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Microheterogeneity of the Carbohydrate Moiety of the Human Erythrocyte Glucose Transporter

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The carbohydrate moiety of the human erythrocyte glucose transporter was isolated using two independent methods: hydrazinolysis and *N*-glycanase treatment. The major structure observed was constituted of complex-type carbohydrate chains carrying repetitive units of *N*-acetyllactosamine. This structure exhibited microheterogeneity: a broad variability in the number of repetitive units, presence of branched structures and substitution by fucosyl residues. Moreover, significant amounts of bi-antennary and hybrid structures were present.

The selective transport of glucose across the erythrocyte membrane is carried out by a 55 kDa glycoprotein, which migrates as band 4.5 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of red blood cell membranes [1]. Mueckler *et al.* [2] described the complete structure of the erythrocyte-type glucose transporter. It is a fully integrated membrane glycoprotein having 12 membrane-spanning α-helices carrying only one *N*-glycosidic carbohydrate chain linked to Asn-45. The structure of this chain has never been completely established, but some results have appeared in the literature. It was shown that treatment of the native glycoprotein with endoglycosidase-F [3] led to a marked change in the electrophoretic pattern: the broad band around 55 kDa shifted to a sharp band at 46 kDa. This finding could imply that the carbohydrate chain includes oligo-mannose or bi-antennary type structures [4]. Alternatively, this degradation could be due to the presence of contaminating *N*-glycanase in the enzyme preparation. On the other hand, treatment of the same glycoprotein with endo-β-galactosidase (*Escherichia freundii*) also caused a similar shift of the electrophoretic band 4.5 [5], suggesting a poly-*N*-acetyllactosamine-type structure. The purified glycoprotein [6] was composed of *N*-acetylglucosamine, galactose, mannose, fucose and sialic acid (17% [6] or 30% [7] of the total weight). Moreover, one galactose residue was found for every two *N*-acetylglucosamine residues [8], which is not compatible with the presence of repetitive *N*-acetyllactosamine units. Nonetheless, the calculated sialic acid composition indicated that each branch of the carbohydrate chain carried many sialyl residues.

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From all these results, we could consider that the carbohydrate chain of the human erythrocyte glucose transporter has a more sophisticated structure than expected and thus we decided to analyze this chain using immobilized lectins, ion-exchange chromatography, gel permeation, and specific glycosidases. Some of these results were reported in a preliminary communication [8].

Materials and Methods

Freshly outdated human erythrocytes from several donors were obtained from a blood bank and band 4.5 was purified according to the method of Baldwin *et al.* [9]. Native glycoprotein was electrophoresed on 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (Pierce, Rockford, IL, USA) as described by Laemmli [10]. and characterized by specific photolabeling using tritiated cytochalasin B [11]. Kaplan and Pedersen's procedure [12] was used to measure protein and Bartlett's method to determine phospholipids [13].

The carbohydrate moiety of the human erythrocyte glucose transporter was isolated using two independent methods, hydrazinolysis and *N*-glycanase treatment.

a). Native glycoprotein (0.4 mg) was dried under vacuum and directly subjected to hydrazinolysis according to the method of Takasaki *et al.* [14]. After removing the excess hydrazine and re-*N*-acetylation, oligosaccharides were purified by descending paper chromatography in *n*-butanol/ethanol/water, 4/1/1 by vol, for two days.

b). Native glycoprotein (0.4 mg) was treated with *N*-glycanase. After a 36 h incubation with 10 units of *N*-glycanase (EC 3.5.1.52) from *Flavobacterium meningosepticum* (Genzyme, Boston, MA, USA) in 240 mM phosphate buffer, pH 8.6 (enzyme concentration, 40 units/ml), the deglycosylated material was spun down by ultracentrifugation at 100,000 x *g* for 30 min at 4°C. The supernatant was desalted by passage through ion-exchange resin columns (AG-50 then AG-3) at 4°C and eluted with water.

