EXHIBIT 40

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Carbohydrate Structure of Erythropoietin Expressed in Chinese Hamster Ovary Cells by a Human Erythropoietin cDNA*

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The proper glycosylation of erythropoietin is essential for its function *in vivo*. Human erythropoietins were isolated from Chinese hamster ovary cells transfected with a human erythropoietin cDNA and from human urine. Carbohydrate chains attached to these proteins were isolated and fractionated by anion-exchange high performance liquid chromatography (HPLC) and HPLC employing a Lichrosorb-NH_2 column. The structures of fractionated saccharides were analyzed by fast atom bombardment-mass spectrometry and methylation analysis before and after treatment with specific exoglycosidases.

Both erythropoietins were found to contain one *O*-linked oligosaccharide/mol of the proteins, and its major component was elucidated to be NeuNAcα2→3Galβ1→3(NeuNAcα2→6)GalNAcOH (where NeuNAc represents *N*-acetylneuraminic acid) in both proteins. The *N*-linked saccharides of recombinant erythropoietin were found to consist of biantennary (1.4% of the total saccharides), triantennary (10%), triantennary with one *N*-acetyllactosaminyl repeat (3.5%), tetraantennary (31.8%), and tetraantennary with one (32.1%), two (16.5%), or three (4.7%) *N*-acetyllactosaminyl repeats. All of these saccharides are sialylated by 2→3-linkages. Tetraantennary with or without polylactosaminyl units are mainly present as disialosyl or trisialosyl forms, and these structures exhibit the following unique features. α2→3-Linked sialic acid and *N*-acetyllactosaminyl repeats are selectively present in the side chains attached to C-6 and C-2 of 2,6-substituted α-mannose and C-4 of 2,4-substituted α-mannose.

We have also shown that the carbohydrate moiety of urinary erythropoietin is indistinguishable from recombinant erythropoietin except for a slight difference in sialylation, providing the evidence that recombinant erythropoietin is valuable for biological as well as clinical use.

Erythropoietin is a glycoprotein which stimulates proliferation and differentiation of erythroid precursor cells to more mature erythrocytes (1). Erythropoietin is primarily produced in adult kidney and fetal liver cells (2–4). Patients with chronic renal failure are anemic as a result of impaired renal function which leads to a decreased production of erythropoietin (5).

Thus, availability of purified erythropoietin in quantity is essential to understand molecular mechanisms of erythropoiesis and for treatment of anemia. However, this has been hampered by the fact that only a very small amount of erythropoietin is present in starting sources, even in such cases as the urine of aplastic anemia patients (6).

In order to overcome this problem, cDNA clones for human erythropoietin have been isolated in several laboratories, and the expression of erythropoietin cDNA clones has been achieved (7–9). Furthermore, the recombinant erythropoietin has been successfully used to reverse the anemia of patients with endo-stage renal disease (10, 11). Interestingly, the erythropoietin produced in *Escherichia coli* or yeast was inactive or very weakly active *in vivo*. On the other hand, the erythropoietin produced in COS cells or Chinese hamster ovary cells was found to be fully active *in vivo*. In agreement with these results, it has been reported that desialylation of partially purified erythropoietin results in inactivation of erythropoietin activity (12–15). Thus, it is apparent that the proper glycosylation is essential for erythropoietin activity *in vivo*.

These results prompted us to analyze carbohydrate structures of erythropoietin produced by transfection of recombinant DNA into Chinese hamster ovary cells. In addition, we compared those structures with carbohydrate units present in erythropoietin purified from human urine.

EXPERIMENTAL PROCEDURES

Erythropoietin—Chinese hamster ovary cells (dihydrofolate reductase$^-$) were transfected with an expression vector which harbors the human erythropoietin cDNA as described (7). This expression vector also contains dihydrofolate reductase minigene so that stable transfects can be grown in the presence of methotrexate (16). Erythropoietin was purified from the spent medium of those cells as described (7). The purification procedure was slightly modified from that of Miyake *et al.* (6), and fractionation on a Vydac C_4 reverse-phase HPLC[1] column (The Separations Group) was included (7). This erythropoietin will be called recombinant erythropoietin hereafter. Erythropoietin was also purified from the urine of aplastic anemia patients according to Miyake *et al.* (6), with a similar modification as for purification of recombinant erythropoietin. The erythropoietin purified from urine will be called urinary erythropoietin hereafter. These erythropoietin samples were provided by Chugai Pharmaceutical Co., Ltd. (Tokyo).

Isolation of N-Linked Glycopeptides and O-Linked Oligosaccharides from Erythropoietins—Glycopeptides were prepared by Pronase digestion of erythropoietin (5 mg of recombinant erythropoietin and

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[1] The abbreviations used are: HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry, Hex, hexose; HexNAc, *N*-acetylhexosamine; NeuNAc, *N*-acetylneuraminic acid; Lac, *N*-acetyllactosaminyl repeat.

1 mg of urinary erythropoietin, respectively) as described (17). The Pronase digest was applied to a small column (1.0 × 45 cm) of Sephadex G-15, which was equilibrated and eluted with water. The gylcopeptides, eluted at and near the void volume, where lyophilized and subjected to alkaline borohydride degradation, as described (17). Briefly, the glycopeptides were dissolved in 500 μl of 0.05 M NaOH, 1 M $NaBH_4$ containing 5 mCi of NaB^3H_4 (9 Ci/mmol) and incubated at 45 °C for 24 h. After treatment, 1–2 ml of methanol which contained 1 drop of acetic acid was added to the sample and evaporated under nitrogen. The alkaline borohydride-treated sample was then applied to the same Sephadex G-15 column for desalting. The glycopeptides and oligosaccharides, which eluted between the void volume and the salt peak, were pooled and applied to a column (1.0 × 140 cm) of Bio-Gel P-4 (200–400 mesh). The column was eluted with 0.1 M NH_4HCO_3 at a flow rate of 6 ml/h, and each fraction contained 1 ml. Under these chromatographic conditions, *N*-linked glycopeptides eluted near the void volume, whereas *O*-linked oligosaccharides eluted in later fractions (see Fig. 2).

Isolation of N-Linked Saccharides from Glycopeptides—The glycopeptide fraction containing *N*-linked saccharides was digested with *Flavobacterium meningosepticum N*-glycanase (peptide-N^4-(*N*-acetyl-β-glucosaminyl)aspargine amidase) (18) which was purchased from Genzyme (Boston, MA). The glycopeptides from 5 mg of erythropoietin were dissolved in 500 μl of 0.1 M sodium phosphate buffer, pH 8.6, containing 20 mM EDTA and 20 mM 2-mercaptoethanol and incubated with 30 m units of the *N*-glycanase, which corresponds to 30 units of the enzyme expressed by Genzyme, at 37 °C for 20 h. When the glycopeptides from 1 mg of urinary erythropoietin were digested with *N*-glycanase, the incubation mixture was scaled down to one-fifth.

The digest was desalted by Sephadex G-15 gel filtration, and the saccharides were reduced at room temperature for 30 min with 5 mCi of NaB^3H_4 dissolved in 200 μl of 0.01 M NaOH followed by 3 mg of $NaBH_4$ for 2 h. After reduction, the sample was neutralized with the addition of methanol containing a small amount of acetic acid and evaporated under nitrogen. The dried sample was dissolved in water, and the supernatant obtained after centrifugation was applied to the Sephadex G-15 column for desalting. The saccharides, radioactively labeled at reducing terminals, were applied to a column (1.0 × 45 cm) of Sephadex G-50 (superfine) eluted with 0.2 M NaCl (see Fig. 2*B*). Each fraction contained 0.5 ml.

Fractionation of Oligosaccharides by Anion-exchange High Performance Liquid Chromatography—Oligosaccharide fractions obtained after Sephadex G-50 gel filtration were subjected to anion-exchange HPLC with a Varian HPLC apparatus (Model 5000, Varian Associates, Inc.). The sample was applied to a Toyo Soda TSK-DEAE column purchased from Kratos Analytical Instruments (Ramsey, NJ). The column (4.6 mm × 24 cm) was equilibrated with 25 mM potassium phosphate buffer, pH 5.0, and after eluting with the same buffer for 10 min, the elution was programed by the linear gradient to 0.4 M potassium phosphate buffer, pH 5.0, over 80 min. The flow rate was constant at 1 ml/min, and each fraction contained 0.3 ml. The elution was monitored by measuring the absorbance at 206 nm with a Beckman 163 detector. Since potassium phosphate has absorbance at 202 nm, the absorbance at 206 nm was measured under these conditions. The base line of the absorbance at 202 nm was increased under these conditions. In order to estimate the elution positions of monosialosyl, disialosyl, trisialosyl, and tetrasialosyl saccharides, IgG (bovine, Sigma), fetuin (19, 20), and α_1-acid glycoprotein saccharides (21, 22) were prepared by hydrazinolysis and subjected to HPLC under the same conditions. The saccharides, separated by TSK-DEAE, were subjected to methylation analysis to confirm their structures. Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAcOH was obtained by extensive digestion of IgG saccharide with β-glactosidase and β-*N*-acetylglucosaminidase.

Fractionation of Neutral Oligosaccharides by HPLC—The saccharides were desialylated by mild acid hydrolysis in 0.01 N HCl at 80 °C for 1 h. The desialylated sample was desalted by Sephadex G-15 gel filtration and evaporated. The neutral saccharides were then fractionated by HPLC with the same apparatus as described above. The saccharides were dissolved in acetonitrile, 10 mM potassium phosphate buffer, pH 4.5 (65:35), and applied to a column (0.4 × 25 cm) of Lichrosorb-NH_2 (5-μm particle size, Merck). The column was eluted with a linear gradient to acetonitrile, 10 mM potassium phosphate buffer, pH 4.5 (39:61), over 60 min. In order to achieve this elution, the first solvent was acetonitrile, 10 mM potassium phosphate buffer, pH 4.5 (65:35, v/v), and the second solvent was 10 mM potassium phosphate buffer, pH 4.5. The ratio of these two solvents was 100:0 at the beginning of the elution and 60:40 at the end of elution. This elution system was used because it was preferable to premix acetonitrile and the buffer before the elution started, otherwise, mixing of two solutions causes cooling down of the solvent. The flow rate was 1 ml/min, and each fraction contained 0.5 ml.

Structural Analysis of Saccharides—Structures of saccharides were analyzed essentially as described previously for other saccharides. These methods include fast atom bombardment-mass spectrometry of permethylated saccharides (17), analysis of partially *O*-methylated monosaccharides after methylation and hydrolysis ("methylation analysis") (17, 23), and exoglycosidase digestion combined with methylation analysis (24). Methylation of saccharides and purification of methylated saccharides for FAB-MS and methylation analysis were carried out as described previously (17, 23). Before methylation of saccharides, all samples were desalted by Sephadex G-15 gel filtration eluted with water.

Glycosidase digestion of saccharides was carried out as follows. For sequential digestion by β-galactosidase and β-*N*-acetylglucosaminidase, the saccharides were incubated with 5 milliunits of *Charonia lampas* β-galactosidase in 40 μl of 0.1 M sodium citrate buffer, pH 4.3, at 37 °C for 24 h. After incubation, the incubation mixture was heated in a boiling water bath for 2 min. The sample was then incubated with 100 milliunits of beef kidney β-*N*-acetylglucosaminidase in 140 μl of 0.1 M sodium citrate buffer, pH 4.3. In order to inhibit a possible contaminating activity of β-galactosidase, 100 mM (final concentration) galactose was added to this incubation mixture. After further incubation at 37 °C for 24 h, the mixture was heated in a boiling water bath for 2 min. The digests were then purified by Sephadex G-50 gel filtration followed by HPLC employing a Lichrosorb-NH_2 column as described above. For extensive digestion by a mixture of β-galactosidase and β-*N*-acetylglucosaminidase, the saccharides were first incubated with *C. lampas* β-galactosidase and then with beef kidney β-*N*-acetylglucosaminidase without heat inactivation of β-galactosidase or the addition of galactose. After total incubation at 37 °C for 48 h, enzymes were inactivated by heating in a boiling water bath for 2 min, and digested saccharides were purified by anion-exchange HPLC. β-Galactosidase from *C. lampas* and β-*N*-acetylglucosaminidase from beef kidney were purchased from Sigma and Boehringer Mannheim, respectively.

