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Note

Studies on structure and heterogeneity of carbohydrate chains of *N*-glycoproteins by use of liquid chromatography. "Oligosaccharide maps" of glycoproteins

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The main difficulty in the investigation of carbohydrate chains of glycoproteins is the necessity to isolate individual oligosaccharide fragments in amounts sufficient for structural studies by chemical and instrumental methods. The solution of this problem is usually complicated by the low availability of many glycoproteins and heterogeneity of their carbohydrate chains. The progress in liquid chromatography under pressure greatly extends our possibilities in this field and facilitates the isolation of individual oligosaccharides. However, liquid chromatography may be utilized not only for the separation of oligosaccharide mixtures, but also for structural information on the basis of chromatographic behavior of oligosaccharides. To realize the latter opportunity, the chromatographic characteristics for as many *N*-linked oligosaccharides as possible should be accumulated and this "bank of chromatographic data" may be used further for structural investigation of carbohydrate chains of "unknown" glycoproteins.

We present herein the chromatographic system and data on separation of a great number of desialylated *N*-oligosaccharides obtained from four well known glycoproteins. A correlation between the structure of the oligosaccharides and their chromatographic behavior is discussed also. In the course of structural studies on *N*-linked nonsialylated carbohydrate chains of influenza virus hemagglutinin[1–3], we have developed a method for the fractionation of oligosaccharides obtained by reductive cleavage of the *N*-glycosylamine linkage of *N*-glycoprotein with lithium borohydride[4]. This new procedure resulted in the formation of a mixture of reduced oligosaccharides corresponding to the native Asn-linked carbohydrate chains of *N*-glycoproteins. We found that their separation may be achieved most efficiently by two consecutive procedures using a reverse-phase (C-8 or C-18) column and an amino column, because some oligosaccharides have identical or very similar elution times on chromatography in the first or the second column.

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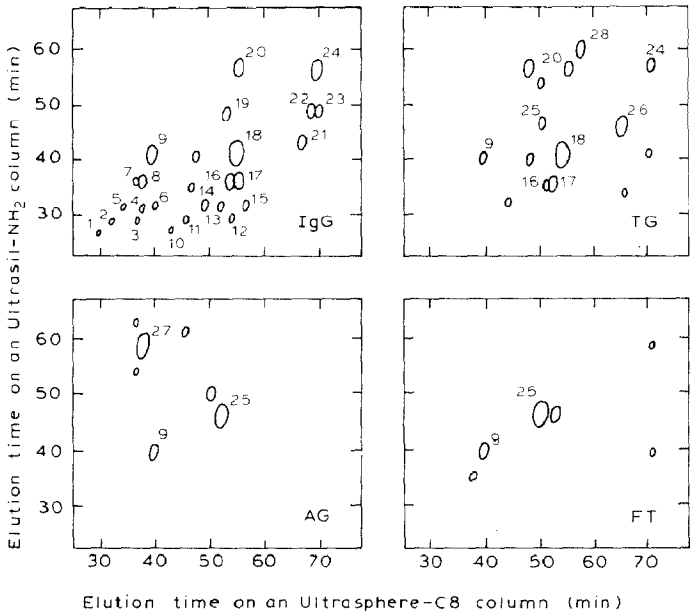