In both cases, the released carbohydrates were labeled by reducing the terminal *N*-acetylglucosamine with tritiated sodium borohydride [14] and purified by descending paper chromatography on Whatman No. 3 paper in the solvent system, ethyl acetate/pyridine/acetic acid/water, 5/5/1/3 by vol, for 36 h.

Neutral and acidic oligosaccharides were separated by DEAE-cellulose ion-exchange chromatography. The gel was equilibrated in 0. 05 M pyridine acetate buffer, pH 6.0; the sample was applied and eluted with this buffer to give neutral compounds. Acidic compounds were eluted with 0.05 M pyridine acetate buffer, pH 5.8 and then pH 4.0.

Affinity chromatography on columns of immobilized lectins was performed as previously described. Concanavalin A (Con A-Sepharose, Pharmacia, Uppsala, Sweden) as described by Cummings and Kornfeld [15], *Datura stramonium* agglutinin (DSA), Cummings and Kornfeld [16] and Yamashita *et al.* [17] , tomato (*Lycopersicon esculentum*) lectin coupled to Sepharose-4B (TL) as described by Merkle and Cummings [18] and potato (*Solanum tuberosum*) lectin coupled to Sepharose-4B (PL) according to Matsumoto *et al.* [19]. The last three immobilized lectins were purchased from BioCarb, Lund, Sweden.

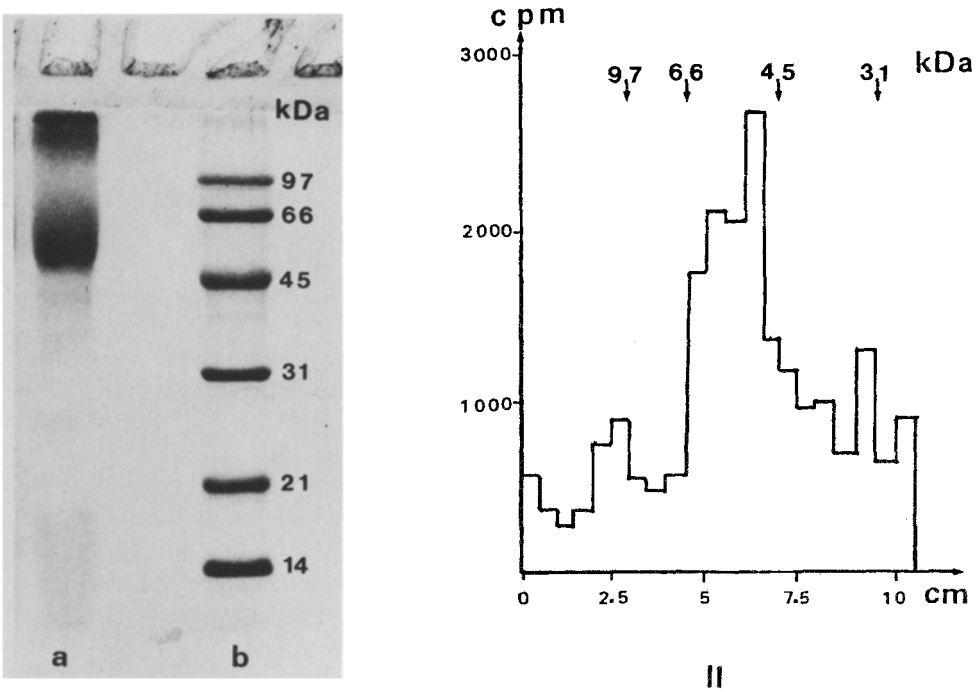

Figure 1. Characterization of the erythrocyte glucose transporter by electrophoresis in 12% polyacrylamide gels containing 0.05% of SDS. Ia; native glycoprotein alkylated as described by Baldwin *et al.* [9]: Ib; standards: II; after photolabeling of the native glycoprotein with tritiated cytochalasin B. The radioactivity per 5 mm slice of the gel is represented.

