrow^{4}_{2}$Manα1→3)Manβ1→4- group.

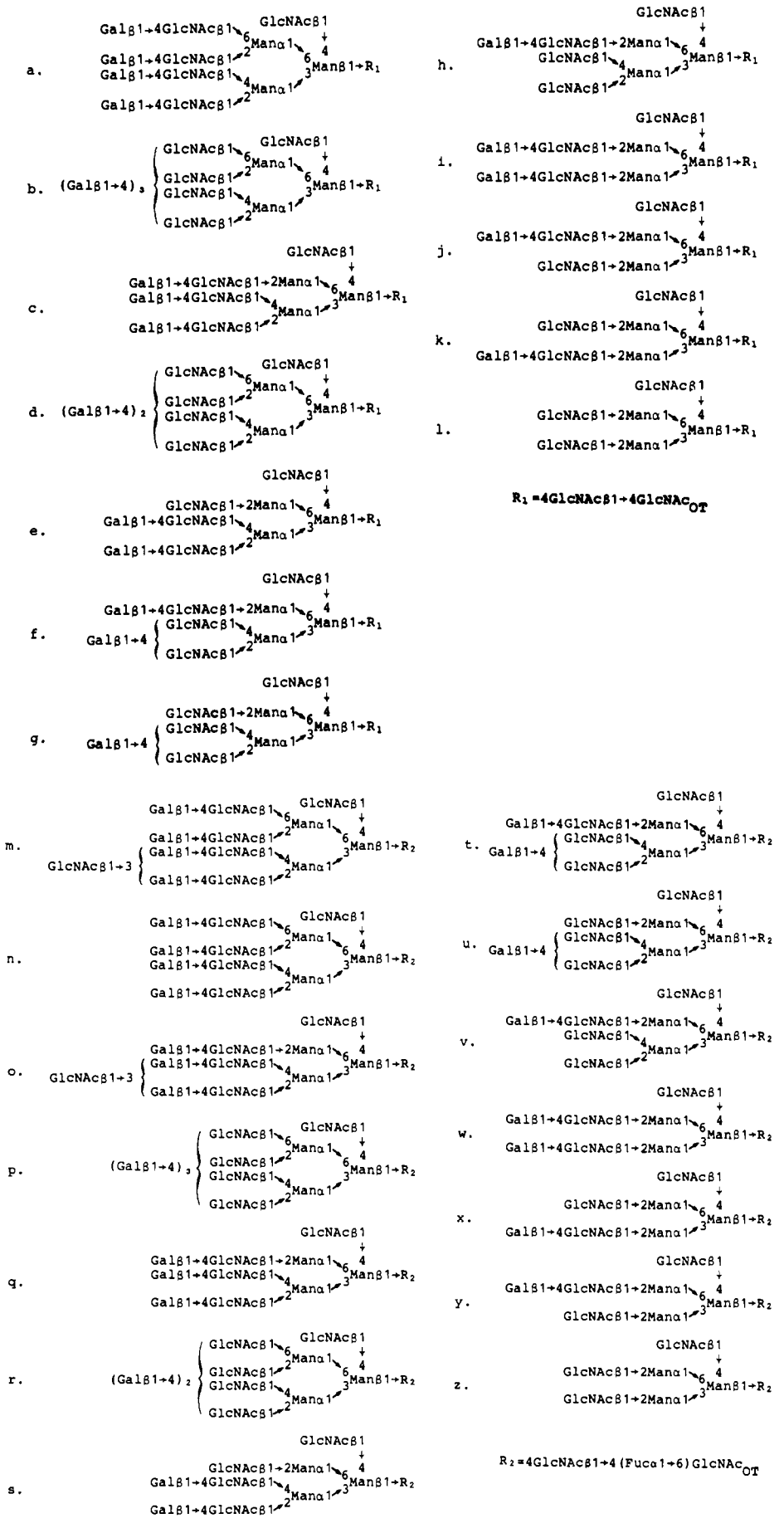


FIGURE 6: Structures of oligosaccharides a–z in Figures 3 and 4. Oligosaccharides galactosylated at the 2,4:2 arm were not included in b and p, and oligosaccharides galactosylated at the 4:2 arm and 2:2 arm were not included in d and r.

structure 2 or 3. These results indicated that oligosaccharides d and r should be mixtures of the four oligosaccharides in Figure 6.