Determination of Carbohydrate Composition— Sialic acid content was determined by the periodate-resorcinol reaction (25). Neutral sugars and hexosamines were determined after methanolysis in 0.5 N hydrochloric acid in anhydrous methanol at 80 °C for 4 h. Inositol was added as an internal standard. After methanolysis, the products were dried under a nitrogen stream and further *in vacuo* under P_2O_5 and NaOH. The dried products were trimethylsilylated with Tri-Sil (Pierce Chemical Co.). The trimethylsilylated derivatives were then analyzed by gas-liquid chromatograph-mass spectrometry as described.[2] In parallel, fetuin was analyzed in order to obtain response factors.

RESULTS

Isolation of N-Linked Saccharides from Recombinant Erythropoietin—Erythropoietin was purified from the spent medium of Chinese hamster ovary cells transfected with a human erythropoietin gene and from human urine of aplastic anemic patients as described under "Experimental Procedures." The purified proteins showed a major band with M_r 38,000 and a faint band with M_r ~80,000 (Fig. 1). The latter band is probably the dimer of erythropoietin. The carbohydrate composition of this molecule is shown in Table I. Glycopeptides were prepared from 5 mg of recombinant erythropoietin by Pronase digestion and isolated by Sephadex G-15 gel filtration. Glycopeptides (1.8 mg) were then treated with alkaline borohydride to release *O*-linked oligosaccharides, and the alkaline borohydride-treated samples were applied to a column of Bio-Gel P-4. As shown in Fig. 2*A*, three peaks were detected in addition to a salt peak. The second (fractions 30–35) and the third (fractions 36–40) peaks were found to

[2] M. Fukuda, M. Lauffenburger, H. Sasaki, E. M. E. Rogers, and A. Dell (1987) *J. Biol. Chem.*, in press.

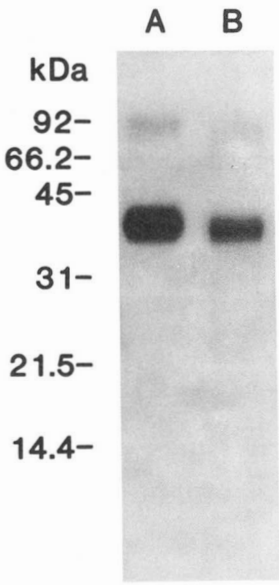


FIG. 1. **Autoradiogram of sodium dodecyl sulfate gel electrophoresis of recombinant erythropoietin (*lane A*) and urinary erythropoietin (*lane B*).** Purified erythropoietins were iodinated with ^{125}I according to Greenwood *et al.* (26). The radioactively labeled proteins were then applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (gel concentration, 15%) according to Laemmli (27), and the gel was directly autoradiographed with Kodak x-ray AR-5 film

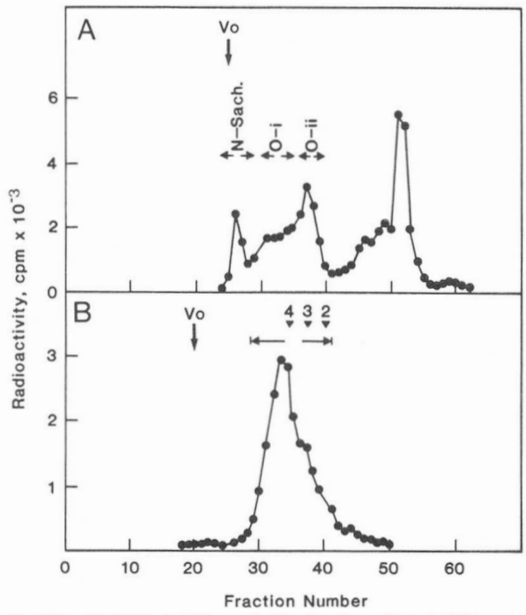


FIG. 2. **Bio-Gel P-4 (*A*) and Sephadex G-50 (*B*) gel filtrations of glycopeptides obtained from recombinant erythropoietin.** Glycopeptides were subjected to alkaline borohydride treatment in the presence of NaB^3H_4 and applied to a column of Bio-Gel P-4 (*A*). The *O*-linked oligosaccharides were eluted in fractions 30–35 (*O-i*) and fractions 36–40 (*O-ii*). The glycopeptides, which contained *N*-linked saccharides (*N-Sach.*), eluted in fractions 24–29 and were digested with *N*-glycanase. The digest was reduced with NaB^3H_4 and subjected to Sephadex G-50 gel filtration (*B*). The fractions, indicated by the *horizontal arrows*, were pooled and subjected to further analysis. The *arrowheads* indicate the elution positions of the trisialosyl saccharide from α_1-acid glycoprotein (*arrowhead 4*), the trisialosyl saccharide from fetuin (*arrowhead 3*), and the asialo form of IgG saccharide (*arrowhead 2*).

TABLE I

Carbohydrate composition of erythropoietin

Numbers are expressed as moles/mole of erythropoietin.

	Urinary erythropoietin	Recombinant erythropoietin			
		Batch 1	Batch 2	Batch 3	Batch 4
Fuc	2.9	4.1	2.9	2.3	3.6
Man	9.2	8.7	9.4	8.2	9.1
Gal	12.9	13.8	14.1	15.5	13.0
GlcNAc	16.3	17.2	18.9	17.3	19.8
GalNAc	0.9	0.9	1.4	0.8	1.4
NeuNAc	10.4	9.5	9.7	11.8	10.8

contain *O*-linked oligosaccharides (see Miniprint),[3] whereas glycopeptides containing *N*-linked saccharides eluted at fractions 25–29.

Fractions 25–29 were pooled and digested with *N*-glycanase. The digest, after reduction with NaB^3H_4, was subjected to Sephadex, G-50 gel filtration as shown in Fig. 2*B*. The saccharides eluting between fractions 29 and 41 were subjected to further analysis.

Methylation Analysis and FAB-MS of N-Linked Saccharides from Recombinant Erythropoietin—In order to determine the carbohydrate structures of *N*-linked saccharides, the saccharides were subjected to FAB-MS and methylation analyses. As shown in Fig. 3*A*, FAB-MS of permethylated *N*-linked saccharides provided fragment ions at *m/z* 376 (and 344), 825, 1274, and 1723, which correspond to $NeuNAc^+$, $NeuNAc\rightarrow Hex\rightarrow HexNAc^+$, $NeuNAc\rightarrow Hex\rightarrow HexNAc\rightarrow Hex\rightarrow HexNAc^+$ and $NeuNAc\rightarrow Hex\rightarrow HexNAc\rightarrow Hex\rightarrow HexNAc\rightarrow Hex\rightarrow HexNAc^+$. These results indicate that *N*-linked saccharides contain *N*-acetyllactosaminyl repeats in the side chains. This conclusion was supported by the detection of a fragment ion at *m/z* 1362 for $Hex_3\cdot HexNAc_3^+$ when desialylated and methylated saccharides were subjected to FAB-MS (Fig. 4*A*). Methylation analysis of *N*-linked saccharides indicates the following features. 1) Galactose is terminal or 3-substituted; 2) all of the *N*-acetylglucosamine residues except the reducing terminal residue are substituted at C-4; 3) 2-substituted mannose (0.16 mol), 2,4-substituted mannose (0.78 mol), 2,6-substituted mannose (1.05 mol), and 3,6-substituted mannose (1.0 mol) were detected (Table II).

After desialylation, a majority of 3-substituted galactose residues were converted to terminal galactose, indicating that sialic acid is linked to galactose through an $\alpha 2\rightarrow 3$-linkage. However, 0.82 mol of 3-linked galactose, which corresponds to 17% of the total galactose derivatives, was still detected after desialylation. This amount of galactose is presumably derived from *N*-acetyllactosaminyl repeats. The same analysis also showed that 85% of reducing terminal *N*-acetylglucosamine is substituted with fucose at C-6, whereas the rest of the reducing terminal *N*-acetylglucosamine contains no fucose.

These results suggest that the *N*-linked saccharides of recombinant erythropoietin are mainly composed of tetraantennary saccharides with or without *N*-actyllactosaminyl repeats.

Isolation of Asialo N-Linked Saccharides—*N*-Linked sac-

[3] Portions of this paper (including part of "Results," Figs. 4, 6, and 8–10, and Table II and IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 87M-1260, cite the authors, and include a check or money order for $6.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

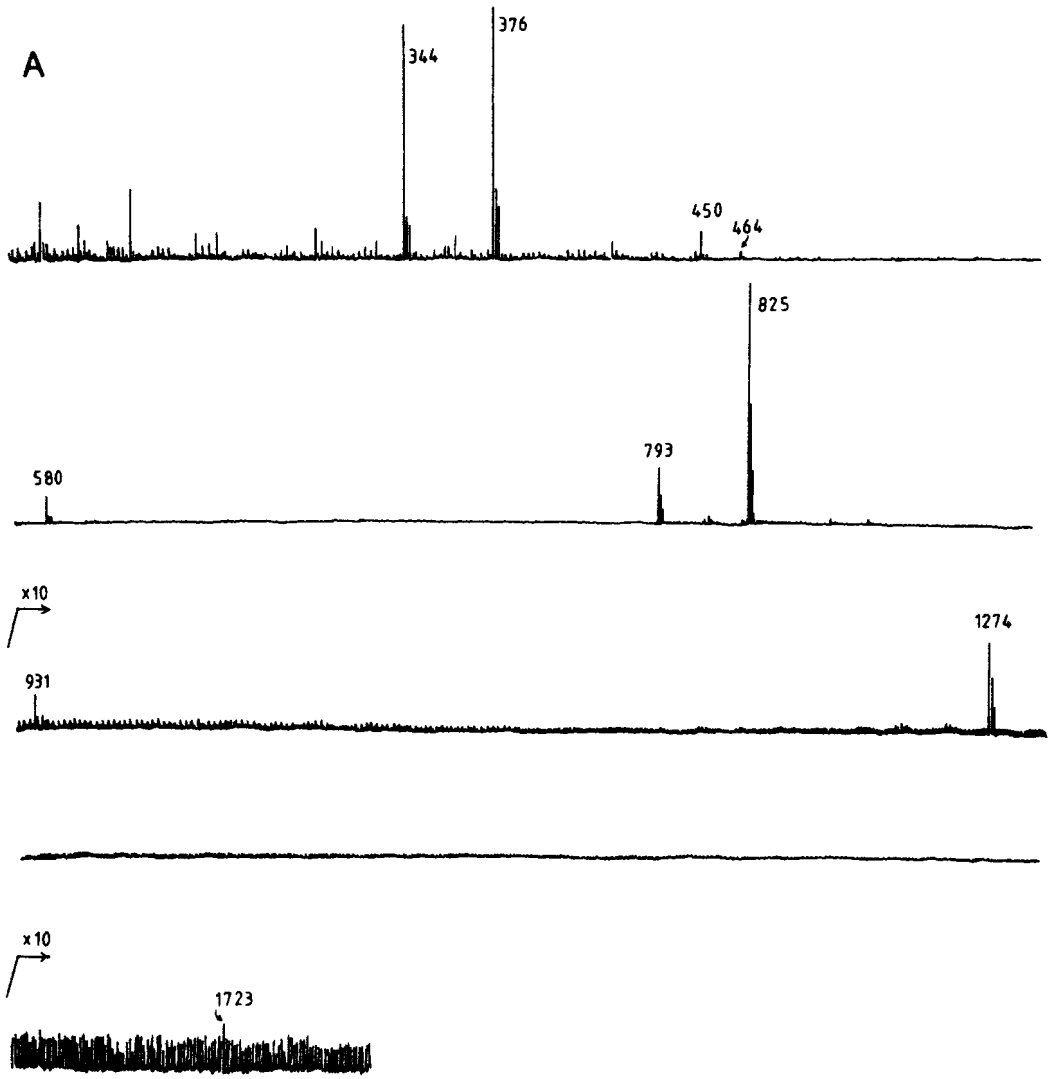


FIG. 3. **Fast atom bombardment-mass spectra of permethylated total *N*-linked saccharides from recombinant erythropoietin (*A*) and urinary erythropoietin (*B*).** The positive spectra were recorded. *A*, fragment ions were detected at *m/z* 376 (and 344) for NeuNAc$^+$, 580 for NeuNAc→Hex$^+$, 825 (and 793) for NeuNAc→Hex→HexNAc$^+$, 1274 for NeuNAc→Hex→HexNAc→Hex→HexNAc$^+$, and 1723 for NeuNAc→Hex→HexNAc→Hex→HexNAc→Hex→HexNAc$^+$. A small peak was detected also at *m/z* 464 for Hex→HexNAc$^+$. *B*, in addition to the ions described above, a minor fragment ion was detected at *m/z* 913 for Hex→HexNAc→Hex→HexNAc$^+$.