Neutral and desialylated acidic oligosaccharide alditols were analyzed using high resolution liquid chromatography on Bio-Gel P-4, as described by Yamashita *et al.* [20], with dextran hydrolysate as the internal standard [21]. Each compound was characterized by its molecular weight expressed in glucose units. Standard oligosaccharides, lactose (eluted at two glucose units) and bi-antennary complex-type carbohydrate chains obtained from transferrin (eluted at 13 glucose units) were used for calibration.

The presence of tritiated *N*-acetylglucosaminitol in the different radioactive fractions was checked after acidic hydrolysis. For that purpose, an aliquot of the radioactive sample was hydrolyzed by 3 N aqueous hydrogen chloride for 4 h at 100°C, reacetylated by acetic anhydride in aqueous alkaline medium and analyzed on a Bio-Gel P-4 column. The radioactive peak of the *N*-acetylglucosaminitol appeared at 2.5 glucose units [20].

Carbohydrate moieties were degraded by endo-β-galactosidase (EC 3.2.1.103) from *Bacteroides fragilis* in 50 mM sodium acetate buffer, pH 5.8, in the presence of 0.2 mg/ml of bovine serum albumin. Other enzymatic degradations, α-L-fucosidase (EC 3.2.1.51) from bovine

epididymis, β-galactosidase (EC 3.2.1.23), β-*N*-acetylhexosaminidase (EC 3.2.1.52) and α-mannosidase (EC 3.2.1.24) from jack bean and sialidase (EC 3.2.1.18) from *Vibrio cholerae* were performed as described previously by us and others [22, 23].

Results and Discussion

Isolation of the Carbohydrate Chain

For each preparation, the human erythrocyte glucose transporter was purified from 250 ml of freshly outdated human erythrocytes from which 50 ml of a liposome suspension containing 50 μg/ml of protein and 200 μg/ml of phospholipids were obtained. The homogeneity of the preparation was checked by SDS-PAGE before and after photolabeling with cytochalasin B as shown in Fig. 1.

Hydrazinolysis and enzymatic cleavage by *N*-glycanase were used independently to isolate the carbohydrate moiety of the glucose transporter. After labeling with tritiated sodium borohydride, the radioactive oligosaccharides were purified by descending preparative paper chromatography. As both methods released *N*-linked chains, the only labeled compound should be *N*-acetylglucosaminitol. To make sure that all the radioactivity of the sample was coming from *N*-acetylglucosaminitol, acid hydrolysis was performed on an aliquot of the radioactive material. More than 80% of this material was constituted of tritiated *N*-acetylglucosaminitol. When the carbohydrate moiety was released from the same quantity of glucose transporter, by either treatment, the same amount of radioactivity was recovered after purification. This indicates that neither cleavage nor incomplete release of the carbohydrate chain occurs during the isolation procedure.

Affinity Chromatography

Purified oligosaccharides obtained from several preparations of glucose transporter were independently analyzed by affinity chromatography on a Con A-Sepharose column. As regards oligosaccharides isolated by hydrazinolysis, 93% of the radioactive material was excluded from the Con A column, 3% eluted in the presence of 0.01 M α-methylglucose (bi-antennary structure) and 4% eluted in the presence of 0.3 M α-methylmannose (hybrid-type and oligo-mannose structures). Results observed for oligosaccharides released by the *N*-glycanase are in the same range of values (92%, 4.5% and 3.5%, respectively). Thus, in both cases, the main part of the oligosaccharides was constituted of tri- or tetra-antennary complex-type carbohydrate chains. Nevertheless, significant amounts of bi-antennary, oligo-mannose and/or hybrid type sugar chains were also present.