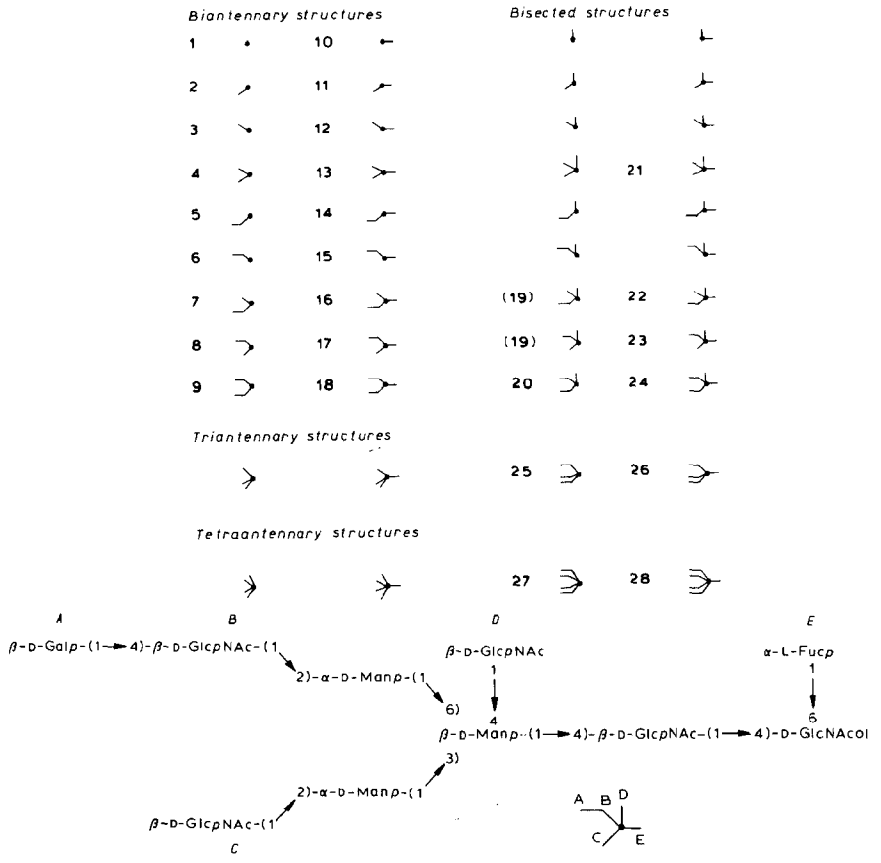
Fig. 1. Oligosaccharide maps of immunoglobulin G (IgG), tyroglobulin (TG), α_1-acid glycoprotein (AG), and fetuin (FT). The size of an oligosaccharide spot qualitatively reflects the relative abundance of the oligosaccharide. The structures of the oligosaccharides enumerated are listed in Scheme 1.

This method was applied further to the study of the chromatographic behavior of oligosaccharides obtained from well characterized glycoproteins, *i.e.*, immunoglobulin G (IgG), thyroglobulin (TG), α_1 acid glycoprotein (AG), and fetuin (FT). The oligosaccharide fractions corresponding to the complex chains of *N*-glycoproteins were obtained by lithium borohydride degradation and purified on a Bio-Gel P-6 column as described previously[3,4]. The mixture of oligosaccharides was fractionated on an Ultrasphere-C8 column (Column I) with u.v. detection, and all the components were collected. Each of them was then fractionated on an Ultrasil-NH_2 column (Column II), and again all the components were collected. As a result, chromatographically homogeneous oligosaccharides were usually obtained, but in some cases repeated chromatography on column I or II was necessary. The oligosaccharides thus isolated were characterized by monosaccharide composition in addition to two elution times on columns I and II.

It is convenient to represent the set of oligosaccharides obtained from each glycoprotein as a two-dimentional map[2,5], the coordinates of which are the elution times on columns I and II. These "oligosaccharide maps" (Fig. 1) give a clear picture of the relative abundance of each oligosaccharide, and of the heterogeneity of carbohydrate chains of a glycoprotein as a whole. When fractionation is carried out under standard conditions, the oligosaccharides having identical chromatographic characteristics and composition, isolated from different glycoproteins, may be considered as identical ones.

The most crucial step is an interpretation of the maps, *i.e.*, the elucidation of the relation between the structure of the oligosaccharide and its location on the map. This was achieved by comparing the literature data on the structure and proportion of carbohydrate chains in the glycoproteins investigated with our data on the relative amounts and composition of the oligosaccharides isolated. Additional proof of the structures assigned was obtained from data of defucosylation or degalactosylation (or both) which resulted in characteristic alteration of the l.c. parameters of the parent oligosaccharides. The interpretation of the oligosaccharide map for IgG may serve as an example.

It is known that IgG contains mainly biantennary and "bisected" chains [β-D-Glc*p*NAc group (1→4)-linked to β-D-Man*p* residue], both fucosylated or not[6]. According to the monosaccharide composition, the major oligosaccharides **9, 18, 20,** and **24** correspond to these chains (Fig. 1). Moreover, each type of chains appeared to be represented by a series of oligosaccharides devoid of some D-



Scheme 1. The structures and symbols of the oligosaccharides. The structure of one of the oligosaccharides and its symbol are at the bottom as an example. The numerals at the symbols correspond to those used for isolated oligosaccharides (see Fig. 1).

