EXHIBIT 34

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Comparative Study of the Asparagine-linked Sugar Chains of Human Erythropoietins Purified from Urine and the Culture Medium of Recombinant Chinese Hamster Ovary Cells*

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The asparagine-linked sugar chains of human erythropoietin produced by recombinant Chinese hamster ovary cells and naturally occurring human urinary erythropoietin were liberated by hydrazinolysis and fractionated by paper electrophoresis, lectin affinity chromatography, and Bio-Gel P-4 column chromatography. Both erythropoietins had three asparagine-linked sugar chains in one molecule, all of which were acidic complex type. Structural analysis of them revealed that the sugar chains from both erythropoietins are quite similar except for sialyl linkage. All sugar chains of erythropoietin produced by recombinant Chinese hamster ovary cells contain only the NeuAcα2→3Gal linkage, while those of human urinary erythropoietin contain the NeuAcα2→6Gal linkage together with the NeuAcα2→3Gal linkage. The major sugar chains were of fucosylated tetraantennary complex type with and without *N*-acetyllactosamine repeating units in their outer chain moieties in common, and small amounts of 2,4- and 2,6-branched triantennary and biantennary sugar chains were detected. This paper proved, for the first time, that recombinant technique can produce glycoprotein hormone whose carbohydrate structures are common to the major sugar chains of the native one.

Erythropoietin is a glycoprotein hormone involved in the regulation of the level of peripheral erythrocytes (1) by stimulating the differentiation of the erythroid progenitor cells into mature erythrocytes (2). The hormone is primarily produced in the kidney of adults (3). Therefore, the decrease of the erythropoietin production by the destruction of kidney mass (from chronic renal failure (4) or some other reasons) causes anemia. Highly purified erythropoietin is expected to be useful in the therapeutic treatment of such a type of anemia (5). Erythropoietin has been purified from urine of patients with severe aplastic anemia (6); however, it is quite difficult to obtain the sufficient amount of human urinary erythropoietin (urinary HuEPO)[1] for the investigation of its chemical and biological properties and for the clinical application.

In order to overcome this problem, a new approach has been developed by using recombinant DNA techniques. So far, two groups reported the cloning of human erythropoietin gene and its nucleotide sequence analysis (7, 8). Lin *et al.* (8) has succeeded in the expression of the erythropoietin gene in Chinese hamster ovary (CHO) cells by recombinant techniques. The rHuEPO has three possible sites for *N*-glycosylation, Asn-*X*-Ser/Thr (7, 8), and is actually sensitive to *N*-glycanase digestion (9). Recently, Lai *et al.* (43) estimated on the basis of amino acid sequencing data that urinary HuEPO also has three *N*-linked sugar chains and one *O*-linked sugar chain. Analysis of the monosaccharide composition of HuEPO performed in our laboratory confirmed the occurrence of one *N*-acetylgalactosamine residue, indicating that one *O*-linked sugar chain is included in recombinant HuEPO (44). Sugar moiety of urinary HuEPO has been suggested to affect biological properties such as turnover rate, antigenicity, and so on (10–14). Therefore, it is important to elucidate the sugar chain structures of erythropoietin.

Several bioactive glycoproteins have been produced in recombinant mammalian cells (15–17). Among them, the sugar chain structure of human γ-interferon produced in CHO cells has been elucidated; however, its natural counterpart has not been analyzed (17). In this paper, we will describe the comparative analysis of the asparagine-linked sugar chain structures of rHuEPO produced in CHO cells and naturally occurring urinary HuEPO.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Paper Electrophoresis of Oligosaccharides Released from Erythropoietin by Hydrazinolysis—Radioactive oligosaccharides obtained from rHuEPO and urinary HuEPO by hydrazinolysis were subjected to paper electrophoresis at pH 5.4. As shown in Fig. 1,[3] both samples contained acidic oligosaccharides composed of at least seven components (A-1 to A-7) but no neutral oligosaccharide. When digested with sialidase from *Arthrobacter ureafaciens*, approximately 90% of the total acidic oligosaccharides from rHuEPO and 80% of those from urinary HuEPO were converted to neutral oligosaccharides (data not shown). The residual oligosaccharides migrated

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[1] The abbreviations used are: urinary HuEPO, human erythropoietin purified from the urine of patients with aplastic anemia; CHO, Chinese hamster ovary; rHuEPO, human erythropoietin produced in recombinant CHO cells; ConA, concanavalin A; DSA, *Daura stramonium* agglutinin; AAL, *Aleuria aurantia* lectin; Fuc, fucose.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 3–7) 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 included in the microfilm edition of the Journal that is available from Waverly Press.

[3] Subscript OT is used to indicate NaB[^{3}H]$_4$-reduced oligosaccharides. All sugars mentioned in this paper were of D-configurations except for fucose, which was of L-configuration.