The fractions excluded from the Con A column were further analyzed. The presence of large carbohydrate chains constituted of repetitive units of *N*-acetyllactosamine was examined by affinity chromatography on specific lectins such as DSA, tomato lectin (TL) and potato lectin (PL). TL had the greatest affinity for oligosaccharides having three *N*-acetyllactosamine units [18], whereas PL exhibited maximum affinity for four units [19]. Chromatography of Con A-excluded oligosaccharides obtained from both methods gave similar results: 59%, 51% and 52% of the oligosaccharides released by *N*-glycanase treatment were retained by DSA, TL

Table 1. DEAE-Cellulose ion-exchange chromatography of samples eluted from a Con A affinity column.
The different samples obtained after Con A affinity chromatography were loaded on a DEAE-cellulose column (2 x 15 cm). The column was equilibrated in 50 mM pyridine acetate buffer, pH 6.3, and eluted with the following buffers: 50 ml of 50 mM pyridine acetate buffer, pH 6.3 (neutral fraction N); 50 ml of 50 mM pyridine acetate buffer, pH 5.3 (acidic fraction A1); 50 ml of 50 mM pyridine acetate buffer, pH 4.0 (acidic fraction A2); 2 ml fractions were collected and elution was monitored by counting the radioactivity in an aliquot of these fractions.

	Fraction N	Fraction A1	Fraction A2
N-Glycanase			
Con A I	70%	21%	9%
Con A II	65%	35%	
Con A III	75%	25%	
Hydrazinolysis			
Con A I	75%	25%	
Con A II	77%	23%	
Con A III	82%	18%	

and PL, respectively; and 63%, 55% and 42% for those released by hydrazinolysis. These results indicate the presence of high amounts of poly-*N*-acetyllactosamine chains in the carbohydrate moiety of the glucose transporter. About 50% of them have three or more repetitive units.

Ion-exchange Chromatography

Each fraction eluted from the Con A column was first desalted by passage through a Bio-Gel P-2 column and then analyzed on a DEAE-cellulose column. The results are shown in Table 1. Acidic compounds were present in all Con A fractions in the following ratios: about one third of the radioactivity in the Con A I and II fractions, and one quarter of the Con A III fraction. Sialidase treatment (*V. cholerae*) of the acidic fractions converted 85% of the radioactivity into neutral fractions, as demonstrated by a second passage through the DEAE-cellulose column (data not shown). The sensitivity of the Con A III fraction to sialidase treatment suggests that this fraction was probably constituted of hybrid-type carbohydrate chains and further analyses were performed to confirm this hypothesis.

For all further analyses, oligosaccharides isolated either by hydrazinolysis or by *N*-glycanase treatment gave similar results and only the data obtained for hydrazinolysed compounds will be given.