charides, obtained from 5 mg of recombinant erythropoietin (Batch 1 in Table I), were desialylated by mild acid hydrolysis and then desalted by Sephadex G-15 gel filtration. Neutral saccharides thus obtained were fractionated by HPLC employing a Lichrosorb-NH_2 column. As shown in Fig. 5*A*, *N*-linked saccharides from recombinant erythropoietin provided six peaks which correspond to biantennary (peak 2), triantennary (peak 3), tetraantennary (peak 4), tetraantennary with one *N*-actyllactosaminyl repeat (peak 5, Lac_1), tetraantennary with two *N*-acetyllactosaminyl repeats (peak 6, Lac_2), and tetraantennary with three *N*-acetyllactosaminyl repeats (peak 7, Lac_3). The identical elution profile was obtained when *N*-linked saccharides were desialylated by clostridial neuraminidase. No significant amount of carbohydrate was detected in other fractions. This result indicates that most of the glycopeptides were digested by *N*-glycanase since the glycopeptides would elute later than saccharides without amino acid residues in a Lichrosorb-NH_2 column.

Structures of Biantennary Saccharides and Triantennary Saccharides—The elution position of the biantennary saccharides (peak 2) was identical to the IgG saccharide which is mainly composed of $Gal_2 \cdot GlcNAc_2 \cdot Man_3 \cdot GlcNAc_2 \cdot Fuc$. In addition, this saccharide bound to a concanavalin A-Sepharose and was eluted by 20 mM methyl-α-glucoside. Those results establish that this fraction (peak 2) is a typical complex saccharide with biantennary side chains.

Methylation analysis (Table II) of the triantennary saccharides (peak 3) provided 0.65 mol of 2,6-substituted mannose (3,4-di-*O*-methylmannose) and 0.35 mol of 2,4-substituted mannose (3,6-di-*O*-methylmannose) in addition to 1 mol each of 2- and 3,6-substituted mannose (Table II). The saccharides were also digested by a mixture of β-galactosidase and β-*N*-acetylglucosaminidase to yield Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAcOH, as judged by HPLC with a Lichrosorb-NH_2 column followed by methylation analysis. These results established the structures of the triantennary saccharides, as shown in Table III.

In order to elucidate which of the outer α-mannosyl residues is disubstituted at C-2 and C-4, the *N*-linked saccharides (fractions 29–41 in Fig. 2*B*) were subjected to periodate oxi-

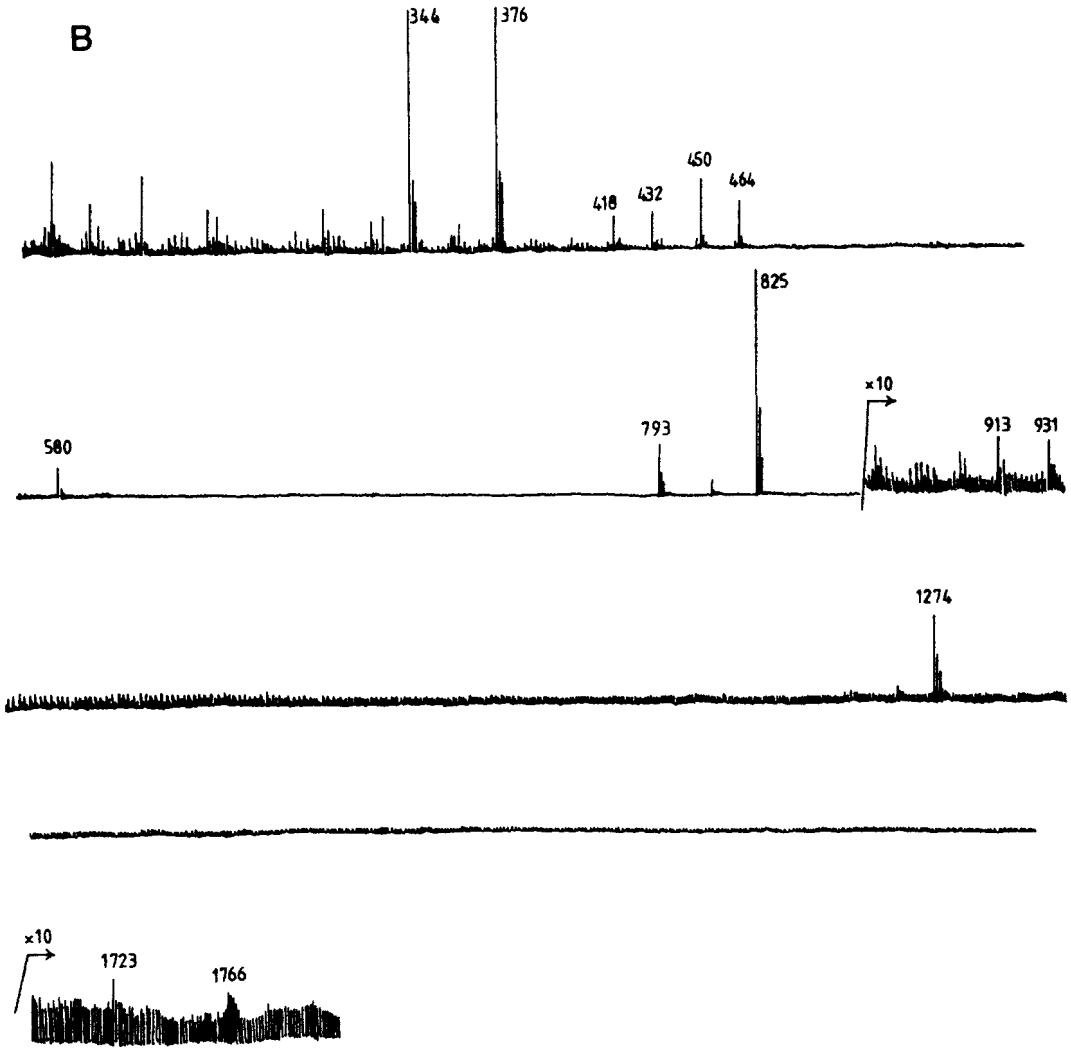


FIG. 3—*Continued*

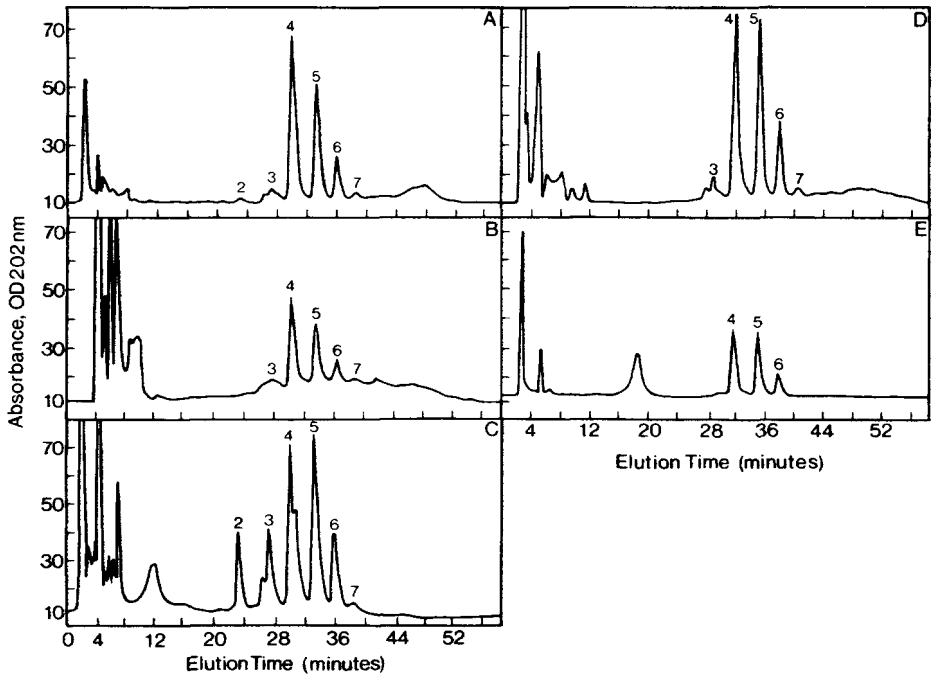


FIG. 5. **HPLC of asialo *N*-linked saccharides derived from erythropoietin.** *N*-Linked saccharides were desialylated by mild acid hydrolysis, applied to a 5-μm Lichrosorb-NH_2 column, as described under "Experimental Procedures." The effluent was monitored by measuring the absorbance at 202 nm. *A* and *B*, total *N*-linked saccharides from recombinant erythropoietin (*A*) and urinary erythropoietin (*B*); *C–E*, *N*-linked saccharides from recombinant erythropoietin separated by TSK-DEAE ion-exchange chromatography as shown in Fig. 7*A*. Monosialosyl (*C*), disialosyl (*D*), and trisialosyl (*E*) fractions were desialylated, applied to the Lichrosorb-NH_2 column, and eluted under the same conditions. *Ordinate*, relative intensity at A_{202} nm; *abscissa*, retention time.

TABLE III

Structures of asialo N-linked saccharides obtained from recombinant erythropoietin

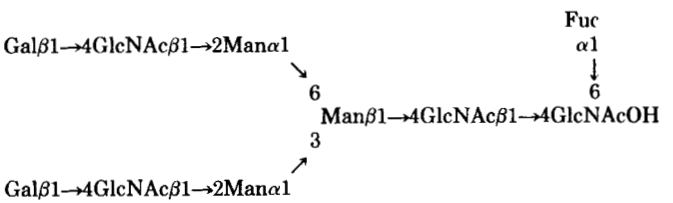
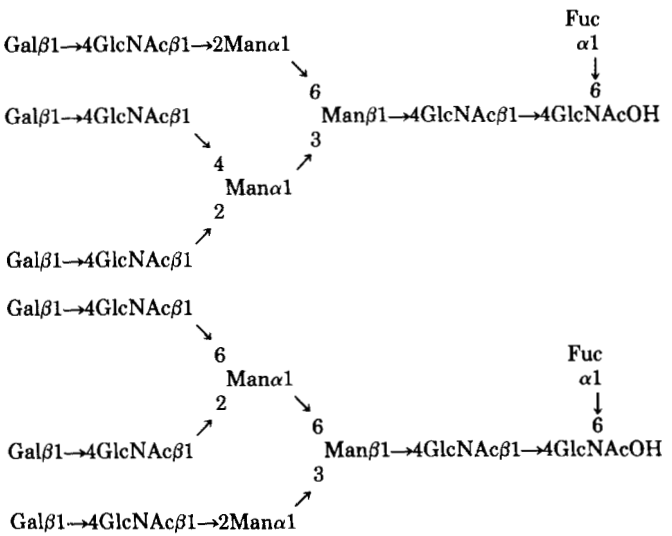
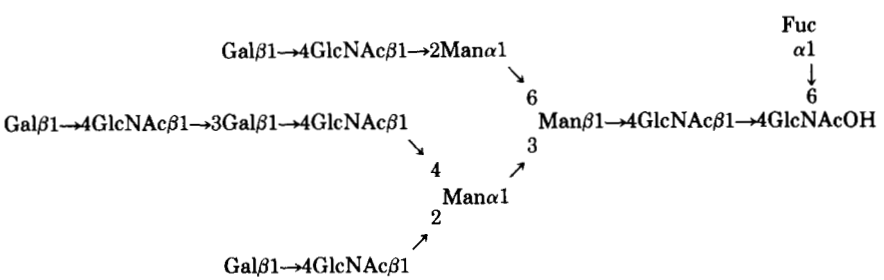
Saccharide	Structure[a]	Relative amount
		%
Biantennary		1.4
Triantennary		3.5
Triantennary Lac_1		6.5
		3.5

TABLE III-*Continued*

Saccharide	Structure[a]	Relative amount %
Tetraantennary Lac_1		3.2
Tetraantennary Lac_2		13.2
Tetraantennary Lac_2		3.3

[a] 15% of the saccharides lack fucose attached to the reducing terminal *N*-acetylglucosamine.

dation followed by reduction and mild acid hydrolysis (Smith degradation) as described (26). Methylation analysis of the product provided 2,4,6-tri-*O*-methylmannose with the concomitant loss of 3,4-di-*O*-methylmannose. These results indicate that the 2,4-disubstituted α-mannose is linked to C-3 of β-mannose, as shown in Table III.