Oligosaccharides e and f are digalactosyl derivatives of component II, and oligosaccharides s and t are digalactosylated derivatives of component VII. Therefore, three isomeric structures could be considered for both groups of oligosaccharides, galactosylated (1) at the 4:2 arm, (2) at the 2:2 arm, and (3) at the 2,4: arm. Because e and s did not interact with an E-PHA–agarose column (Table III), oligosaccharides with structure 3 should be included in them. In contrast, f and t should contain oligosaccharides with structures 1 and/or 2 because they were retarded in an E-PHA–agarose column (Table III). Upon DSA–Sepharose column chromatography, radioactive oligosaccharides e and s gave a single retarded fraction, while f and t completely passed through the column without interaction (Table IV). These results also supported the assumption described above. Accordingly, these four oligosaccharides should have the structure shown in Figure 6.

Oligosaccharides g and h are monogalactosyl derivatives of component II, and oligosaccharides u and v are monogalactosyl derivatives of component VII. Therefore, three isomeric structures could be considered, galactosylated (1) at the GlcNAcβ1→2Manα1→3 group, (2) at the GlcNAcβ1→4Manα1→3 group, and (3) at the GlcNAcβ1→2Manα1→6 group. Because oligosaccharides h and v were retarded in an E-PHA–Sepharose column (Table III), they should have structure 3. In contrast, oligosaccharides g and u passed through the column, indicating that oligosaccharides with structures 1 and/or 2 are included. These results indicated that the four oligosaccharides have the structure shown in Figure 6.

Oligosaccharides j and k are monogalactosyl derivatives of component III, and oligosaccharides x and y are monogalactosyl derivatives of component VIII. Therefore, two isomeric structures could be considered, galactosylated (1) at the Manα1→3 arm and (2) at the Manα1→6 arm. Because oligosaccharides j and y were retarded in an E-PHA–agarose column (Table III), they should have structure 2. In contrast, oligosaccharides k and x should have structure 1 since they passed through an E-PHA–agarose column without interaction (Table III). Therefore, oligosaccharides j, k, x, and y should have the structures as shown in Figure 6.

The data in Table IV indicated that oligosaccharides m and o are the trigalactosyl derivative of component IV and the digalactosyl derivative of component V, respectively. It was assumed that the GlcNAcβ1→3Galβ1→4GlcNAc outer chain is not galactosylated further because radioactive oligosaccharide o completely passed through a DSA–Sepharose column without any interaction (Table IV). As reported previously (Yamashita et al., 1987), complex-type oligosaccharides with the Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→ outer chain bind strongly to the column. The GlcNAcβ1→2Manα1→6 group of component V in oligosaccharide o should always be galactosylated because oligosaccharide o was retarded in an E-PHA–agarose column (Table III). Therefore, oligosaccharide o should have the structure as shown in Figure 6. Whether the GlcNAcβ1→3Galβ1→4GlcNAcβ1→ outer chain is linked only at the C-2 or C-4 position or randomly distributed to the two positions on the Manα1→3 residue remains to be confirmed. On the basis of the structural rule found in oligosaccharide o, the structure of oligosaccharide m was deduced to be as shown in Figure 6.

FIGURE 7: Proposed structures of sugar chains included in rat kidney aminopeptidase N and dipeptidylpeptidase IV. Possibly, the distribution of the GlcNAcβ1→3Galβ1→4GlcNAc outer chain is limited on the Manα1→3 arm of the tri- and tetraantennary oligosaccharides.