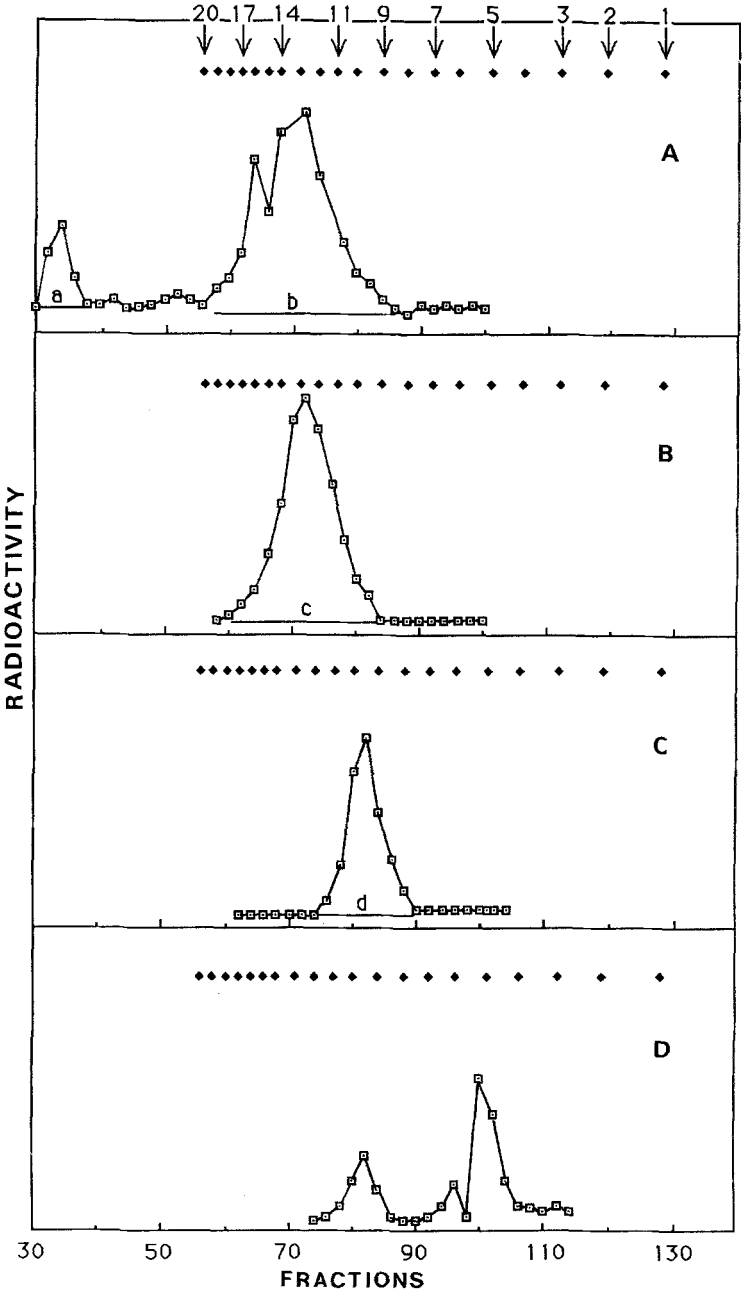

Figure 2. High resolution Bio-Gel P-4 chromatography of the neutral Con A III fraction. The column (2.3 x 100 cm) was kept at 55°C and eluted with water at a flow rate of 0.5 ml/min. The sample was co-chromatographed with dextran hydrolysate which was monitored by differential refractometry. Black arrows in the upper panel of the Figure indicate the number of glucose units. Fractions (2.8 ml) were collected and their radioactivity was determined by liquid scintillation on an aliquot.
A: native fraction. B: treatment of "b" in A with α-mannosidase. C: treatment of "c" in B with a mixture of β-galactosidase, β-*N*-acetylglucosaminidase and α-fucosidase. D: treatment of "d" in C with a mixture of α-mannosidase and β-*N*-acetylglucosaminidase.

Analysis of Neutral and Desialylated Con A II and III Fractions

The neutral and desialylated Con A III fractions were analyzed on a Bio-Gel P-4 column and for both fractions two areas can be delineated (data for the neutral fraction are given in Fig. 2A), one of which was excluded (a) and the other eluted at 17-10 glucose units (b). Acidic hydrolysis performed on an aliquot of the void peak (a) showed that it is devoid of carbohydrate material. Treatment of (b) with jack bean α-mannosidase caused a slight shift of this peak to give (c) (Fig. 2B), but in no instance the appearance of a peak at five glucose units, which would reveal the presence of α-mannosidase-sensitive carbohydrate structures (oligo-mannose). On the contrary, treatment of (c) by a mixture of β-galactosidase, β-*N*-acetylhexosaminidase and α-fucosidase leads to a peak (d) at 10 glucose units, whereas the same treatment performed without α-fucosidase results in a peak at 12 glucose units (data not shown). When the radioactive material in (d) was treated with a mixture of β-*N*-acetylhexosaminidase and α-mannosidase, two peaks appeared at 6.5 and 5.5 glucose units (Fig. 2D), corresponding to the tritiated ManGlcNAcGlcNAc-ol and ManGlcNAc(Fuc)GlcNAc-ol structures. These results suggest that oligosaccharides of the Con A III fractions are constituted of fucosylated and non-fucosylated hybrid-type oligosaccharides having five or four mannose residues, respectively [20, 24], which contribute to the microheterogeneity of the carbohydrate moiety.