Structure of Asialo Tetraantennary Saccharides and Triantennary Saccharides with One N-Acetyllactosaminyl Repeat—FAB-MS of the saccharides which eluted at peak 4 provided a molecular ion at *m/z* 3135 corresponding to $(Fuc_1 \cdot Hex_7 \cdot HexNAc_6)_R$ (Fig. 4*B*). This ion was associated with A-type ions at *m/z* 2668 for $Hex_7 \cdot HexNAc_5^+$ and at *m/z* 464 (and 432) for Hex→HexNAc$^+$. In addition, an ion at *m/z* 913 corresponding to Hex→HexNAc→Hex→HexNAc$^+$ was detected. The latter result suggests that this peak contains a triantennary saccharide with one *N*-acetyllactosaminyl repeat. The presence of a triantennary saccharide was confirmed by methylation analysis (Table II). A small amount (0.11 mol) of 2-substituted mannose (3,4,6-tri-*O*-methylmannose) was detected as well as 1 mol of 2,4-substituted and 0.9 mol of 2,6-substituted mannose. This result indicates that a triantennary saccharide with 2,4- and 2-substituted α-mannose is present in this saccharide fraction. This triantennary saccharide presumably contains one *N*-acetyllactosaminyl repeat. The presence of 3-substituted galactose supports the conclusion that this triantennary saccharide contains one *N*-acetyllactosaminyl repeat (Table II).

The saccharides were sequentially digested with β-galactosidase and β-*N*-acetylglucosaminidase and subjected to HPLC. As shown in Fig. 6*A*, the products provided two peaks: the first peak eluted at 12 min corresponds to $Man_3 \cdot GlcNAc \cdot (\pm Fuc)GlcNAcOH$, and the second peak at 15 min corresponds to $Gal \cdot GlcNAc \cdot Man_3 \cdot GlcNAc \cdot (\pm Fuc)GlcNAcOH$. The ratio of two peaks was found to be 1.0:0.19. These results indicate that about 15% of the saccharides are triantennary saccharides with one *N*-acetyllactosaminyl repeat, whereas 85% of the saccharides are tetraantennary saccharides. These two molecular species, however, will provide the same molecular ion at *m/z* 3135 on FAB-MS analysis.

In order to elucidate which side chains were elongated to form the *N*-acetyllactosaminyl repeat, the saccharides which eluted at 15 min in Fig. 6*A* (denoted as 1) were methylated. The saccharides provided 1 mol of 4-substituted mannose (2,3,6-tri-*O*-methylmannose), 3,6-substituted mannose (2,4-di-*O*-methylmannose), terminal mannose (2,3,4,6-tetra-*O*-methylmannose), terminal galactose (2,3,4,6-tetramethylgalactose), 2 mol of 4-substituted *N*-acetylglucosamine (3,6-di-*O*-methyl-*N*-methylacetylglucosamine), and 1 mol of 4,6-substituted *N*-acetylglucosamine (1,3,5-tri-*O*-methyl-*N*-methylacetylglucosaminitol). These results indicate that the *N*-acetyllactosaminyl repeat is linked to C-4 of α-mannose, as shown in Table III.

The structure of the saccharide "core" (see Fig. 6*A*) was confirmed by methylation analysis and FAB-MS to be Man1→6(Man1→3)Man1→4GlcNAc1→4(Fuc1→6)GlcNAc-OH (89%) and Man1→6(Man1→3)Man1→4GlcNAc1→4GlcNAcOH (11%). Combining these results with exoglycosidase digestion, the structure of the core was elucidated to be Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4($\pm$Fucα1→6)GlcNAcOH. All of the saccharides including tetraantennary with or without *N*-acetyllactosaminyl repeats were converted to this core saccharide when they were digested with a mixture of β-galactosidase and β-*N*-actylglucosaminidase.

Structure of Asialo Tetraantennary Saccharides with One N-Acetyllactosaminyl Repeat (Lac_1)—Methylation analysis of peak 5 in Fig. 5*A* provided 1 mol of 2,4-substituted mannose, 2,6-substituted mannose, and 3,6-substituted mannose (Table II), indicating that peak 5 is composed of tetraantennary saccharides. The presence of 3-substituted galactose (0.8 mol) in this asialo form indicates that the saccharides contain one *N*-acetyllactosaminyl repeat. FAB-MS of the saccharides after permethylation yielded a signal at *m/z* 913 for Hex→HexNAc→Hex→HexNAc$^+$ (Fig. 4*C*), which is indicative of one *N*-acetyllactosaminyl repeat.

The saccharides of peak 5 were sequentially digested by β-galactosidase and β-*N*-acetylglucosaminidase and subjected to HPLC. As shown in Fig. 6*B*, the major product eluted at 15 min, which corresponds to $Gal \cdot GlcNAc \cdot Man_3 \cdot GlcNAc \cdot (\pm Fuc)GlcNAcOH$. The methylation analysis of this product provided 0.1 mol of 2-substituted mannose, 0.1 mol of 4-substituted mannose, 0.8 mol of 6-substituted mannose, and 1 mol each of terminal mannose and 3,6-substituted mannose as mannose derivatives. These results indicate that 80% of the *N*-acetyllactosaminyl repeat is attached to C-6 of 2,6-substituted mannose, 10% is attached to C-4 of 2,4-substituted mannose, and 10% is attached to C-2 of either 2,6- or 2,4-substituted mannose (Table III).

Structure of Tetraantennary Saccharides with Two N-Acetyllactosaminyl Repeats (Lac_2)—Methylation analysis of peak 6 in Fig. 5*A* provided 1.7 mol of 3-substituted galactose, 4 mol of terminal galactose, and 1 mol each of 2,4-substituted, 2,6-substituted, and 3,6-substituted mannose, in addition to other derivatives (Table II). The results indicate that this saccharide fraction consists of tetraantennary saccharides with two *N*-acetyllactosaminyl repeats. FAB-MS of this oligosaccharide fraction supported the above conclusion since a fragment ion at *m/z* 913 corresponding to Hex→HexNAc→Hex→HexNAc$^+$ was detected (Fig. 4*D*).

The saccharides were digested sequentially by β-galactosidase and β-*N*-acetylglucosaminidase to yield a major peak at 19 min (peak 2 in Fig. 6*C*), which corresponds to $(Gal \cdot GlcNAc)_2 \cdot Man_3 \cdot GlcNAc \cdot (\pm Fuc)GlcNAcOH$, confirming that the starting sample contains tetraantennary saccharides with two *N*-acetyllactosaminyl repeats. Methylation analysis of this product provided 1 mol of terminal mannose, 0.8 mol of 2,6-substituted mannose, and 0.2 mol each of 6- and 4-substituted mannose, in addition to 1 mol each of 3,6-substituted mannose, terminal galactose, and reducing terminal *N*-acetylglucosamine and 2 mol of 4-substituted *N*-acetylglucosamine. However, 2,4-substituted mannose or 3-substituted galactose was not detected. These results indicate that each side chain arising from 2,6-substituted mannose was elongated by one *N*-acetyllactosaminyl repeat in 80% of the saccharides. In addition, 20% of the molecules have *N*-acetyllactosaminyl repeats in side chains elongating from C-6 and C-4 (Table III).

Structure of Tetraantennary Saccharides with Three N-Acetyllactosaminyl Repeats (Lac_3)—The saccharides in peak 7 eluted at the position where saccharides containing seven *N*-acetyllactosaminyl units are expected to elute. This is because the difference of the elution time between peaks 7 and 6 is the same as that between peaks 6 and 5. The saccharides were sequentially digested with β-galactosidase and β-*N*-acetylglucosaminidase, and the products were analyzed by HPLC. As shown in Fig. 6*D*, a major peak eluted at 24 min, which corresponds to $(Gal \cdot GlcNAc)_3 \cdot Man_3 \cdot GlcNAc(\pm Fuc)GlcNAcOH$. However, about 35% of the products eluted at the position corresponding to $(Gal \cdot GlcNAc)_2 \cdot Man_3 \cdot GlcNAc(\pm Fuc)GlcNAcOH$.

These results suggest that 65% of the peak 7 saccharides are tetraantennary saccharides with three *N*-acetyllactosaminyl repeats (Lac_3), whereas 35% of the saccharides are

tetraantennary saccharides with two *N*-acetyllactosaminyl repeats (Lac_2). These results were confirmed by methylation analysis, as shown in Table II. Methylation analysis of the saccharides provided 2.4 mol of 3-substituted galactose, 4 mol of terminal galactose, and 1 mol each of 2,4-, 2,6-, and 3,6-substituted mannose. The same analysis indicates that about 45% of the reducing terminal *N*-acetylglucosamine was unreduced judging from the amount of 3-*O*-methyl-*N*-acetylglucosamine. This compound is produced when the reducing terminal *N*-acetylglucosamine is incompletely reduced before methylation.

In order to elucidate which side chains were elongated to form the *N*-acetyllactosaminyl repeat, the saccharides which eluted at 24 min (peak 3 in Fig. 6*D*) were methylated. The saccharides provided 0.6 mol of 2,6-substituted mannose, 0.4 mol of 6-substituted mannose, 0.3 mol of 4-substituted mannose, 0.3 mol of terminal mannose, 0.4 mol of 2-substituted mannose, and 1 mol of 3,6-substituted mannose as mannose derivatives. These results indicate that each side chain arising from 2,6-substituted mannose and the side chains arising from C-2 or C-4 of 2,4-substituted mannose were elongated by *N*-acetyllactosaminyl units.

Since FAB-MS of the starting materials afforded a fragment ion at *m/z* 1723 for NeuNAc→Hex→HexNAc→Hex→HexNAc→Hex→HexNAc$^+$ (see Fig. 3*A*), it is likely that Lac_3 saccharides contain three *N*-acetyllactosaminyl units in one of the side chains which are attached to C-2 or C-6 of 2,6-substituted mannose or C-4 of 2,4-substituted mannose. This was confirmed by the fact that peak 3 in Fig. 6*D* provided a small amount (0.1 mol) of 3-substituted galactose on methylation analysis.

Structures of Asialo N-Linked Saccharides from Recombinant Erythropoietin—The results obtained above are summarized in Table III. By measuring radioactivity in each fraction, the relative yields of saccharides were calculated. In some cases, it was necessary to obtain the ratio after exoglycosidase digestion. For example, the ratio of triantennary with one *N*-acetyllactosaminyl repeat and tetraantennary in peak 4 was obtained in Fig. 6*A*.