galactose and 2-acetamido-2-deoxy-D-glucose residues ("incomplete" chains). Thus, oligosaccharides **1–9** constitute a series of structures ranging from the core pentasaccharide up to complete biantennary chain (Fig. 1 and Scheme 1). That oligosaccharides **4, 7,** and **8** represent the totally or partially degalactosylated fragments of **9** was proved by digestion of the latter with β-D-galactosidase and analysis of the products by liquid chromatography. An analogous series but with an additional L-fucose residue in each structure includes oligosaccharides **10–18**. In addition to sugar analysis, the structures were confirmed by mild acid hydrolysis of **10–18** to give **1–9**, respectively, which were detected by l.c. Similarly, oligosaccharides **19** and **20**, and **21, 22, 23,** and **24** correspond to two well distinguished series containing bisected, and bisected and fucosylated chains; as just mentioned, their structural interrelation was proved by mild acid hyrolysis and β-D-galactosidase treatment.

Similarly, the oligosaccharide maps of TG[7,8], AG[9], and FT[10] were interpreted on the basis of the known structures of the carbohydrate chains (Fig. 1 and Scheme 1). Thus, bi-, tri-, and tetra-antennary complex chains, fucosylated (**18, 26,** and **28** from TG) or nonfucosylated (**9, 25,** and **27** from AG) were observed; FT contained mainly nonfucosylated bi- and tri-antennary chains (**9** and **25**). The isolation of oligosaccharides identical both in their chromatographic characteristics and com-

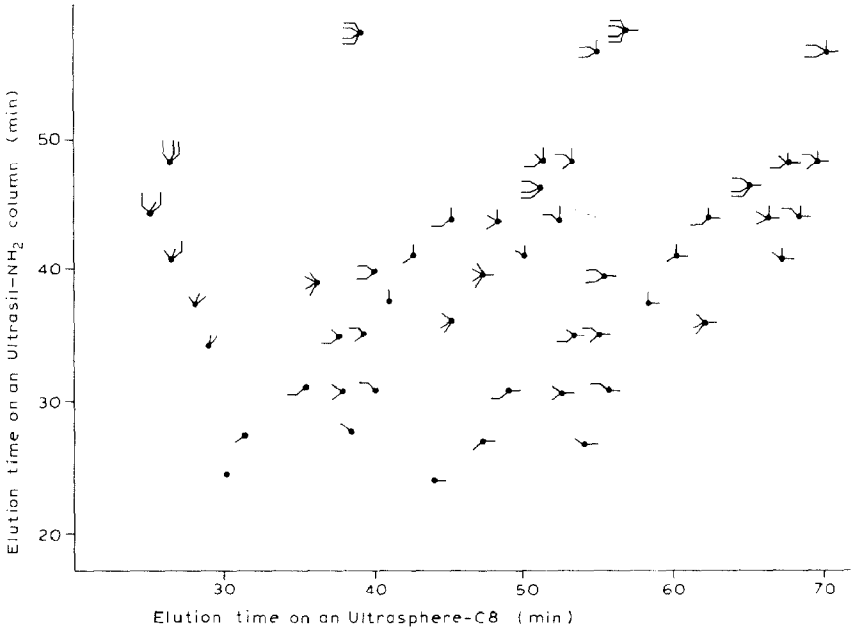


Fig. 2. The generalized oligosaccharide map showing dependence of chromatographic behavior of the oligosaccharide from its structure (*cf.*, Scheme 1). Localization of the isomers was made according to Tomija *et al.*[11]. The symbols differing from those in Scheme 1 by a clockwise rotation of 90° refer to oligomannoside chains.

position from different glycoproteins (**9, 18, 24,** and **25**) proves essentially our conclusions.

We were not able to attribute any definite structure for some minor oligosaccharides not marked on the maps. However, according to monosaccharide composition, the oligosaccharide from FT having coordinates 53/46 may be considered as a triantennary chain with an isolactosamine instead of a "normal" lactosamine residue[10], and the oligosaccharide 46/61 from AG may contain five lactosamine residues[9]. It is interesting that the sequence of elution of all the oligosaccharides on column I, determined by us, and that found by separation of 2-aminopyridine derivatives of fifty oligosaccharides on a Shim-pack CLC-ODS column[11,12] are the same.