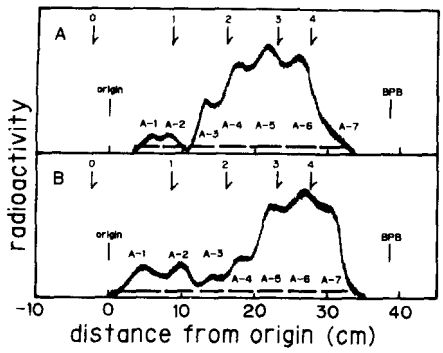

FIG. 1. **Paper electrophoresis of the radioactive oligosaccharides obtained from rHuEPO and urinary HuEPO.** The oligosaccharides released from rHuEPO (*panel A*) and urinary HuEPO (*panel B*) by hydrazinolysis were subjected to paper electrophoresis at a potential of 73 V/cm for 90 min. The *arrows* indicate the position of authentic oligosaccharides: *0*, *1*, *2*, *3*, and *4* indicate non-, mono-, di-, tri-, and tetrasialylated $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$, respectively

TABLE I

Fractionation of asialo-oligosaccharides released from rHuEPO and urinary HuEPO by serial lectin affinity column chromatography

Fractions[a]	Molar percent of the total asialo-oligosaccharide fraction	
	rHuEPO	Urinary HuEPO
$ConA^+AAL^+$	4.1	5.3
$ConA^+AAL^-$	1.9	3.7
$ConA^-DSA^rAAL^+$	4.8	2.4
$ConA^-DSA^rAAL^-$	4.1	1.2
$ConA^-DSA^+AAL^+$	76.6	74.5
$ConA^-DSA^+AAL^-$	8.5	12.9

[a] The symbols represent the bound (+), the passed-through (−), and the retarded (r) fractions, respectively.

around the area corresponding to monosialyl oligosaccharides and were then converted to neutral oligosaccharides by mild methanolysis (0.05 N HCl-methanol, 37 °C, 4 h). From the analysis of each acidic fraction the oligosaccharides, which were partially sensitive to sialidase and susceptible to the methanolysis, were shown to be derived from the small parts of A-3 to A-6 (6–10% of each fraction) and from the major part of A-7 (95% of this fraction) (data not shown). Thus, the result suggested the presence of small amounts of oligosaccharides containing both sialic acid residues and possibly sulfate group. The structures of their neutral portions were common to those of sialidase-sensitive acidic oligosaccharides (data not shown). However, no detailed analysis of them to assign the location of sialic acid and sulfate residues was performed because of the limited amounts of samples.

Fractionation of Asialo-oligosaccharides—The neutral oligosaccharide fractions obtained by sialidase digestion were fractionated by a serial lectin affinity column chromatography. The sample was first applied to a column of ConA-Sepharose. The fraction which passed through the column was then separated into a retarded fraction and a bound fraction by passing through a DSA-Sepharose column. Each of these three fractions, thus obtained, was finally fractionated by a column of AAL-Sepharose. The proportion of each fraction obtained from rHuEPO and urinary HuEPO was summarized in Table I. There were quite similar points between two samples. The first is that the major parts of oligosaccharides were recovered in the $ConA^-DSA^+$ fraction in common: 85.1%

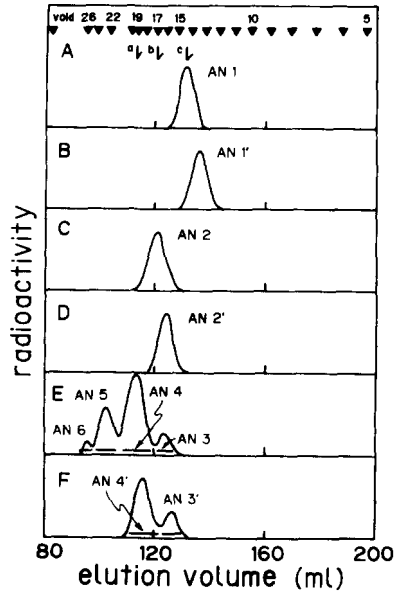

FIG. 2. **Bio-Gel P-4 column chromatography of asialo-oligosaccharides.** Asialo-oligosaccharides of rHuEPO, fractionated with lectin columns as shown in Table I, were analyzed by Bio-Gel P-4 column chromatography. *Panel A*, the $ConA^+AAL^+$ fraction; *panel B*, the $ConA^+AAL^-$ fraction; *panel C*, the $ConA^-DSA^rAAL^+$ fraction; *panel D*, the $ConA^-DSA^rAAL^-$ fraction; *panel E*, the $ConA^-DSA^+AAL^+$ fraction; *panel F*, the $ConA^-DSA^+AAL^-$ fraction. *Arrows a*, *b*, and *c* indicate the elution positions of authentic $Gal_4 \cdot GlcNAc_4 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$, 2,4-branched $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$, and $Gal_2 \cdot GlcNAc_2 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$, respectively. *Black triangles* indicate the elution positions of glucose oligomers (the *numbers* indicate the glucose units).

of the total asialo-oligosaccharides for rHuEPO and 87.4% for urinary HuEPO. The AAL-bound (AAL^+) fraction was also predominant in both samples: 85.6% for rHuEPO and 82.2% for urinary HuEPO, indicating that large parts of oligosaccharides are fucosylated at their core.

Each fraction was then analyzed by a column of Bio-Gel P-4. Since no qualitative difference in elution profile was detected between the two samples, only the results obtained from rHuEPO were shown in Fig. 2. Four fractions except for the DSA^+ fraction were eluted as single components (Fig. 2, *A–D*). The $ConA^-DSA^+AAL^+$ and AAL^- fractions were composed of four and two components, respectively (Fig. 2, *E* and *F*). The components thus separated were termed as AN1 to AN6 for the AAL^+ fractions and as AN1′ to AN4′ for the AAL^- fractions as shown in Fig. 2.

Structural Analysis of the Sugar Chains Obtained from rHuEPO and Urinary HuEPO—Structures of the oligosaccharides were studied by exoglycosidase digestions, Smith degradation, and methylation analysis as described in detail in the Miniprint Section of this paper and proposed as listed in Table II.