Fractionation of Intact N-Linked Saccharides by TSK-DEAE Ion-exchange Chromatography—In order to determine how these saccharides are sialylated, intact *N*-linked saccharides were fractionated by HPLC employing a TSK-DEAE column. As shown in Fig. 7*A*, sialylated saccharides were essentially separated into four fractions: monosialosyl (fraction I) (7% of the total saccharide), disialosyl (fraction II) (41%), trisialosyl (fraction III) (48%), and tetrasialosyl (fraction IV) (4%) saccharides. No detectable amount of carbohydrate was present in other fractions.

After desialylation, monosialosyl saccharides (fraction I) were found to contain biantennary (12% of the total monosialosyl saccharides), triantennary (17%), tetraantennary (plus triantennary with one *N*-acetyllactosamine repeat) (28%), tetraantennary with one *N*-acetyllactosamine repeat (29%), tetraantennary with two *N*-acetyllactosamine repeats (11%), and tetraantennary with three *N*-acetyllactosamine repeats (3%) (Fig. 5*C*). Disialosyl saccharides (fraction II) were found to consist of triantennary (7% of the total disialosyl saccharides), tetraantennary (plus triantennary with one *N*-acetyllactosamine repeat) (43%), and tetraantennary saccharides with one (36%), two (12%), or three (2%) *N*-acetyllactosamine repeats (Fig. 5*D*). Trisialosyl saccharides (fraction III), however, contain only tetraantennary (47% of the total tetrasialylated saccharides) and tetraantennary saccharides with one (39%), two (12%), or three (2%) *N*-acetyllactosaminyl repeats (Fig. 5*E*). The amount of tetrasialylated saccharides was significantly low (4.2% of the total). Those saccharides were found to contain tetraantennary saccharides and tetraantennary saccharides with one or two *N*-acetyllactosaminyl repeats (data not shown).

These results indicate that 1) the biantennary saccharide is almost exclusively in a monosialylated form; 2) the triantennary saccharides are in monosialylated or disialylated forms; 3) the tetraantennary saccharides are mostly in disialylated or trisialylated forms; and 4) the tetraantennary saccharides with one, two, or three *N*-acetyllactosamine repeats are mostly in disialylated or trisialylated forms. These results suggest that one of the side chains in the saccharides is almost alway terminated without a sialic acid residue (see below).

In order to know whether any difference exists among different batches of recombinant erythropoietin, two additional batches (Batches 3 and 4 in Table I) of recombinant erythropoietin were subjected to analysis. Interestingly, these samples contained more highly sialylated saccharides: the disialosyl form is 18–21% of the total saccharides; the trisialosyl form is 64–67%, the tetrasialosyl form is 10–13%; and the monosialosyl form is less than 6%. However, the relative ratios of asialo biantennary, triantennary, tetraantennary, and tetraantennary saccharides with one, two, or three *N*-acetyllactosaminyl repeats were almost identical among different samples. These results indicate that sialylation may vary among different batches of recombinant erythropoietin but their backbone structures are the same.

Separation of Saccharides with Different Backbone Structure but with the Same Number of Sialic Residues in Side Chains—The results of Fig. 7*A* suggested to us that each peak in the monosialosyl, disialosyl, or trisialosyl fraction may represent saccharides with different backbone structures but with the same number of sialic acid residues. In order to test this possibility, another 5 mg of recombinant erythropoietin (Batch 2 in Table I) was treated to yield *N*-linked saccharides, and these saccharides were fractionated by TSK-DEAE ion-exchange chromatography. This sample provided an elution profile almost identical to that in Fig. 7*A*. Saccharides were divided into four (fraction II) or three (fraction III) fractions, desialylated, and subjected to another HPLC employing a Lichrosorb column. Fraction II-1, which eluted earliest in TSK-DEAE chromatography, provided tetraantennary saccharides with two or three *N*-acetyllactosaminyl repeats (Fig. 8*A*, see Miniprint), whereas the last peak (fraction II-4) mainly consists of triantennary and tetraantennary saccharides (Fig. 8*D*). Similarly, the earliest peak in the trisialosyl fraction (fraction III-1) provided Lac_2 and a small amount of Lac_1 and Lac_3 (Fig. 8*E*), whereas the last peak (fraction III-3) provided almost exclusively tetraantennary saccharides (Fig. 8*G*). These results indicate that the saccharides with higher numbers of *N*-acetyllactosamine units elute earlier than those with smaller numbers of *N*-acetyllactosamine units in TSK-DEAE ion-exchange chromatography.

Localization of α2→3-Linked Sialic Acid in the Side Chains—In order to know which side chains are preferentially sialylated, fractions II-2, II-3 and III-3 were digested extensively with a mixture of β-galactosidase and β-*N*-acetylglucosaminidase, and the products were purified by Sephadex G-50 gel filtration followed by TSK-DEAE chromatography. The purified products were then subjected to methylation analysis, and the results are summarized as follows.

Disialosyl Tetraantennary Saccharides with One or Two N-Acetyllactosaminyl Repeats (Fraction II-2)—Methylation analysis on the exoglycosidase product of fraction II-2 provided the following mannose derivatives: 0.9 mol each of 2,6-

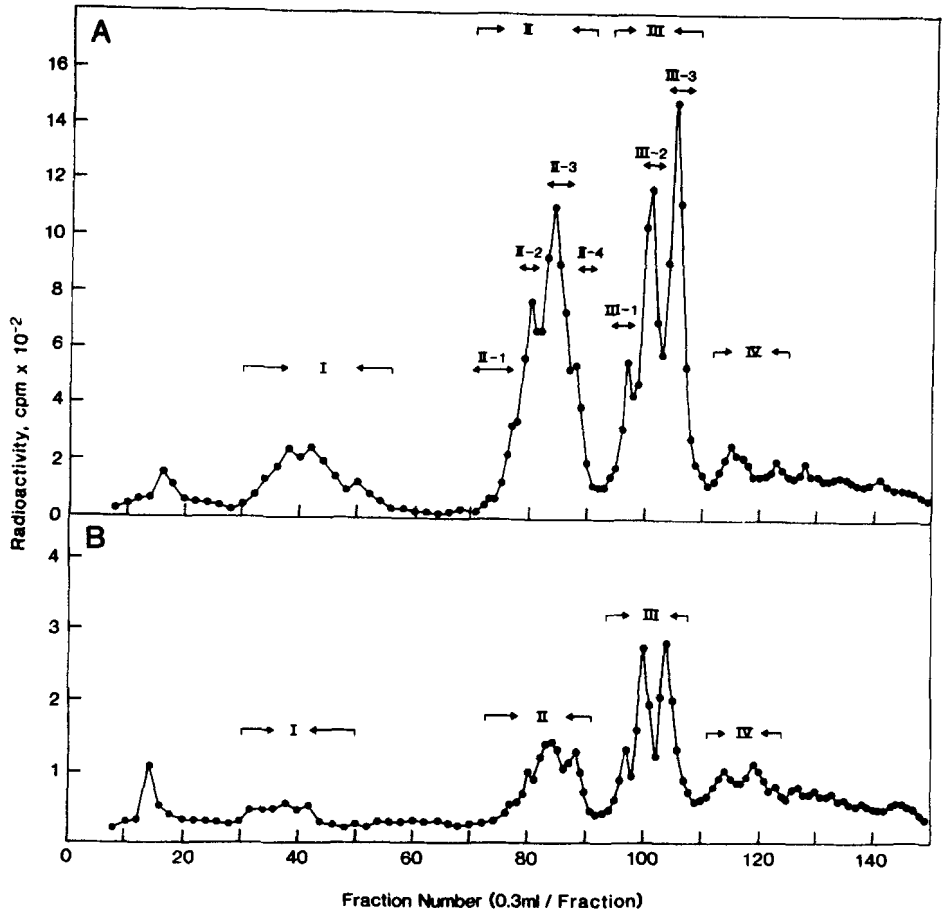


FIG. 7. **Ion-exchange HPLC of *N*-linked saccharides obtained from recombinant erythropoietin (*A*) and urinary erythropoietin (*B*).** *N*-Linked oligosaccharide fractions obtained after Sephadex G-50 gel filtration (fractions 29–41 in Fig. 2*B*) were applied to a column of TSK-DEAE SW2 equilibrated with 25 mM potassium phosphate buffer, pH 5.0. After washing with the same buffer for 10 min, the column was eluted with a linear gradient from the same buffer to 400 mM potassium phosphate buffer, pH 5.0. A portion of the fractions indicated by the *horizontal arrows* were pooled and subjected to HPLC with a Lichrosorb column, as shown in Figs. 5 (*C–E*) and 8.

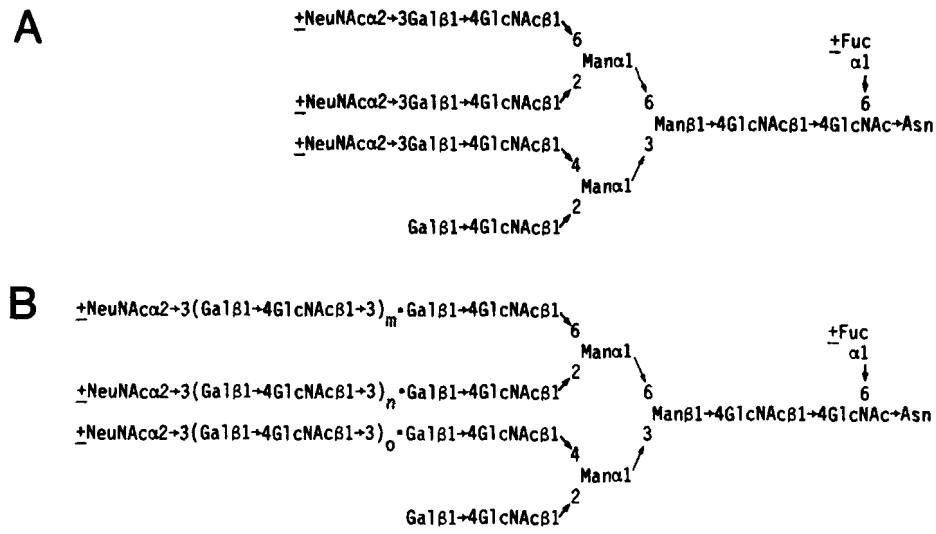


FIG. 11. **Proposed structures of tetraantennary saccharides (*A*) and tetraantennary saccharides with *N*-acetyllactosaminyl repeats (*B*) obtained from recombinant erythropoietin.** *A*, sialylation takes place preferentially at the side chain arising from C-6 and then from the side chain at C-2 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose. *B*, the saccharides with one *N*-acetyllactosaminyl repeat ($m = 1$, $n = 0$, and $o = 0$, in 90% of the molecules, and $o = 1$ and $m = n = 0$ in 10% of the molecules); the saccharides with two *N*-acetyllactosaminyl repeats ($m = n = 1$ and $o = 0$ in 60% of the molecules, and $m = o = 1$ and $n = 0$ in 40% of the molecules); and the saccharides with three *N*-acetyllactosaminyl repeats ($m + n + o = 3$).

substituted and terminal mannose, 0.1 mol of 4- and 6-substituted mannose, and 1 mol of 3,6-substituted mannose. No other mannose derivatives, including 2,4-substituted mannose, were detected. The results indicate that the 2 sialosyl residues are almost exclusively linked to the side chains arising from C-2 and C-6 of 2,6-substituted mannose (see Fig. 11).

Disialosyl Tetraantennary Saccharide (Fraction II-3)—Methylation analysis of the exoglycosidase product of fraction II-3 provided the following mannose derivatives: 0.6 mol of terminal mannose, 0.3 mol of 2-substituted mannose, 0.4 mol of 4-substituted mannose, 0.1 mol of 6-substituted mannose, 0.6 mol of 2,6-substituted mannose, and 1 mol of 3,6-substituted mannose. The results indicate that 60% of the molecules are sialylated in both chains elongating from C-2 and C-6 of 2,6-substituted mannose, and 40% of the molecules are sialylated in the side chain arising from C-4 of 2,4-substituted mannose and that from C-6 or C-2 of 2,6-substituted mannose.