Analysis of the neutral and desialylated Con A II fractions on the Bio-Gel P-4 column also showed two areas (data for the neutral fraction are given in Fig. 3A), one of which was excluded from the column (a) and the other eluted at 17-11 glucose units (b). As above, acidic hydrolysis of the void peak (a) did not reveal the presence of any oligosaccharide. The fraction eluted at 17-11 glucose units (b) was treated with jack bean β-galactosidase and two areas (c and d) were obtained on a Bio-Gel P-4 column (Fig. 3B). The first one (c) was eluted with a maximum at 14.5 glucose units, and corresponds to non-degraded material. The presence of fucosyl residues on the carbohydrate chain could explain this absence of degradation. Treatment of the material in (c) by a mixture of β-galactosidase, β-*N*-acetylhexosaminidase and α-fucosidase resulted in a shift of this peak to 10.5 glucose units (Fig. 3C). The same treatment performed without fucosidase did not result in any degradation. The second area (d) has a maximum at 11 glucose units, which could result from the elimination of two galactose residues. This latter peak treated with jack bean β-*N*-acetylhexosaminidase gave a new peak eluting at seven glucose units (Fig. 3D), corresponding to the pentasaccharide $Man_3GlcNAc_2$. From these results, we could conclude that Con A II fractions contained bi-antennary carbohydrate chains, some of them having fucose residues on their antennae.

Analysis of Neutral and Desialylated Con A I Fractions

All the Con A I fractions were excluded from the Bio-Gel P-4 column. They are supposed to be constituted of poly-*N*-acetyllactosamine carbohydrate chains which generally exhibit an affinity for DSA. Nevertheless, as we showed above, only 50% of the radioactive material was retained by this lectin column. Such loss of affinity for DSA could be attributable to some structural parameters, and to investigate this possibility we decided to analyse separately DSA-retained and non-retained fractions. Each isolated fraction was further submitted to endo-β-galactosidase treatment.