There was no qualitative difference in the structures of asialo-oligosaccharides between rHuEPO and urinary HuEPO. Both samples contain tetraantennary sugar chains (AN4 and AN4′) as major components: 46% for rHuEPO and 59.9% for urinary HuEPO. The tetraantennary sugar chains with *N*-acetyllactosamine repeating units (AN5 and AN6) were also detected in both samples, but their contents were much higher in rHuEPO (34.5%) than in urinary HuEPO (7.5%). The smaller portion of oligosaccharides (17.7% for rHuEPO and 32.6% of urinary HuEPO) had biantennary and 2,4-branched and 2,6-branched triantennary sugar chain structures.

TABLE II

Structures of the asparagine-linked sugar chains of rHuEPO and urinary HuEPO

Fucα1
↓
6

R = GlcNAcβ1→4GlcNAc$_{OT}$. R′ = GlcNAcβ1→4GlcNAc$_{OT}$. *, urinary HuEPO had α2→3 and α2→6 linkages; **, *the locations of the N*-acetyllactosamine repeating units in the sugar chains of urinary HuEPO were not determined.

Structures	Asialooligosaccharide fraction	Percent molar ratio			
		rHuEPO		Urinary HuEPO	
		R	R′	R	R′
*(NeuAcα2→3)$_{1-2}$ Galβ1→4GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1→2Manα1↗3 Manα1→4R/R′	AN1/1′	4.1	1.9	5.3	3.7
*(NeuAcα2→3)$_{1-3}$ Galβ1→4GlcNAcβ1→2Manα1↘6 / Galβ1→4GlcNAcβ1↘4 Manα1↗3 / Galβ1→4GlcNAcβ1↗2 Manα1→4R/R′	AN2/2′	4.8	4.1	2.4	1.2
*(NeuAcα2→3)$_{1-3}$ Galβ1→4GlcNAcβ1↘6 Manα1↘6 / Galβ1→4GlcNAcβ1↗2 / Galβ1→4GlcNAcβ1→2Manα1↗3 Manβ1→4R/R′	AN3/3′	2.4	2.1	15.1	4.9
*(NeuAcα2→3)$_{1-4}$ Galβ1→4GlcNAcβ1↘6 Manα1↘6 / Galβ1→4GlcNAcβ1↗2 / Galβ1→4GlcNAcβ1↘4 Manα1↗3 / Galβ1→4GlcNAcβ1↗2 Manβ1→4R/R′	AN4/4′	39.6	6.4	51.9	8.0
*(NeuAcα2→3)$_{1-3}$ **Galβ1→4GlcNAcβ1→3 { Galβ1→4GlcNAcβ1↘6 Manα1↘6 / Galβ1→4GlcNAcβ1↗2 / Galβ1→4GlcNAcβ1↘4 Manα1↗3 / Galβ1→4GlcNAcβ1↗2 } Manβ1→4R	AN5	30.2	0	6.9	0
*(NeuAcα2→3)$_{1-2}$ { **Galβ1→4GlcNAcβ1→3 Galβ1→4GlcNAcβ1↘6 Manα1↘6 / **Galβ1→4GlcNAcβ1→3 Galβ1→4GlcNAcβ1↗2 / Galβ1→4GlcNAcβ1↘4 Manα1↗3 / Galβ1→4GlcNAcβ1↗2 } Manβ1→4R	AN6	4.3	0	0.6	0

The number of sialic acid residues in each acidic component was analyzed as follows. When sialidase digests of the acidic fractions as shown in Fig. 1 were separately subjected to Bio-Gel P-4 column chromatography, it was shown that the tetraantennary oligosaccharide AN4 is included in the asialo-oligosaccharides of A-2, -3, -5, and -6, respectively (data not shown). Thus, the result and the mobility of each acidic fraction relative to authentic oligosaccharide standards indicated that one to four sites of AN4 are sialylated. Other oligosaccharides were also analyzed in the same way (data not shown). Methylation analysis of the oligosaccharide fraction of rHuEPO before (A) and after (AN) sialidase treatment indicated that almost all sialyl residues are linked at the C-3 position of galactosyl residues (Table III). This is in agreement with the fact that all the sialic acid residues of rHuEPO were susceptible to Newcastle disease virus sialidase (data not shown), which specifically cleaves the Siaα2→3Gal linkage. In contrast, approximately 40% of sialyl linkages were resistant to this viral sialidase in the case of urinary HuEPO (data not shown), indicating that other linkages are involved. Methylation analysis of oligosaccharide mixtures obtained from urinary HuEPO before and after sialidase treatment revealed that sialic acids in these oligosaccharides are linked at the C-3 and C-6 positions of galactose residues (data not shown). Therefore, the sialyl linkages resistant to the viral sialidase are considered to occur as the NeuAcα2→6Gal group.

DISCUSSION

Several glycoproteins have recently been produced by using recombinant techniques (15–17). To our knowledge, however, this is the first case to have analyzed comparatively the naturally occurring and the biotechnologically produced glycoproteins on the fine structural basis.