Trisialosyl Tetraantennary Saccharide (Fraction III-3)—Methylation analysis after exoglycosidase digestion of fraction III-3 provided the following mannose derivatives: 1 mol each of 2,6- and 3,6-substituted mannose and 0.5 mol each of 2,4- and 4-substituted mannose. However, no other mannose derivative was detected. The results indicate that the nonsialylated chain almost exclusively arises from C-2 of 2,4-substituted α-mannose.

Structure of Sialylated Tetraantennary Saccharides with or without N-Acetyllactosamine Repeats—Based on the results described above, the structure of intact tetraantennary saccharides and those with *N*-acetyllactosaminyl repeats, which represent 85% of the total saccharides, can be proposed as shown in Fig. 11. The tetraantennary saccharides are mainly present as disialosyl or trisialosyl forms, and 2→3-linked sialic acid is attached to the side chains arising from C-6 and C-2 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose. In the tetraantennary saccharides with *N*-acetyllactosaminyl repeats, sialic acid residues are always present in the side chain which contains *N*-acetyllactosaminyl repeats. This conclusion was supported by FAB-MS analysis. As shown in Fig. 3*A*, all of the fragment ions containing polylactosaminyl units are sialylated.

In order to delineate this further, trisialylated saccharides (fraction III) were extensively digested by β-galactosidase and β-*N*-acetylglucosaminidase. The methylation analysis of this product showed that more than 90% of the side chain attached to C-2 of 2,4-substituted mannose was terminated without sialic acid. These combined results support the proposed structures shown in Fig. 11.

Structure of Carbohydrate Units of Urinary Erythropoietin—Since only a limited amount of urinary erythropoietin was available, the following experiments were carried out to analyze carbohydrate units of urinary erythropoietin. Glycopeptides, prepared by Pronase digestion of urinary erythropoietin, were subjected to alkaline borohydride treatment. The alkaline borohydride-treated sample was then applied to Bio-Gel P-4 gel filtration. Urinary erythropoietin saccharides provided almost the same elution profile as Fig. 2*A*. The glycopeptides containing *N*-linked saccharides (fractions 24–29) were digested by *N*-glycanase, and the digest was subjected to Sephadex G-50 gel filtration. Again, the elution profile of *N*-linked saccharides from urinary erythropoietin was almost identical to that from recombinant erythropoietin (see Fig. 2*B*).

Methylation analysis of *N*-linked saccharides (fractions 29–41 after Sephadex G-50 gel filtration) provided partially *O*-methylated monosaccharide derivatives, which are almost identical to those produced from highly sialylated Batch 3 of recombinant erythropoietin (Table II). FAB-MS of permethylated *N*-linked saccharides provided fragment ions for NeuNAc$^+$ (*m/z* 376 and 344), NeuNAc→Hex→Hex NAc$^+$ (*m/z* 825), NeuNAc→Hex→HexNAc→Hex→HexNAc$^+$ (*m/z* 1274), and NeuNAc→Hex→HexNAc→Hex→HexNAc→Hex→HexNAc$^+$ (*m/z* 1723) (Fig. 3*B*). These results are essentially the same as those obtained on recombinant erythropoietin (compare Fig. 3, *A* and *B*).

N-Linked saccharides were then desialylated and subjected to HPLC employing a Lichrosorb-NH_2 column. As shown in Fig. 5*B*, urinary erythropoietin saccharides provided triantennary, tetraantennary, and tetraantennary saccharides with one, two, or three *N*-acetyllactosaminyl units. The relative proportion among these saccharides is almost identical to that obtained on recombinant erythropoietin except that urinary erythropoietin apparently lacks biantennary saccharides. In order to determine the relative amounts of sialylated *N*-linked saccharides, intact *N*-linked saccharides were subjected to TSK-DEAE ion-exchange chromatography. Fig. 7*B* shows that *N*-linked saccharides from urinary erythropoietin contain disialosyl (27% of the total saccharides), trisialosyl (56%), and tetrasialosyl (17%) saccharides. These results indicate that urinary erythropoietin and recombinant erythropoietin have an almost identical set of *N*-linked saccharide units but with slightly different sialylation depending upon the batches of recombinant erythropoietin (see above and Table I).

DISCUSSION

This paper reports the detailed structures of the carbohydrate moiety of human erythropoietin produced by recombinant DNA. The protein analyzed was produced in Chinese hamster ovary cells which were transfected with human erythropoietin cDNA (7). As far as we are aware, this is the first report on the detailed carbohydrate structure of a glycoprotein produced by recombinant DNA in comparison with the glycoprotein of natural origin. Although Mutsaers *et al.* (28) reported the carbohydrate structure of human γ-interferon produced in Chinese hamster ovary cells, their studies did not investigate those of naturally occurring human γ-interferon. The carbohydrate composition (Table I) showed that erythropoietin contains three *N*-linked saccharides and one *O*-linked saccharide, and these conclusions are consistent with the recent report on the amino acid sequence of human urinary erythropoietin (29).

The present study revealed that a large proportion of the carbohydrate moiety of recombinant erythropoietin is composed of tetraantennary saccharides with one (32.1% of the total saccharides), two (16.5%), and three (4.7%) *N*-acetyllactosaminyl repeats. The localization of these polylactosaminyl units was elucidated by sequential exoglycosidase digestion followed by methylation analysis, and the results are summarized as follows (see also Table III).

When the saccharides contain one *N*-acetyllactosaminyl repeat, more than 70% of this repeat is preferentially attached to the side chain arising from C-6 of 2,6-substituted mannose, and 19% of the repeat is attached to that from C-4 of 2,4-substituted mannose. When the saccharides contain two *N*-acetyllactosaminyl repeats, these repeats are attached to C-2 and C-6 of 2,6-substituted mannose in 80% of the molecules. The rest of the molecule contains *N*-acetyllactosaminyl repeats in the side chains arising from C-6 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose. These results indicate that *N*-acetyllactosaminyl repeats are most preferentially added to C-6 of 2,6-substituted mannose and then to C-2 of 2,6-substituted mannose. These conclusions are consistent with previous reports on several cellular glycoproteins. For example, Cummings and Kornfeld (30) reported that the mouse lymphoma BW5147 cell line expressed a significant amount of polylactosaminoglycan, whereas its mutant, which lacks the side chain arising from C-6 of 2,6-substituted mannose, expresses a minimum amount of polylactosaminoglycan. Li *et al.* (31) isolated polylactosaminoglycan from Chinese hamster ovary cells in which polylactosaminyl units are attached to C-2 and C-6 of 2,6-substituted mannose. Similarly, polylactosaminyl units were found in triantennary and tetraantennary saccharides of various origins (24, 32–34). These results appear to establish that *N*-acetyllactosaminyl repeats are preferentially added to C-6 of 2,6-substituted mannose and then to C-2 of 2,6-substituted mannose. These 2,6-substituted mannose residues are usually linked to the C-6 side of β-mannose. In human erythrocytes, polylactosaminyl elongation can be found in the side chain arising from C-2 of α-mannose which is linked to C-6 of β-mannose (23, 35). It is likely that human erythroid cells contain very little activity of the *N*-acetylglucosaminyltransferase which forms a GlcNAcβ1→6Man branch. As a result, *N*-acetyllactosamine repeats are formed on the secondary preferable side chain, which is attached to C-2 of α-mannose linked to C-6 of β-mannose, in these erythroid cells.

This study showed that human erythropoietin exclusively

contains α2→3-linked sialic acid. This fact allowed us to elucidate the localization of 2→3-linked sialic acid residues among different side chains. This was achieved by extensive digestion of intact saccharides with β-galactosidase and β-*N*-acetylglucosaminidase followed by methylation analysis of the products. These results can be summarized as follows. 1) When typical triantennary or tetraantennary saccharides contain 2 sialic acid residues, they are attached to C-2 and C-6 of 2,6-substituted mannose or C-6 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose. 2) When saccharides with *N*-acetyllactosaminyl repeats contain 2 sialic acid residues, they are attached almost exclusively to C-2 and C-6 of 2,6-substituted mannose. This localization is essentially identical to that of *N*-acetyllactosamine repeats. Thus, it is apparent that polylactosamine is preferably sialylated through an α2→3-linkage.

These results are consistent with our previous results obtained on polylactosaminoglycans from chronic myelogenous leukemia cells; α2→3-linked sialic acid is present on side chains arising from C-6 and C-2 of 2,6-substituted mannose and C-4 of 2,4-substituted mannose, and those side chains are longer than that terminating with 2→6-linked sialic acid (24). Similar results were obtained in human erythrocyte Band 3 polylactosaminoglycans; polylactosaminyl side chains arising from the C-6 side of β-mannose are sialylated through a 2→3-linkage, whereas the shorter chain arising from the C-3 side is sialylated through a 2→6-linkage (23, 35). Similarly, Yamashita *et al.* (33) and Markle and Cummings (36) found that longer polylactosaminyl side chains are almost exclusively sialylated through a 2→3-linkage. Interestingly, short polylactosamine chains in thyroid cell glycoprotein Gp-1 (37) and BW5147 (36) are terminated with 2→6-linked sialic acid. Our results also showed that almost no 2→3-linked sialic acid is attached to the side chain arising from C-2 of 2,4-substituted mannose. It is noteworthy that this side chain was found to be exclusively sialylated through a 2→6-linkage in many glycoproteins including normal and leukemic granulocyte polylactosaminoglycans (19–21, 24, 34, 38–41).

By using a bovine colostrum α2→6-sialyltransferase, Joziasse *et al.* (42) have shown that preferential sialylation takes place first on C-2 of 2,4-substituted mannose and then on C-4 of 2,4-substituted mannose. This branch (or side chain) specificity appears to be opposite to the distribution of 2→3-linked sialic acid. Thus, it is likely that α2→3-sialyltransferase and α2→6-sialyltransferase have complementary specificity toward different side chains. Furthermore, our results raise the possibility that the side chains containing polylactosaminyl units would be preferable sites for 2→3-linked sialylation.

This study demonstrated that the carbohydrate moiety of human erythropoietin isolated from human urine is indistinguishable from that of recombinant erythropoietin except for a difference in degree of sialylation. Urinary erythropoietin has a similar degree of sialylation as the highly sialylated batch of recombinant erythropoietin (Tables I and II). The recombinant erythropoietin was produced in Chinese hamster ovary cells, and urinary erythropoietin is presumably derived from human kidney cells. The results therefore suggest two possibilities. 1) Chinese hamster ovary and human kidney cells contain similar glycosyltransferases. 2) The protein acceptor itself influences glycosylation even when a similar set of glycosyltransferases are not present in two cell types. It will be interesting to see if the carbohydrate moiety of erythropoietin produced in other mammalian cells is similar to those elucidated in this study. This study also demonstrated that the major carbohydrate units of erythropoietin are tetraantennary saccharides with or without *N*-acetyllactosamine repeats. It has been shown that rat liver cells uptake the asialo form of glycoproteins which contain tri- or tetraantennary saccharides (43). It is therefore reasonable that the asialo form of erythropoietin is taken up by liver cells through a galactose-binding protein (15). Our preliminary studies showed that a portion of intact erythropoietin of both recombinant and urinary origins is taken up by rat liver cells, presumably because of the incomplete sialylation. It will be interesting to test if sialylation by α2→6-sialyltransferase elongates the serum concentration of erythropoietin and sustains *in vivo* activity longer than the starting erythropoietin.

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SUPPLEMENTAL MATERIAL
TO
CARBOHYDRATE STRUCTURE OF ERYTHROPOIETIN EXPRESSED IN CHINESE HAMSTER OVARY CELLS BY A HUMAN ERYTHROPOIETIN cDNA

By

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Isolation of O-linked oligosaccharides from recombinant erythropoietin - The O-linked oligosaccharides were separated from N-linked glycopeptides by Bio-Gel P-4 gel filtration as shown in Fig. 2A. The fractions from 30 to 35 (designated O-i) and those from 36-40 (designated O-ii) were separately pooled and subjected to TSK-DEAE ion exchange chromatography as shown in Fig. 9.

