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Complex-type Carbohydrates of Apolipoprotein-B of Human Plasma Low-density Lipoproteins

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Two fractions of glycopeptides containing *N*-glycosidic asparagine-linked glycans were isolated by concanavalin-A-Sepharose affinity chromatography from Pronase digests of apolipoprotein-B of human low-density lipoproteins. Methylation analysis indicated that the non-binding fraction contains about 0.5 mol complex-type tri- or tetra-antennary oligosaccharides per mol lipoprotein. Structures containing a bisecting *N*-acetylglucosamine were also detected in this fraction. The weakly binding fraction from the chromatography contains the majority of the complex oligosaccharides of apolipoprotein-B, i.e. 5-6 mol of bi-antennary chains of the transferrin-type per mol of lipoprotein. In addition to sialylated structures, branches containing a terminal *N*-acetylglucosamine residue were detected on the complex-type glycans of apolipoprotein-B.

Apolipoprotein-B (Apo-B) synthesized by the liver is a major structural determinant of very-low-density lipoproteins and their catabolic derivatives, low-density lipoproteins (LDL), in which Apo-B, also designated Apo-B 100 [1], is the only protein component [2]. The majority of LDL is removed from plasma into a variety of cells by a specific receptor-mediated endocytosis, whereafter the lipoproteins are degraded in lysosomes [3]. Apo-B has been shown to mediate the recognition of the lipoprotein by its receptor [2]. The catabolic receptor-mediated pathway of LDL is physiologically important because the majority of the plasma cholesterol is transported in LDL. Defective removal of plasma LDL has been shown to lead to hypercholesterolemia and accelerated atherosclerosis [2].

Apo-B is a glycoprotein, whose polypeptide and carbohydrate moieties have not been totally characterized. In LDL, Apo-B has a mass of about 500 kDa, which is about 25% of the weight of the lipoprotein particle [2]. Apo-B contains 4-9 % carbohydrate *N*-glycosidically-linked to asparagine, and consists of *N*-acetylneuraminic acid, *N*-acetylglucosamine, galactose and mannose [4-10]. Two main carbohydrate structures have been described on Apo-B, i.e. a high-mannose [6-10] and a complex-type oligosaccharide [6].

Abbreviations: Apo-B, apolipoprotein B; LDL, low-density lipoprotein.

Apo-B has been estimated to contain five oligomannosidic chains, which vary in the number of mannose residues from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ [10]. A previous report by Swaminathan and Aladjem [6] suggests that the major complex-type carbohydrate of Apo-B contains two *N*-acetylneuraminic acid, two galactose, three *N*-acetylglucosamine and five mannose residues, of which the mannose residues and one *N*-acetylglucosamine form the core of the oligosaccharide.

In order to understand the structural characteristics of Apo-B and its interaction with cell surface binding sites it is important to determine the carbohydrate structures in detail. In this work complex-type carbohydrates of Apo-B were further studied. Fractionation and separation of these structures was based on their different binding affinities to concanavalin-A lectin [11]. Two main types of complex structures were detected on Apo-B: a bi-antennary oligosaccharide and a tri- or tetra-antennary chain, the latter of which has not been described on Apo-B previously.

Materials and Methods

Reagents

Concanavalin-A-Sepharose 4B, Sephadex G-25 and G-50 Fine gels were from Pharmacia, Uppsala, Sweden. Pronase (EC 3.4.24.4), bovine serum albumin, glycoprotein β-D-galactosyltransferase (EC 2.4.1.38) from bovine milk and UDP-galactose were purchased from Sigma Chemical Co. (St Louis, MO, USA). UDP-[^{14}H]-galactose (309 Ci/mol) was from Amersham International (Amersham, UK). For fluorography Kodak X-O mat AR films were used.

Glycopeptides

Low-density lipoproteins were prepared by sequential ultracentrifugation [12] from plasma of human donors after fasting for 16 h, followed by gel filtration on a Bio-Gel A-15m column (1.5 × 60 cm; Bio-Rad, Richmond, CA, USA) as described earlier [10]. Apo-B was delipidated by extraction of LDL with chloroform/methanol/water, 2/2/1.8 by vol [13]. The glycopeptides were prepared by digestion of Apo-B with pronase and purified on a Sephadex G-25 Fine gel [10]. The glycopeptides were fractionated on a concanavalin-A-Sepharose column (2 × 10 cm) by eluting the gel with 0.1 M NaCl, 0.1 M NaCl/0.02 M α-methyl glucoside, and 0.1 M NaCl/0.2 M α-methyl glucoside as described in detail previously [10, 11]. The non-binding fraction (A) and weakly binding fraction (B) eluting with 0.1 M NaCl and 0.1 M NaCl/0.02 M α-methyl glucoside, respectively, were isolated and purified on a Sephadex G-50 Fine gel (1 × 50 cm) for further characterization.