Both rHuEPO and urinary HuEPO contained tetraantennary oligosaccharides as major components. The residual oligosaccharides had biantennary and triantennary sugar chain structures. Both samples were also rich in fucosylated oligosaccharides. In addition to these similarities, some differences were also found in the sugar chains of the two erythropoietin samples. The total amount of complex-type oligosaccharides with *N*-acetyllactosamine repeating units in their outer chain moieties accounted for 34.5% in the case of rHuEPO, which was approximately five times higher than that of urinary HuEPO (7.5%). All sialic acid residues in the sugar chains of rHuEPO occur as the NeuAcα2→3Gal group, while about 60% of those of urinary HuEPO occur as the

TABLE III

Methylation analysis of acidic (A) and asialo-oligosaccharide (AN) fractions derived from rHuEPO

	Molar ratio[a]	
	A	AN
Fucitols		
2,3,4-Tri-*O*-methyl (1,5-di-*O*-acetyl)	0.8	0.9
Galactitols		
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	0.8	3.3
2,4,6-Tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	3.0	0.6
Mannitols		
3,4,6-Tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	0.2	0.3
3,6-Di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	0.9	0.9
3,4-Di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	0.8	0.8
2,4-Di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.0	1.0
2-N-Methylacetamido-2-deoxyglucitols		
1,3,5,6-Tetra-*O*-methyl (4-mono-*O*-acetyl)	0.2	0.2
3,6-Di-*O*-methyl (1,4,5-tri-*O*-acetyl)	5.0	5.0
1,3,5-Tri-*O*-methyl (4,6-di-*O*-acetyl)	1.0	1.0

[a] Numbers were calculated by taking the value for 2,4-di-*O*-methylmannitol as 1.00.

NeuAcα2→3Gal group and the others occur as the NeuAcα2→6Gal linkages. Despite these dissimilarities, the most important evidence is that all the oligosaccharides found in rHuEPO were included in urinary HuEPO. The absence of unusual sugar chains in rHuEPO is favorable for the clinical application of this hormone, since we do not need to take any account of antigenicity on its sugar moiety. It has been shown that sialidase digestion of urinary HuEPO results in the loss of its biological activity *in vivo* because of hepatic clearance (10, 11). Therefore, the fact that rHuEPO contained no neutral oligosaccharides might also be important for its clinical application.

The functional role of the sugar moiety of erythropoietin has not been resolved well, although its physiological significance was suggested by several studies. Sialic acid residues of erythropoietin are important not only for escaping from the hepatic clearance system of asialoglycoproteins but also may contribute to the conformational stabilization, since asialoerythropoietin becomes sensitive to heat denaturation and trypsin digestion (12). Desialylation does not decrease the *in vitro* activity of EPO but rather stimulates it when assayed at low concentration (12, 13). Thus, it is possible that the degree of sialylation affects the physical and biological properties of this glycoprotein. Recently, Dordal *et al.* (14) have shown that digestion of erythropoietin with endoglycosidase F or mixed glycosidases from *Diplococcus pneumoniae* results in the complete loss of its *in vivo* activity, but approximately 50% of its activity *in vitro* and immunoreactivity still remain. It has been suggested that the sugar chains are located at or near the binding domain of erythropoietin for its target cells (39). Therefore, it is likely that the sugar moiety of erythropoietin contributes to its biological function. Availability of the sufficient amount of rHuEPO and the structural information of its sugar moiety as obtained in this study will help us to resolve the functional roles of the sugar moiety of erythropoietin in the future.

Glycosylation of proteins primarily depends on the level of glycosyltransferases in the cells and is also affected by their primary amino acid sequences. The difference in sialyl linkages detected in the two erythropoietin samples may be the result of the former situation. In accordance with rHuEPO, G protein of vesicular stomatitis virus grown in CHO cells (44, 45) and recombinant γ-interferon produced in CHO cells (17) have been shown to contain only the NeuAcα2→3Gal linkage. Therefore, the exclusive expression of this sialyl linkage may indicate that CHO cells lack CMP-NeuAc:Galβ1→4GlcNAcα2→6sialyltransferase. All of the oligosaccharides detected in rHuEPO were also found in urinary HuEPO. This results also suggests the similarity of biosynthetic background between CHO cells and erythropoietin-producing cells in the human kidney. Alternatively, large parts of sugar chain structures may be regulated by the primary amino acid sequence of peptide. The major oligosaccharides of rHuEPO have tetraantennary structure, and considerable amounts of *N*-acetyllactosamine repeating structures were detected. However, such structures have been detected neither in γ-interferon produced in CHO cells (17) nor in vesicular stomatitis virus grown in CHO cells (40, 41). Instead, γ-interferon exclusively expressed biantennary structure, and vesicular stomatitis virus expressed biantennary and 2,6-branched triantennary structures. On the basis of these results, it seems likely that the primary amino acid sequence of polypeptide moiety is another important factor controlling the synthesis of sugar chains.

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Supplemental Material to: Comparative Study of the Asparagine-linked Sugar Chains of Human Erythropoietins Purified from Urine and the Culture Medium of Recombinant Chinese Hamster Ovary Cells

by

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EXPERIMENTAL PROCEDURES

Chemicals, Enzymes, and Lectins———NaB[^{3}H]$_4$ (340 mCi/mmol) was purchased from New England Nuclear, Boston, MA. NaB[^{2}H]$_4$ and sialidase from *Arthrobacter ureafaciens* (18) were purchased from Nakarai Chemicals, Co. Ltd., Kyoto. β-Galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase were purified from jack bean meal by the method of Li and Li (19). Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were purified from the culture fluid of *Diplococcus pneumoniae* according to the method of Glasgow *et al.* (20). Endo-β-galactosidase from *Flavobacterium keratolyticus* (21), α-mannosidase II from *Aspergillus saitoi* (22), and α2→3 sialidase from Newcastle disease virus (23) were purified according to the cited references. *Aleuria aurantia* lectin (AAL) and *Datura stramonium* agglutinin (DSA) were purified by the method of Kochibe *et al.* (24) and Yamashita *et al.* (25), respectively, and immobilized by coupling to Sepharose 4B (26). The amounts of AAL and DSA coupled to 1 ml of the gel were 5 mg and 4 mg, respectively. Con A-Sepharose 4B were purchased from Pharmacia, Uppsala.