The O-i fraction provided disialosyl oligosaccharides and trisialosyl oligosaccharides, whereas the O-ii fraction provided mainly monosialosyl oligosaccharides, as judged by FAB-MS. The fractions corresponding to monosialosyl-, disialosyl-, and trisialosyl-saccharides from the O-i and O-ii fractions were pooled and subjected to methylation analysis and FAB-MS analysis.

Structures of O-linked oligosaccharides isolated from recombinant erythropoietin - As shown in Fig. 1DA, FAB-MS of the permethylated monosialosyl oligosaccharide afforded a molecular ion of m/z 873 for $(NeuNAc\cdot Hex\cdot HexNAc)_R$. These ions were associated with fragment ions at m/z 580 corresponding to $NeuNAc\cdot Hex^+$, m/z 376 and 344 corresponding to $NeuNAc^+$ and $(NeuNAc\text{-methanol})^+$.

The oligosaccharide was digested by clostridial sialidase to yield Galβ1-3GalNAcOH as described (17). These results, together with methylation analysis (Table IV) indicated that the monosialosyl oligosaccharide is NeuNAcα2-3Galβ1-3GalNAcOH.

FAB-MS of the disialosyl oligosaccharide provided a molecular ion of m/z 1234 for $(NeuNAc_2\cdot Hex\cdot HexNAc)_R$ (Fig. 1DB). This ion was accompanied by A-type fragment ions of $NeuNAc\cdot Hex^+$ (m/z 580) and $NeuNAc^+$ (376 and 344), and a B-cleavage ion of m/z 859. In addition, an ion at m/z 637 was produced by β-elimination of NeuNAc-Hex from the parent molecule. (The detailed mechanisms for β-cleavage and β-elimination will be described elsewhere.[2]). The oligosaccharide was sequentially digested by clostridial sialidase and Escherichia coli β-galactosidase as described previously (17). These results, together with methylation analysis (Table IV) indicate that the disialosyl oligosaccharide is NeuNAcα-2+3Galβ1+3(NeuNAcα2+6)GalNAcOH.

The trisialosyl oligosaccharide provided a molecular ion of m/z 1595 for $(NeuNAc_3\cdot Hex\cdot HexNAc)_R$ (Fig 1DC). In addition, molecular ions at m/z 1218 and m/z 1234 for non-reduced and reduced forms of $NeuNAc_2\cdot Hex\cdot HexNAc$ were also detected in this sample. Since the amount of these components was low, further analysis to elucidate the complete structure could not be made. However, it is possible that the trisialosyl oligosaccharide from the recombinant erythropoietin has the same structure found recently in human erythrocyte glycophorins.[2]

Structures of O-linked oligosaccharides from urinary erythropoietin - The O-linked oligosaccharides from urinary erythropoietin were recovered in fraction 30-40 in Bio-Gel P-4 gel filtration (see Fig. 2A). Initial attempts to further fractionate O-linked oligosaccharides failed and no significant amount of O-linked oligosaccharides were detected after TSK-DEAE ion exchange chromatography. This was probably due to a low quantity of the O-linked oligosaccharides from urinary erythropoietin. Therefore, fractions from 30-40 were pooled and directly analyzed by methylation analysis and FAB-MS analysis.

As shown in Fig. 1DD, FAB-MS of the oligosaccharides provided a molecular ion of m/z 1234 for $NeuNAc_2\cdot Hex\cdot HexNAc_2$. In addition, fragment ions for $NeuNAc^+$ (376 and 344) were detected. A fragment ion at m/z 825 corresponding to $NeuNAc\cdot Hex\cdot HexNAc^+$ may be produced from N-linked saccharides which were present as contaminants in this oligosaccharide fraction. These results, together with methylation analysis, indicated that the major O-linked oligosaccharide from urinary erythropoietin is the disialosyl saccharide, which is probably NeuNAcα2-3Galβ1+3(NeuNAcα2+6)GalNAcOH.

TABLE II. Relative proportions of methylated sugars obtained from N-linked saccharides of recombinant and urinary erythropoietins.

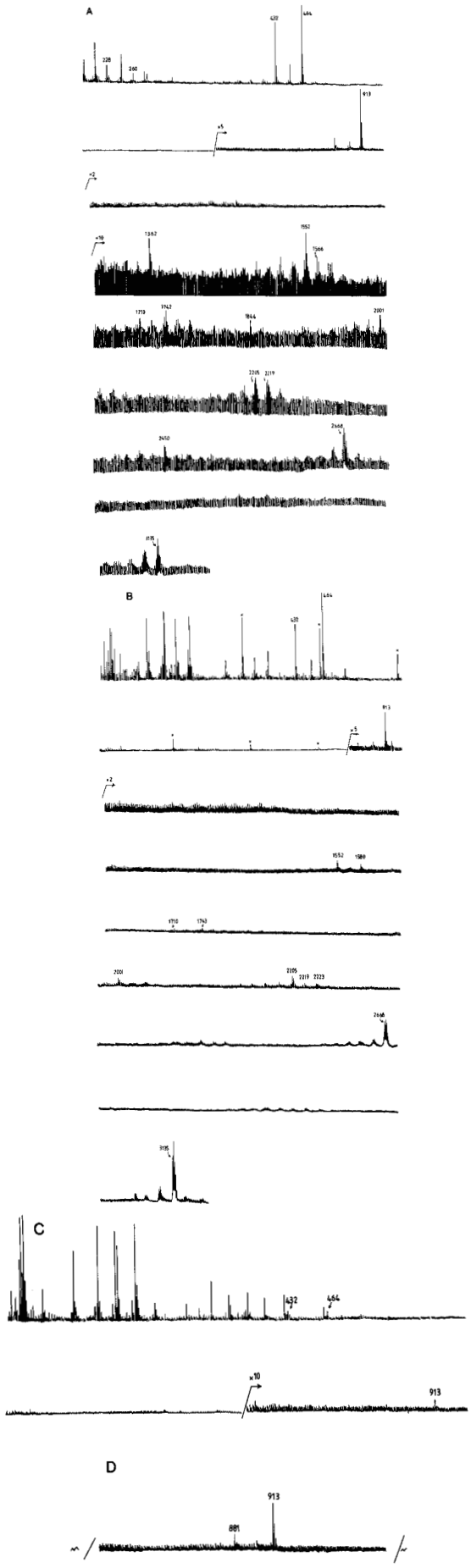
Methylated Sugars	N-saccharides							
	Recombinant batch 1	batch 3	Urinary	3	4	5	6	7
Fucitol								
2,3,4-tri-O-methyl	0.9	0.9	0.9	0.9	0.95	0.9	0.9	0.9
Galactitol								
2,3,4,6-tetra-O-methyl	1.34	0.47	0.38	3.0	3.90	4.0	4.0	4.0
2,4,6-tri-O-methyl	3.52	4.30	4.11	0	0.11	0.8	1.7	2.4
Mannitol								
3,4,6-tri-O-methyl	0.16	0.12	0.26	1.00	0.11	0	0	0
3,6,-di-O-methyl	0.78	0.81	0.76	0.35	1.00	1.0	1.0	0.9
3,4-di-O-methyl	1.05	1.07	0.98	0.65	0.90	1.0	1.0	1.1
2,4-di-O-methyl	1.00	1.00	1.00	1.00	1.00	1.0	1.0	1.0
2-N-methylacetamido-2-deoxyglucitol								
1,3,5,6-tetra-O-methyl	0.10	0.12	0.14	trace	0.05	trace	0.05	0.10
1,3,5-tri-O-methyl	0.80	0.81	0.81	0.9	0.9	0.9	0.85	0.55
3,6-di-O-methyl	5.82	5.76	5.54	4.0	5.0	6.0	6.7	7.4
3-O-methyl	0.10	0.07	0.05	0.1	0.05	0.05	0.10	0.35

TABLE IV. Relative proportions of methylated sugar obtained from O-linked saccharides of recombinant and urinary erythropioetins.

	Recombinant			
	Monosialosyl	Disialosyl	Trisialosyl	Urinary
Galactitol[a]				
2,4,6-tri-O-methyl	1.0	1.0	1.0	1.0
2-N-methylacetamido-2-deoxygalactitol				
1,4,5,6-tetra-O-methyl	0.8	trace	0	0.2
1,4,5-tri-O-methyl	0.1	1.0	0.9	0.7
4-mono-O-methyl[b]	0.1	0	0.1	0.1

[a] A small amount of mannose derivatives (less than 0.1 mole) was detected in all of the fractions.

[b] This derivative was probably produced because of incomplete reduction.



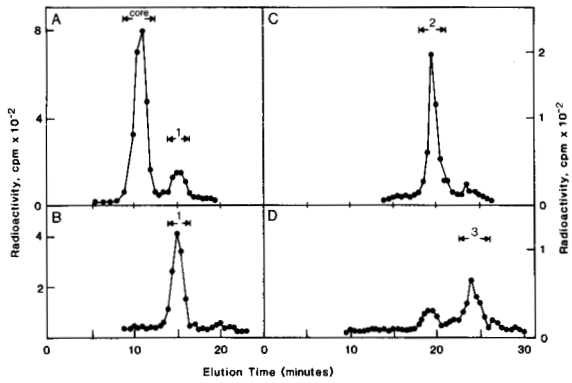


Figure 6. HPLC of exoglycosidase digests of N-linked saccharides derived from recombinant erythropoietin. N-linked saccharides, separated by HPLC with a column of Lichrosorb-NH_2 as shown in Fig. 5A, were digested sequentially by β-galactosidase and β-N-acetylglucosaminidase and resubjected to the same HPLC. The chromatographic conditions are the same as in Fig. 5 except that the chromatograms were monitored by measuring radioactivity.

A. The digest of saccharide 4.

B. The digest of saccharide 5, Lac_1.

C. The digest of saccharide 6, Lac_2.

D. The digest of saccharide 7, Lac_3.

Figure 4. Fast atom bombardment-mass spectra of asialo N-linked saccharides from recombinant erythropoietin. The positive spectra were taken on the permethylated samples.

A. Total N-linked saccharides (fractions 29-41 in Fig. 2B) after desialylation. A series of fragment ions derived from lactosaminyl repeats were detected: m/z 464 (and 432) for $Hex_1{\cdot}HexNAc^+$, 913 for $Hex{\cdot}HexNAc{\cdot}Hex{\cdot}HexNAc^+$, 1362 for $Hex{\cdot}HexNAc{\cdot}Hex{\cdot}HexNAc{\cdot}Hex{\cdot}HexNAc^+$. A prominent molecular ion was detected at m/z 3135 corresponding to $(Fuc_1{\cdot}Hex_7{\cdot}HexNAc_6)_2$. This ion is accompanied by an A-type fragment ion at m/z 2668 for $Hex_5{\cdot}HexNAc_5^+$, and a β-cleavage ion at m/z 2205 for $Hex_4{\cdot}HexNAc_4^+$. The A-type ion for tri-antennary saccharides was detected at m/z 2219 for $Hex_5{\cdot}HexNAc_4^+$. These A-type ions (m/z 2668 and 2219) were produced by a cleavage between two N-acetylglucosamine residues in the chitobiosyl core. β-cleavage ions derived from tri-antennary and bi-antennary saccharides were detected at m/z 2001 for $Hex_5{\cdot}HexNAc_4^+$, and 1552 for $Hex_4{\cdot}HexNAc_3^+$, respectively.

B. Desialylated tetra-antennary and tri-antennary with one N-acetyllactosamine (saccharide 4 in Fig. 5A). Almost all fragment ions detected in A were found in this saccharide, except for the following. The A-type ions derived from bi-antennary or tri-antennary saccharides (m/z 2219 and 1770) were absent or barely detected. Although the fragment ion at m/z 913 was prominent, the ion at 1362 for $Hex_3{\cdot}HexNAc_3$ was not detected.