Structures of Acidic Oligosaccharides. As described already, the A1, A2, and A3 fractions obtained from aminopeptidase N and dipeptidylpeptidase IV contain mono-, di-, and trisialyl oligosaccharides, respectively. Comparison of the methylation data of the Con A(−) fractions before and after sialidase treatment revealed that significant changes were detected only in the galactose residues (Table II). 2,3,4-Tri-*O*-methylgalactitol, which was detected in the original oligosaccharide samples from the two enzymes, vanished completely after desialylation. 2,4,6-Tri-*O*-methylgalactitol in the original sample from dipeptidylpeptidase IV also completely vanished after desialylation. In contrast, a small but consistent amount of 2,4,6-tri-*O*-methylgalactitol in the original sample from aminopeptidase N remained after desialylation. This tri-*O*-methylgalactitol is considered to be derived from oligosaccharides m and o (Figure 6), which were absent in dipeptidylpeptidase IV. These results indicated that the sialic acid residues in the acidic oligosaccharides of the two enzymes are linked at the C-3 and C-6 positions of galactose residues. From the results so far described, the whole structures of oligosaccharides released from the two enzymes by hydrazinolysis were proposed as summarized in Figure 7.

DISCUSSION

The data reported in this paper indicated that the structures of sugar chains of rat kidney aminopeptidase N and of dipeptidylpeptidase IV are very similar to those of rat kidney γ-glutamyltranspeptidase reported previously (Yamashita et al., 1983a). The characteristic features common to all three enzymes are that many of the outer-chain moieties of their complex-type sugar chains are incompletely galactosylated. This rather unusual phenomenon might be caused by the addition of a bisecting *N*-acetylglucosamine residue. As reported by Schachter et al. (1983), addition of this residue during the processing of complex-type sugar chains will prohibit the action of many glycosyltransferases, including β-galactosyltransferase. Therefore, enhanced expression of *N*-acetylglucosaminyltransferase III, responsible for the addition of a bisecting *N*-acetylglucosamine residue to sugar chains, in the epithelial cells of kidney may inhibit the completion of the galactosylation of outer-chain moieties. As in the case of γ-glutamyltranspeptidase, the triantennary sugar chains of aminopeptidase N and of dipeptidylpeptidase IV are all 2,4:2 branching and no triantennary sugar chains with 2:2,6 branching are included. Therefore, the *N*-acetylglucosaminyltransferase IV activity of these cells, which makes the GlcNAcβ1→4Manα1→3 group, might be stronger than *N*-

acetylglucosaminyltransferase V, which is responsible for the formation of the GlcNAcβ1→6Manα1→6 group. It is of interest that the GlcNAcβ1→3Galβ1→4GlcNAcβ1→ outer chain, which was found in the complex-type sugar chains of rat kidney γ-glutamyltranspeptidase, was not detected in the sugar chains of dipeptidylpeptidase IV, although it was detected in a small portion of the complex-type sugar chains of aminopeptidase N.

The evidence that the three apical enzymes of epithelial cells have a similar set of sugar chains favors a hypothesis that the carbohydrate moieties of these glycoproteins are playing a role in transporting themselves to the same segment of the plasma membrane. However, structural study of the sugar chains of glycoproteins in the basolateral side membrane is essential for experimental proof of the hypothesis. Because aminopeptidase N and dipeptidylpeptidase IV were purified by rather simple steps with high yields (Matsuda et al., 1983), the data reported in this paper should cover almost all features of their sugar-chain structures. It was found that rat kidney γ-glutamyltranspeptidase solubilized with proteinase contained 92% of the total hexose of γ-glutamyltranspeptidase solubilized with detergent (Yamashita et al., 1983a). Although no Asn-X-Ser or Thr sequence was included in the anchor peptide fragment, a possible presence of an O-linked sugar chain in this peptide cannot be denied. because neither primary amino acid sequences nor the papain cleavage points of aminopeptidase N and dipeptidylpeptidase IV are known, it is hard to discuss the sugars remaining in their anchor peptides. However, this possibility must be kept in mind in discussing the trafficking function of the sugar chains of the two enzymes.

The data reported here indicated that the use of a series of immobilized lectin columns is very effective for the structural study of the complex-type sugar chains, especially for those with extreme heterogeneity. Without this technique, structural studies of the sugar chains of the kidney enzymes described in this paper could not be performed. In combination with glycosidases with narrow aglycon specificities, these lectin columns will facilitate the structural elucidation of the sugar chains present in limited amounts.

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