Digestion with Newcastle disease virus sialidase, which specifically cleaves the sialyl-α2→3-Gal linkage, was performed in 50 μl of 0.1 M cacodylate buffer, pH 6.0, containing 1–10 × 10^4 cpm of acidic oligosaccharides and 28 munits of the enzyme. After incubation at 37°C for 2 h, the reaction was stopped with the addition of 500 μl ethanol. Other exoglycosidase digestions were performed as reported in the previous papers (27, 28).

Preparation of EPO———rHuEPO was purified from the media of recombinant CHO cells culture by the method of Davis (45). Urinary HuEPO was concentrated from urine of aplastic anemia patients by the phenol p-amino salicylate treatment followed by ethanol precipitation according to the method of Miyake *et al.* (6), and purified by affinity chromatography using an immobilized monoclonal antibody column. Each preparation showed single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Release of the Asparagine-Linked Sugar Chains as Oligosaccharides from HuEPO——— Ten milligrams of rHuEPO, and three milligrams of urinary HuEPO were subjected to hydrazinolysis as described previously (29). The oligosaccharide fractions were freed from the mucin-type oligosaccharides by paper chromatography using solvent II. One-fifth of the oligosaccharide fraction from rHuEPO and a half of that from urinary HuEPO were labelled with tritium by NaB[^{3}H]$_4$ reduction. The remaining oligosaccharide fraction from rHuEPO was reduced by NaB[^{2}H]$_4$ in order to obtain samples for methylation analysis.

Analytical Methods———High voltage paper electrophoresis (30), Bio-Gel P-4 (minus 400 mesh) column chromatography (31), periodate oxidation (30), affinity chromatographies on an AAL-Sepharose 4B (32), a Con A-Sepharose 4B (33) and a DSA-Sepharose 4B column (25), and mild methanolysis (42) were performed according to the cited references. Methylation analysis of oligosaccharides was performed as described in a previous paper (34), with use of a JEOL DX-300 gas chromatograph-mass spectometer. Paper chromatography was carried out by using three solvent systems: solvent I, buthanol-1:ethanol:water (4:1:1, V/V); solvent II, ethyl acetate:pyridine:acetic acid:water (5:5:1:3); or solvent III, buthanol-1:acetic acid:water (12:3:5). Identification of sialic acid released from HuEPO by Arthrobacter sialidase treatment was performed by paper chromatography using solvent III after NaB[^{3}H]$_4$ reduction (30), and identified as *N*-acetylneuraminic acid.

Oligosaccharides———The following oligosaccharides were obtained according to the cited references. Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα1→6(3)[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3(6)]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$[3](Gal$_4$·GlcNAc$_4$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$), Galβ1→4GlcNAcβ1→2Manα1→6(3)[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3(6)]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$](2,4-branched Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$), Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$(Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) and Galβ1→4GlcNAcβ1→2Manα1→6 and 3(Manα1→3 and 6)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$(Gal·GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) were prepared from hamster melanoma tyrosinase (35). GlcNAcβ1→2Manα1→6(3)[GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3(6)]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$(2,4-branched GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) and GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6(3)[GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3(6)]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$(GlcNAc$_4$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) were prepared by jack bean β-galactosidase digestion of Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ and Gal$_4$·GlcNAc$_4$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$, respectively. Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$(Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) and Manα1→6(3)[GlcNAcβ1→4Manα1→3(6)]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$(GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) were also prepared from Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ and 2,4-branched Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ by digestion with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase, respectively. GlcNAcβ1→2(GlcNAcβ1→6)Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$(2,6-branched GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) was from rat α$_1$-acid glycoprotein (36). Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→4 and 3GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$(Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) was obtained from fetuin (37).

RESULTS

Methylation Analysis———Since the amounts of oligosaccharides were limited, methylation analysis of each oligosaccharide could not be performed. In order to confirm the rationality to use the substrate specificities of exoglycosidases for the structural studies of oligosaccharides, methylation analysis of the neutral oligosaccharide fraction (fraction AN) obtained by sialidase treatment of the total oligosaccharides released from rHuEPO and urinary HuEPO were performed. Deuterium-labeled oligosaccharide mixture was digested exhaustively with *Arthrobacter ureafacience* sialidase and then subjected to paper electrophoresis at pH 5.4. Oligosaccharides in the area of neutral oligosaccharide fraction were eluted with water and subjected to methylation analysis. As summarized in Table III, ten partially-*O*-methylated alditols and aminoalditols were detected. The data indicated that all fucose residues of the neutral oligosaccharides occur as non-reducing termini. Most of the galactose residues are also located at non-reducing end of oligosaccharides but small amount of them are included within the sugar chain by →3Galβ1→ linkage, suggesting the presence of *N*-acetyllactosamine repeating units in some oligosaccharides. Mannose residues occur in four forms: →2Manα1→, $^{\rightarrow 6}_{\rightarrow 2}$Manα1→, $^{\rightarrow 6}_{\rightarrow 3}$Manα1→ and $^{\rightarrow 6}_{\rightarrow 3}$Manβ1→. Complete absence of 2-mono-*O*-methyl mannitol indicated that no bisected complex-type oligosaccharide was included. *N*-Acetylglucosamine residues occur in three forms: →4GlcNAc$_{OD}$, $^{\rightarrow 6}_{\rightarrow 4}$GlcNAc$_{OD}$ and →4GlcNAc1→. These data indicated that the oligosaccharides in the neutral fraction contain no unusual structural linkage.