C. Desialylated tetra-antennary with one N-acetyllactosaminyl repeat, Lac_1 (saccharide 5 in Fig. 5A).

D. Desialylated tetra-antennary with two N-acetyllactosaminyl repeats, Lac_2 (saccharide 6 in Fig. 5A). These saccharides were contaminated by other components including detergent. However, significant signals at m/z 913 for $Hex{\cdot}HexNAc{\cdot}Hex{\cdot}HexNAc^+$ (both C and D) and 464 for $Hex{\cdot}HexNAc^+$ (C) were detected.

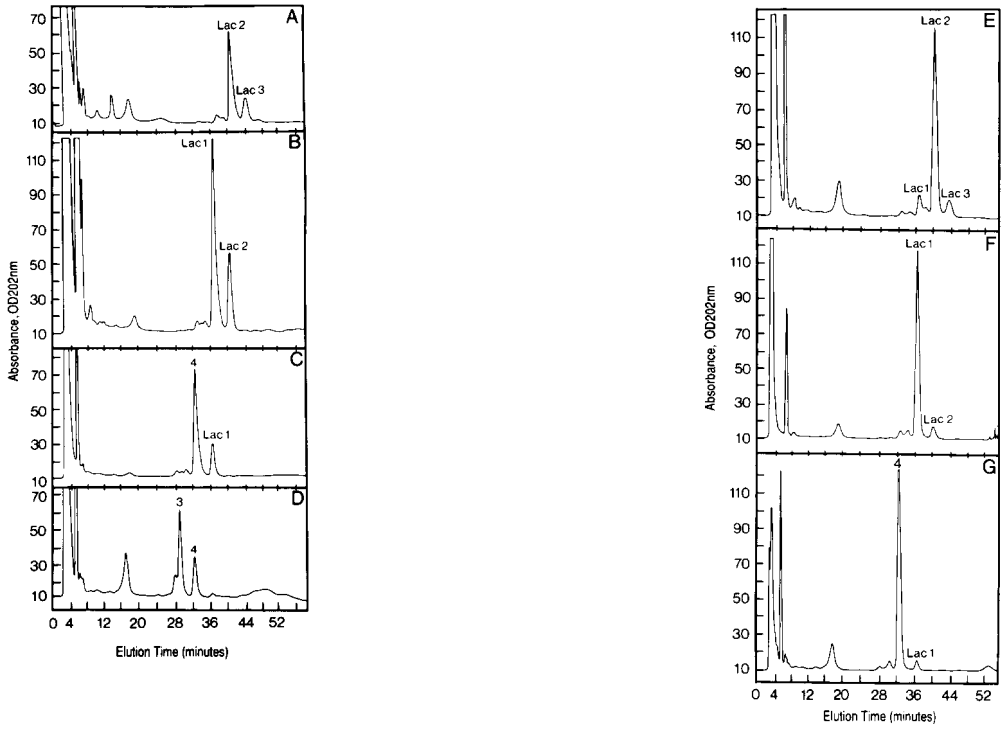


Figure 8. HPLC of each peak obtained by TSK-DEAE ion exchange chromatography. N-linked saccharides from recombinant erythropoietin were separated by TSK-DEAE ion exchange chromatography as shown in Fig. 7A. Each peak obtained was desialylated and subjected to HPLC with a Lichrosorb-NH_2 column as the same condition of Fig. 5.

A. II-1 (fractions 70-77, in Fig. 7A).

B. II-2 (fractions 78-81 in Fig. 7A).

C. II-3 (fractions 82-87 in Fig. 7A).

D. II-4 (fractions 88-91 in Fig. 7A).

E. III-1 (fractions 94-98 in Fig. 7A).

F. III-2 (fractions 99-102 in Fig. 7A).

G. III-3 (fractions 103-108 in Fig. 7A).

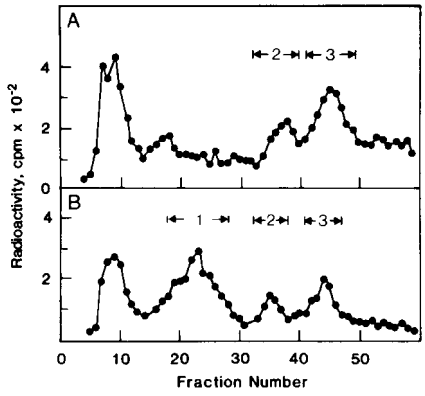


Figure 9. Ion-exchange HPLC of O-linked oligosaccharides obtained from recombinant erythropoietin. Oligosaccharides released with alkaline borohydride treatment were isolated by Bio-Gel P-4 gel filtration and those saccharides were separated by TSK-DEAE ion exchange chromatography, as described in "Experimental Procedures" except for the following. The column was equilibrated with 25 mM potassium phosphate buffer, pH 5.0. After washing with the same buffer for 5 min, the column was eluted with a linear gradient from the same buffer to 400 mM potassium phosphate buffer, pH 5.0 over 40 min. The flow rate was 1 ml/min and each fraction contained 0.5 ml. The horizontal arrows indicate the fractions pooled. No carbohydrate was detected in other fractions.

A. O-i (fractions 30-35 in Fig. 2A).

B. O-ii (fractions 36-40 in Fig. 2A).

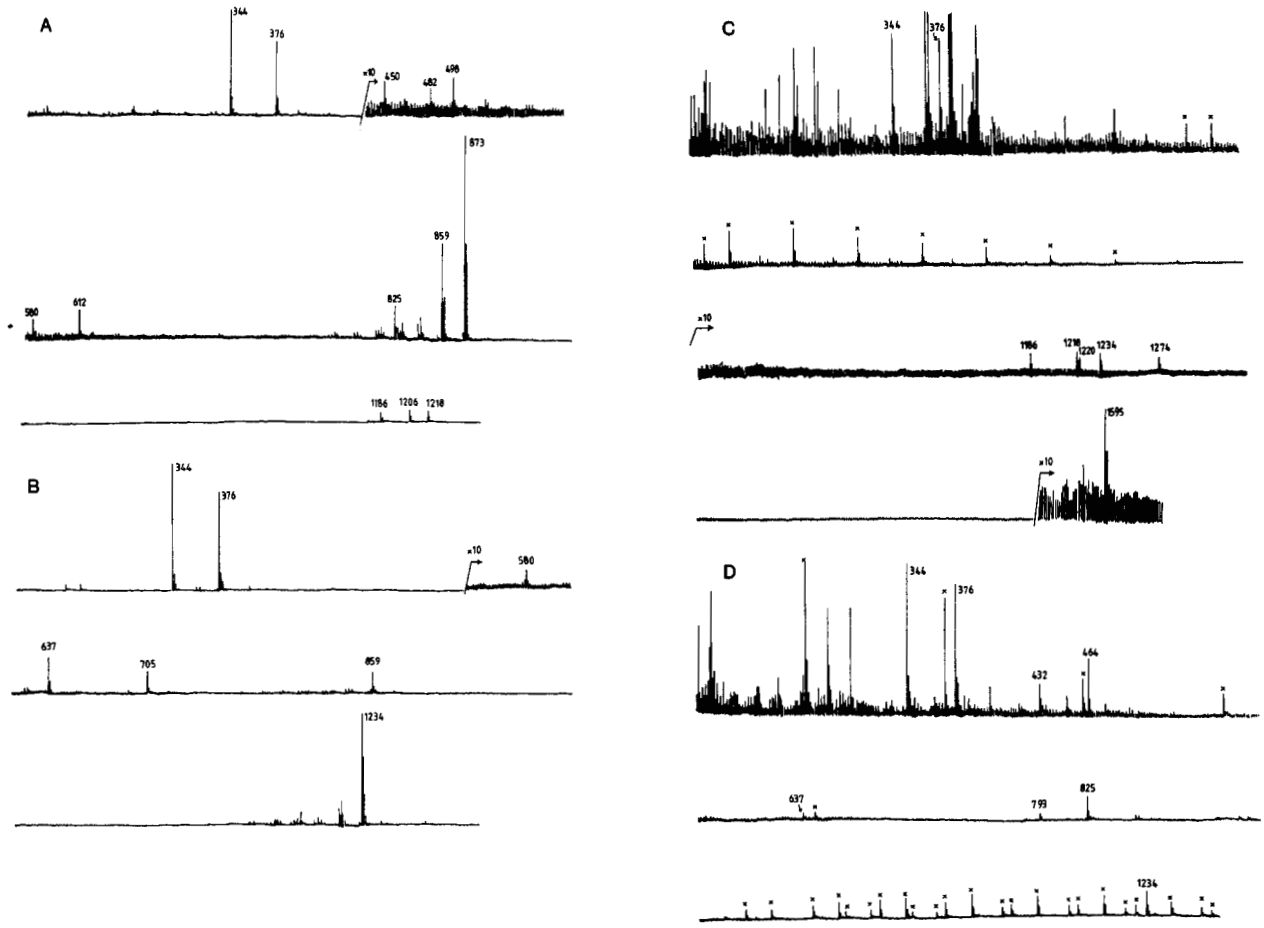


Figure 10. Fast atom bombardment-mass spectra of O-linked oligosaccharides from recombinant erythropoietin (A, B, C) and urinary erythropoietin (D). The positive spectra were obtained on permethylated samples.

A. Fractions 18-27 from Fig. 9B. A prominent molecular ion was detected at m/z 873 corresponding to NeuNAc·Hex·$HexNAc_2$. These molecular ions are accompanied by fragment ions at m/z 580 for NeuNAc+Hex, and 376 (and 344) for $NeuNAc^+$. Ions at m/z 1206 and 1218 correspond to di-undermethylated form of $NeuNAc_2$·Hex·$HexNAc_2$ and the methylglycoside of $NeuNAc_2$·Hex·$HexNAc_2$, respectively. A signal at m/z 1186 is a A-type fragment ion for $NeuNAc_2$·Hex·$HexNAc^+$. A minor ion at m/z 612 is probably due to the methylglycoside of $NeuNAc^+$Hex.

B. Fractions 33-40 in Fig. 9A plus fractions 33-38 in Fig. 9B. A prominent molecular ion was detected at m/z 1234 for $NeuNAc_2$·Hex·$HexNAc_2$. This ion was associated with A-type fragment ions at m/z 580 for NeuNAc+Hex and 376 (and 344) for $NeuNAc^+$. The ion at m/z 637 was produced by β-elimination of NeuNAc+Hex from the parent molecule whereas the ion at m/z 859 corresponds to a β-cleavage product lacking one sialic acid residue.

C. Fractions 41-49 in Fig. 9A plus fractions 41-47 in Fig. 9B. Molecular ions at m/z 1595 and 1234 correspond to $(NeuNAc_2$·Hex·$HexNAc)_2$ and $(NeuNAc_2$·Hex·$HexNAc)_1$. These ions were associated with an ion at m/z 376 and 344 for $NeuNAc^+$. This fraction also provided fragment ions at m/z 1218 and 1186 which are probably derived from unreduced $NeuNAc_2$·Hex·HexNAc and A-type fragment ion for $NeuNAc_2$·Hex·$HexNAc^+$. A minor signal at 1274 corresponds to NeuNAc·Hex_2·$HexNAc_2$ and is probably derived from contaminating N-linked saccharides.

D. O-linked oligosaccharides from urinary erythropoietin (fractions 30-40 in Bio-Gel P-4, see also Fig. 2A). A molecular ion was detected at m/z 1234 corresponding to $(NeuNAc_2$·Hex·$HexNAc)_2$. This ion is accompanied by fragment ions at m/z 376 (and 344) for $NeuNAc^+$. An ion at m/z 825 corresponding to NeuNAc·Hex·$HexNAc^+$ is probably derived from contaminating N-linked saccharides. A cluster of ions 44 mass units apart (marked by crosses) are detergent ions.