The localization, on a map, of a great number of oligosaccharides corresponding to desialylated *N*-linked carbohydrate chains of glycoproteins[3,5,11–13] is illustrated in Fig. 2. The analysis of the regularities, based on the relation between structure and l.c. parameters allowed us to estimate the contribution of each monosaccharide to the overall chromatographic behavior of an oligosaccharide. The most essential effect is exerted by L-fucose and by the "bisecting" 2-acetamido-2-deoxy-β-D-glucopyranosyl group, the former greatly increasing the elution time of an oligosaccharide from column I only (*cf.*, **9** and **18**), and the latter from both columns (*cf.*, **9** and **20**). Other monosaccharides had a lesser but quite noticeable effect. It is of interest that not only the configuration of a monosaccharide residue, but its position in the chain is also important. Thus, the oligosaccharides having 2-acetamido-2-deoxy-D-glucose or *N*-acetyllactosamine residues in chains linked (1→6) to the β-D-mannopyranosyl residue had larger elution times from column I than the isomeric structures linked (1→3) (*cf.*, **2** and **3, 5** and **6**, etc.)[11].

Oligomannoside chains having 5, 6, 7, 8, and 9 mannose residues are also shown on the map (Fig. 2). The oligosaccharides were isolated from influenza virus hemagglutinin and their structures were elucidated by ^{1}H-n.m.r.[1,13]. As shown on the map, these chains are well separable from various types of complex chains. Theoretically, two more isomers may exist for each of the chains having 7 and 8 mannose residues. Some of these isomers differing from those reported in Fig. 2 were observed upon analysis of oligomannoside fractions of TG and H1 hemagglutinin[2], but their structures have not been elucidated as yet.

Tri- and tetra-antennary chains (fucosylated and nonfucosylated) are represented on the map only by complete chains and by chains that do not contain any D-galactose residues. The partially galactosylated variants of these chains would amount to 6 and 15 structures, respectively. We have observed the appearance of these chains after digestion by β-D-galactosidase of oligosaccharides **25, 26, 27,** and **28**, but it was difficult to localize each of them on the map. However, the approximate positions of the chains that lack some of the three or four D-galactose residues in complete structures could be extrapolated from the aforementioned regularities.

Thus, our results demonstrate the great efficiency of a two-column l.c. for the separation of *N*-oligosaccharide mixtures, as well as for the structural analysis of

carbohydrate chains of *N*-glycoproteins. The use of strict standard conditions for elution and of columns calibrated with standard oligosaccharides gives substantial informations about the structure of *N*-oligosaccharides from their location on the oligosaccharide map. Moreover, direct identification of an oligosaccharide with a reference compound also may be carried out. Of course, in some cases, an unambiguous conclusion about the structure of an oligosaccharide based only on two chromatographic parameters and composition may not be possible, but the data obtained will allow the rejection of numerous structures and the selection of a few variants. For selecting a single variant, other chromatographic systems, such as affinity chromatography on lectins, may be used. Thus, this exclusively chromatographic approach will permit to exclude or greatly reduce the use of traditional destructive chemical methods in structural investigation of oligosaccharides obtained from glycoproteins and other sources.

EXPERIMENTAL

Materials. — Fetuin was purchased from Sigma Chemical Co. (St. Louis, MO); other glycoproteins were prepared by the usual methods[5]. Carbohydrate chains were obtained from glycoproteins by treatment with alkaline $LiBH_4$ in aq. *tert*-butyl alcohol, followed by fractionation as described[3,4]. The oligosaccharide fraction was desialylated with formic acid (pH 2.1, 1.5 h at 80°). After gel chromatography on a Bio-Gel P-6 column, a mixture of oligosaccharides corresponding to complex chains of *N*-glycoproteins was obtained[1].

Methods. — L.c. was carried out on a Bio-Rad instrument, equipped with columns; (1 × 25 cm) containing Ultrasphere-C8 (10 μm) (column I) and Ultrasil-NH_2 (10 μm) (Column II); and with u.v. detection at 210 nm. Elution was performed with: for Column I, water at 0.5 mL/min for 15 min, at 1 mL/min for 16–35 min, aq. methanol 0→2.5% at 2 mL/min for 36–55 min, and 2.5→25% at 2 mL/min for 56–95 min; for Column II, 75% aq. methanol at 1 mL/min for 45 min, and then at 2 mL/min. Monosaccharide composition of oligosaccharides was determined after hydrolysis with 3M trifluoroacetic acid for 6 h at 100° by use of a sugar analyzer Biotronic LC-2000 and detection with copper bicinchoninate[14]. L-Fucose was selectively removed by mild acid hydrolysis with 0.05M trifluoroacetic acid for 3 h at 100°, followed by *N*-acetylation. D-Galactosyl groups were removed by β-D-galactosidase from *E. coli* (Aldrich).

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