Structural Analysis of Oligosaccharides in Fractions AN1 and AN1'———Sequential digestion with jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase converted asialo-oligosaccharide AN1 to the trimannosyl core oligosaccharide, Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$, by releasing two residues each of galactose and *N*-acetylglucosamine, respectively (data not shown). The same hexasaccharide core was also obtained after digestion with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase, indicating that the structure of the oligosaccharide in fraction AN1 is the fucosylated biantennary complex-type as shown in Table II. When radioactive AN1' was digested either sequentially with jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase or with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase, it was converted to another trimannosyl core, Man$_3$·GlcNAc·GlcNAc$_{OT}$ (data not shown). Therefore, the oligosaccharide in this fraction should have the structure as shown in Table II. The structural relationship found between AN1 and AN1' was also observed between AN2 to 4 and AN2' to 4', respectively (data not shown). Therefore, only the results obtained from the studies of AN2 to 6 will be described in the following part of this paper.

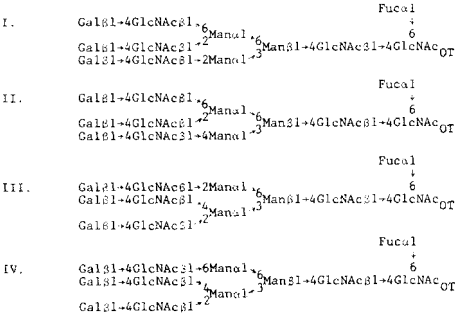
Structural Analysis of Oligosaccharide in Fraction AN2———Asialo-oligosaccharide AN2 was eluted at the same position as authentic 2,4-branched Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ upon Bio-Gel P-4 column chromatography (Fig. 2C). When AN2 was digested with jack bean β-galactosidase, three galactosyl residues were removed (Fig. 3A). The resulting oligosaccharide was finally converted to the fucosylated trimannosyl core with the release of two *N*-acetylglucosamine residues by diplococcal β-*N*-acetylhexosaminidase digestion (Fig. 3B) and then one *N*-acetylglucosamine residue by jack bean β-*N*-acetylhexosaminidase digestion (Fig. 3C). On the basis of the substrate specificity that diplococcal β-*N*-acetylhexosaminidase cleaves GlcNAcβ1→2 linkages in the GlcNAcβ1→2Man group and the GlcNAcβ1→2(GlcNAcβ1→4)Man group but not in the GlcNAcβ1→2(GlcNAcβ1→6)Man group (38), AN2 is supposed to have the 2,4-branched triantennary sugar chain structure. That AN2 was recovered as the retarded fraction from a DSA-Sepharose column also supported this structure, because 2,4-branched triantennary oligosaccharide retards in the DSA-Sepharose column while 2,6-branched triantennary oligosaccharide strongly binds to the column (25).

The location of the 2,4-branch was determined by two cycles of Smith degradation. By the first cycle of Smith degradation, radioactive AN2 was converted to radioactive GlcNAcβ1→2(GlcNAcβ1→4)Manα1→3 or 6Manβ1→4GlcNAcβ1→4 GlcNAc$_{OT}$. It was then converted to Man$_2$·GlcNAc·XylNAc$_{OT}$ by the second cycle of Smith degradation (data not shown). These results indicated that the 2,4-branch is located on the Manα1→3 side. Therefore, the structure of AN2 should be as shown in Table II.

Structural Analysis of Oligosaccharide in Fraction AN3———Asialo-oligosaccharide AN3 was supposed to have 2,6-branched triantennary sugar chain structure on the basis of its elution position upon Bio-Gel P-4 column chromatography (Fig. 2E) and of its strong affinity to a DSA-Sepharose column. This assumption was confirmed by the following experiments. When AN3 was digested with jack bean β-galactosidase, three galactose residues were released (Fig. 4A). The radioactive product was then digested with diplococcal β-*N*-acetylhexosaminidase. As shown in Fig. 4B, one *N*-acetylglucosamine residue was released by this treatment. The resulting radioactive oligosaccharide was finally converted to the fucosylated trimannosyl core after digestion with jack bean β-*N*-acetylhexosaminidase by releasing two *N*-acetylglucosamine residues (Fig. 4D). These results coincide with the digestion pattern of 2,6-branched triantennary sugar chains as already reported (38). When the radioactive product in Fig. 4B was digested with α-mannosidase II from *Aspergillus saitoi*, one mannose residue was removed (Fig. 4C). Since this enzyme remove a mannose from the GlcNAcα 1→6(GlcNAcβ1→2)Manα1→6(Manα1→3)Manβ1→4 GlcNAc--- but not from the GlcNAcβ1→6(GlcNAcβ1→2)Manα1→3(Manα1→6)Manβ1→4 GlcNAc--- (22), it was concluded that the 2,6-branch is exclusively located on the Manα1→6 arm of the trimannosyl core.