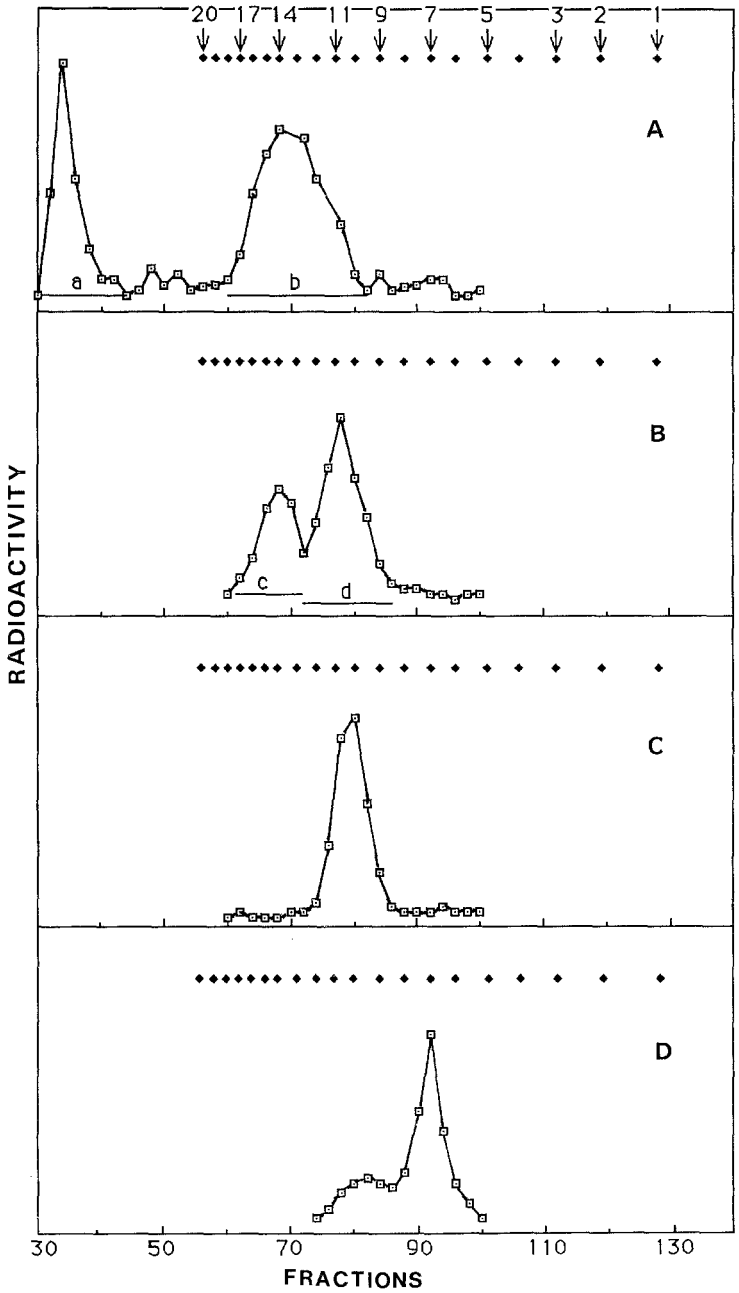

Figure 3. High resolution Bio-Gel P-4 chromatography of the neutral Con A II fraction. The experimental conditions are the same as those reported in Fig. 2.
A: native fraction. B: treatment of "b" in A with β-galactosidase. C: treatment of "c" in B with a mixture of β-galactosidase, β-*N*-acetylglucosaminidase and α-fucosidase. D: treatment of "d" in B with β-*N*-acetylglucosaminidase.

The neutral fraction excluded from the DSA column (Fig. 4A) was partially degraded (27%) by treatment with the endo-β-galactosidase alone. Treatment of the enzyme-resistant material with a mixture of α-fucosidase and endo-β-galactosidase led to a partial degradation, as shown in Fig. 4A, suggesting the presence of highly fucosylated structures in this fraction. In contrast, 81% of the desialylated, DSA excluded fraction was degraded. This degree of degradation was comparable to that observed for the neutral and desialylated DSA-retained fractions (94 and 92%., respectively). A second treatment of the material in the excluded peaks with endo-β-galactosidase did not change the elution profile on the Bio-Gel P-4 column.

After endo-β-galactosidase treatment, two types of peaks were observed on the Bio-Gel P-4 column. The first one exhibiting a maximum at about 13 glucose units was observed for the DSA-excluded fractions and the second having a maximum at 10/11 glucose units was observed for the two DSA-retained fractions. The higher molecular weight of the DSA-excluded fractions could be related to their fucosylation. The elution volume observed for enzyme-treated fractions suggests that they could result from the degradation of bi-antennary carbohydrate chains. If we consider that all the native fractions were excluded from the Con A column, it is possible that the presence of polylactosaminoglycans causes the loss of affinity for the Con A lectin.

Conclusion

The results obtained from several independent experiments using either *N*-glycanase treatment or hydrazinolysis are very similar. The major structure observed for the carbohydrate moiety of the human erythrocyte glucose transporter is constituted of a poly-antennary complex-type sugar chain carrying repetitive units of *N*-acetyllactosamine. Although the erythrocyte glucose transporter has only one glycosylation site, a large structural microheterogeneity is observed for its carbohydrate moiety. Only 50% of the material included more than three repetitive units of *N*-acetyllactosamine. Moreover, the presence of fucosyl residues or of branching structures [GlcNAc β(1-6)-linked] results in a partial resistance to the action of endo-β-galactosidase. Lastly, significant amounts of bi-antennary and hybrid-type carbohydrate chains were observed in different glucose transporter preparations. Such heterogeneous structures were previously observed in desialylated erythrocyte glycopeptides treated with endo-β-galactosidase [25] and in the carbohydrate moiety of the anion transporter (erythrocyte band 3).

What is the biological significance of this microheterogeneity?. Does it play only a decorative role or is it an essential factor in the modulation of transport activity?. In order to obtain some answers to these questions, we are now studying the glucose transport activities of the native, and the partially and fully deglycosylated proteins.

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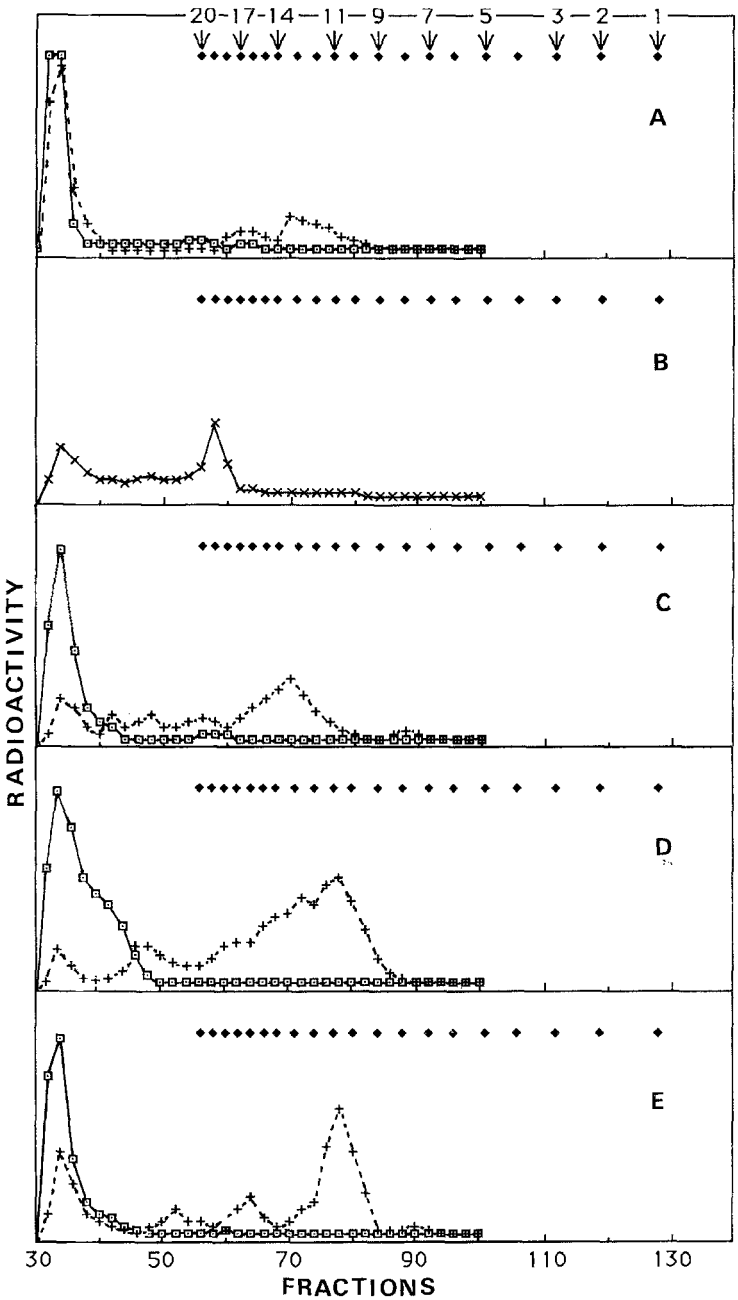

Figure 4. High resolution Bio-Gel P-4 chromatography of the Con A I fractions. The experimental conditions are the same as those reported in Fig. 2.
Elution profiles before and after treatment of these fractions with endo-β-galactosidase. (□); native fractions: (+); endo-β-galactosidase-treated fractions: (x); treatment with a mixture of α-fucosidase and endo-β-galactosidase. A and B; neutral, DSA-excluded fraction. C; desialylated, DSA-excluded fraction. D; neutral, DSA-retained fraction. E; desialylated, DSA-retained fraction.

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