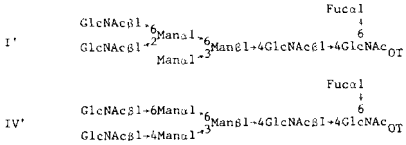
Structural Analysis of Oligosaccharide in Fraction AN4———Jack bean β-galactosidase digestion of AN4 resulted in the release of four galactosyl residues (Fig. 5A). The radioactive product was then converted to the fucosylated trimannosyl core with release of one and three *N*-acetylglucosamine residues by sequential digestion with diplococcal and jack bean β-*N*-acetylhexosaminidase, respectively (Fig. 5B and C). These results indicated that the structure of AN4 is Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα1→6 or 3(Galβ1→4 GlcNAcβ1→4(GlcNAcβ1→4GlcNAcβ1→2)Manα1→3 or 6)Manβ1→4 GlcNAcβ1→4(Fucα1→6) GlcNAc$_{OT}$. Two cycles of Smith degradation of AN4 gave the same radioactive products as in the case of AN2 (data not shown), indicating that the 2,4-branch is exclusively located on the Manα1→3 arm of the trimannosyl core. Therefore, AN4 should have the structure as shown in Table II.

Structural Analysis of Oligosaccharides in Fraction AN5———Sequential digestion of AN5 with jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase released four residues each of galactose and *N*-acetylglucosamine (data not shown). The radioactive product at this stage showed the same mobility in Bio-Gel P-4 column as authentic Gal·GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ (data not shown). It was then converted to the fucosylated trimannosyl core by the second cycle of sequential digestion with the two enzymes releasing one residue each of galactose and *N*-acetylglucosamine (data not shown). These results indicated that AN5 is a tetraantennary complex-type oligosaccharide with the fucosylated trimannosyl core, an outer chain of which is the Galβ 1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→. A trisaccharide residue: Galβ1→4GlcNAcβ1→3 Gal was released from this di-*N*-acetyllactosamine moiety by endo-β-galactosidase digestion (Fig. 6A). The newly exposed *N*-acetylglucosamine residue was then removed by jack bean β-*N*-acetylhexosaminidase digestion (Fig. 6B). The following four possible structures were considered for the radioactive product in Fig. 6B.



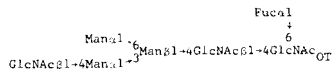
Digestion of the radioactive product in Fig. 6B with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase produced two radioactive components α and β in a molar ratio of 1:2 (Fig. 6C). Both of these components were converted to the fucosylated trimannosyl core by jack bean β-*N*-acetylhexosaminidase digestion releasing two and one *N*-acetylglucosamine residue, respectively (Fig. 6D).

Based on the substrate specificity of diplococcal β-*N*-acetylhexosaminidase (38), the radioactive component α in Fig. 6C should be either one or both of the following two oligosaccharides I' and IV', which were to be derived from oligosaccharides I and IV, respectively.



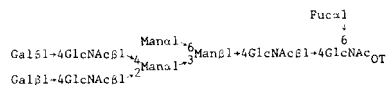
Digestion with jack bean α-mannosidase (2 units) released no mannose from the component α (data not shown), indicating that it contains oligosaccharide IV' but no oligosaccharide I'.

The radioactive component β should have the following structure, which were to be derived from oligosaccharide III.



These results indicated that the Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→ outer chain is located at the C-6 position and the C-2 position of the Manα1→6 arm of the trimannosyl core as shown in Table II.

Structural Analysis of Oligosaccharide in Fraction AN6———By sequential digestion with jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase, four residues each of galactose and *N*-acetylglucosamine were released from radioactive AN6 (data not shown). The product was then converted to the fucosylated trimannosyl core by the second cycle of sequential digestion with the two enzymes releasing two residues each of galactose and *N*-acetylglucosamine (data not shown). These results indicated that the fraction AN6 contains tetraantennary oligosaccharides with two Galβ1→4GlcNAcβ1→3Galβ1→4 GlcNAcβ1→ outer chains. When AN6 was digested with endo-β-galactosidase, its size was decreased by approximately 9 glucose units indicating that two Galβ1→4GlcNAcβ1→3Gal groups were released by this treatment (Fig. 7A). Two residues of newly exposed *N*-acetylglucosamine were then removed by digestion with jack bean β-*N*-acetylhexosaminidase (Fig. 7B). The radioactive product at this stage showed the same size as authentic Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc· Fuc·GlcNAc$_{OT}$. Because one mannose residue was removed from the radioactive product by incubation with 2 units of jack bean α-mannosidase (Fig. 7C), but not by incubation with α-mannosidase II from *Aspergillus saitoi* which cleaves non-substituted Manα1→3 linkge but not non-substituted Manα1→6 linkage (22), the structure of the radioactive peak in Fig. 7B was estimated as follow:



This estimation was further confirmed by the following experimental evidences. Digestion of the radioactive product in Fig. 7B with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase released two galactose residues and one *N*-acetylglucosamine residue (Fig. 7D), and the remaining one *N*-acetylglucosamine residue was removed by incubation with jack bean β-*N*-acetylhexosaminidase to produce the fucosylated trimannosyl core (Fig. 7E).

Based on the data so far described, it was concluded that the two Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→ outer chains are located at the C-2 and the C-6 positions of the Manα1→6 arm as shown in Table II.

Structures of Acid Oligosaccharides———In order to obtain the information as to the sialyl linkages, methylation analysis of the total acidic oligosaccharide fraction (fraction A) was performed. As shown in Table III1, the fraction gave almost the same results as asialo-oligosaccharide fraction (AN) except for galactitol derivatives. Approximately 20% of the galactose of fraction A occur as non-reducing termini and the remainder as →3Gal1→. The amount of the 2,4,6-tri-O-methyl galactitol was prominently decreased after desialylation. The decrease was compensated by the increase of 2,3,4,6-tetra-O-methyl galactitol. These results indicated that almost all sialic acid are linked at the C-3 position of the terminal galactose residues of the neutral oligosaccharides, the structures of which were elucidated by the studies described already (Table II).

Methylation analysis of the oligosaccharide fraction obtained from urinary HuEPO before and after sialidase digestion revealed that difference was detected only in galactose derivatives: 2,3,4- and 2,4,6-tri-O-methyl galactitols were converted to 2,3,4,6-tetra-O-methyl galactitols (data not shown). These results indicated that sialic acids in this sample are linked both at the C-3 and C-6 positions of the terminal galactose.

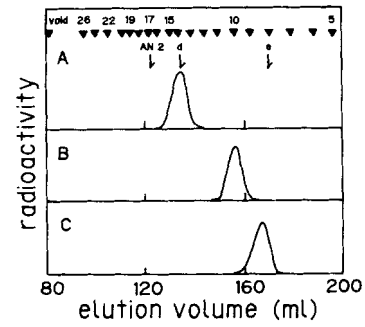


Fig. 3. Sequential glycosidase digestion of AN2. The elution patterns of AN2 from a Bio-Gel P-4 column after sequential digestions with jack bean β-galactosidase (panel A), diplococcal β-*N*-acetylhexosaminidase (panel B), and jack bean β-*N*-acetylhexosaminidase (panel C) are shown. The elution positions of AN2, 2,4-branched GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ (d), and Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ (e) are indicated by arrows. Black triangles are the same as in Fig. 2.

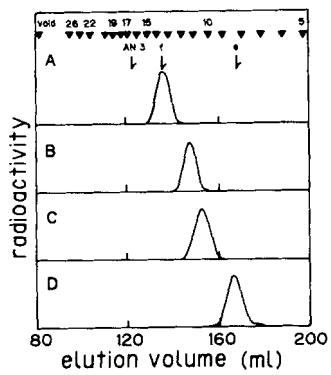


Fig. 4. Sequential glycosidase digestion of AN3. The elution patterns of AN3 from a Bio-Gel P-4 column after sequential digestions with jack bean β-galactosidase (panel A), diplococcal β-*N*-acetylhexosaminidase (panel B), and jack bean β-*N*-acetylhexosaminidase (panel D) are shown. Panel C indicates the elution pattern of the radioactive product in panel B after digestion with aspergillus α-mannosidase II. The elution positions of AN3 and 2,6-branched GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ (f) were indicated by arrows. Black triangles and the arrow with symbol e are the same as in Fig. 3.

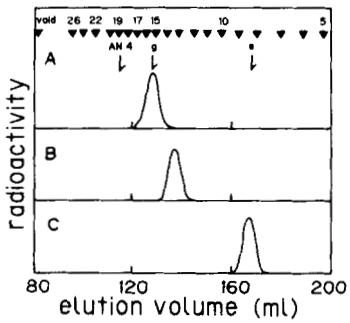


Fig. 5. Sequential glycosidase digestion of AN4. The elution patterns of AN4 on Bio-Gel P-4 column after sequential digestions with jack bean β-galactosidase (panel A), diplococcal β-*N*-acetylhexosaminidase (panel B), and jack bean β-*N*-acetylhexosaminidase (panel C) are shown. The elution positions of AN4 and $GlcNAc_4 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$ (g) were indicated by arrows. Black triangles and the arrow with symbol e are the same as in Fig. 3.

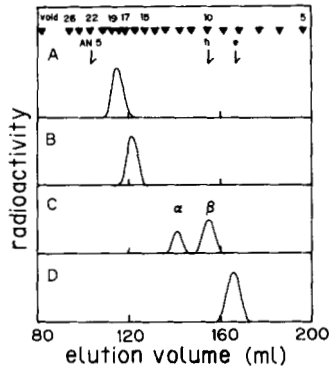


Fig. 6. Sequential glycosidase digestion of AN5. The elution patterns of AN5 from a Bio-Gel P-4 column after sequential digestions with endo-β-galactosidase (panel A), jack bean β-*N*-acetylhexosaminidase (panel B), a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase (panel C), and jack bean β-*N*-acetylhexosaminidase (panel D) are shown. The peaks in panel C were named α and β as indicated. The elution positions of AN5 and $GlcNAc \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$ (h) were indicated by arrows. Black triangles and the arrow with symbol e are the same as in Fig. 3.

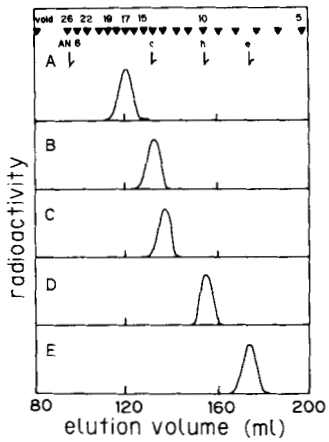


Fig. 7. Sequential glycosidase digestion of AN6. The elution patterns of AN6 from a Bio-Gel P-4 column after sequential digestions with endo-β-galactosidase (panel A), jack bean β-*N*-acetylhexosaminidase (panel B), a mixutre of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase (panel D) and jack bean β-*N*-acetylhexosaminidase (panel E) are shown. Panel C indicates the elution pattern of the radioactive product in panel B after digestion with 2 units of jack bean α-mannosidase. The elution position of AN6 was indicated by an arrow. Black triangles and arrows with symbols c, e and h are the same as in Figs. 2 and 6.