Compositional and Structural Analysis of Oligosaccharides

The monosaccharide composition of the glycopeptides was determined after methanolysis as trimethylsilyl derivatives by GLC according to the method of Bhatti *et al.* [14].

In the methylation analysis, oligosaccharides were permethylated by the modified [15] method of Hakomori [16]. Neutral sugars and hexosamines were determined by GC-MS

(Hewlett-Packard 3992 A) after acetolysis/acid hydrolysis. Hexoses were analyzed as partially methylated alditol acetate derivatives on a 3% OV-210 column using a temperature gradient from 184°C to 210°C at a rate of 1°C/min, and detected at m/z values of 117, 161, 189, and 233 [15]. Detection of the partially methylated hexosaminitols was carried out at m/z values of 116, 158, and 170 after separation on a 2.2% OV-101 column at 290°C [15, 17]. *N*-Acetylneuraminic acid derivatives were released from the methylated glycans by methanolysis [15] and detected at m/z of 201, 330, and 376 using a 2.2% OV-101 column at 246°C.

Labeling of Apolipoprotein-B by Galactosyltransferase

Native LDL (50 μg of Apo-B) in 50 μl of a solution containing 0.15 M NaCl, 50 mM Tris-HCl pH 7.5, 10 mM $MnCl_2$, 1 mM NaN_3, and 0.01% albumin was labeled with [^{14}C]-galactose by adding galactosyltransferase (1.2 mU) and a mixture of UDP-galactose and UDP-[^{14}C]-galactose (specific radioactivity 6.5 mCi/mmol) to a final concentration of 0.3 mM [18]. The incubation was carried out at 37°C up to 4 h. Subsequently, Apo-B was delipidated by extraction with chloroform/methanol/water [13]. The delipidated protein was dissolved in 200 μl of Soluene 350 (Packard, Dovners Grove, IL, USA) and the radioactivity was determined using Pico-Fluor 30 (Packard) scintillation liquid.

Polyacrylamide Gel Electrophoresis

Samples of Apo-B labeled by galactosyltransferase as described above were analyzed by 7.5% polyacrylamide gel electrophoresis according to the method of Laemmli [19]. The ^{14}C-radioactivity on the gel was visualized by fluorography.

Results and Discussion

Apo-B prepared for these studies contained 4.4% carbohydrate. As described previously [10] about 5% and 58% of Apo-B glycopeptides are recovered in the non-binding (A) and weakly binding (B) fractions, respectively, upon chromatography on concanavalin-A-Sepharose. The remaining sugars which bind tightly to the lectin consist of high-mannose carbohydrate chains [10]. Thus, the complex-type sugars account for up to 63% of Apo-B oligosaccharides. Table 1 shows the molar monosaccharide composition of fractions A and B as determined by GLC. The most remarkable finding is that fraction A structures contain relatively more acidic residues and galactose than those in fraction B. GC-MS of the alditol acetate derivatives of the methylated neutral sugars revealed in fraction A 2,4-di-*O*- and 2,6-di-*O*-substituted mannose residues, whereas fraction B sugars lack these substitutions (Table 2). Fraction A glycans also contain a small amount of 3,4,6-tri-*O*-substituted mannose. No unsubstituted (terminal) mannose was detected in either fraction. The amount of terminal galactose in fraction A and B is relatively low as compared to the 3-*O*- and 6-*O*-substituted galactose residues. Both fraction A and B contained only terminal (unsubstituted) *N*-acetylneuraminic acid (Table 2). Analysis of partially methylated hexosaminitols revealed terminal and 4-*O*-substituted *N*-acetylglucosamine in both fractions. However, relatively more terminal *N*-acetylglucosamine was found in the structures of fraction A (Table 2).

Table 1. Monosaccharide composition of the glycopeptide fractions isolated from apolipoprotein-B by chromatography on concanavalin-A-Sepharose.

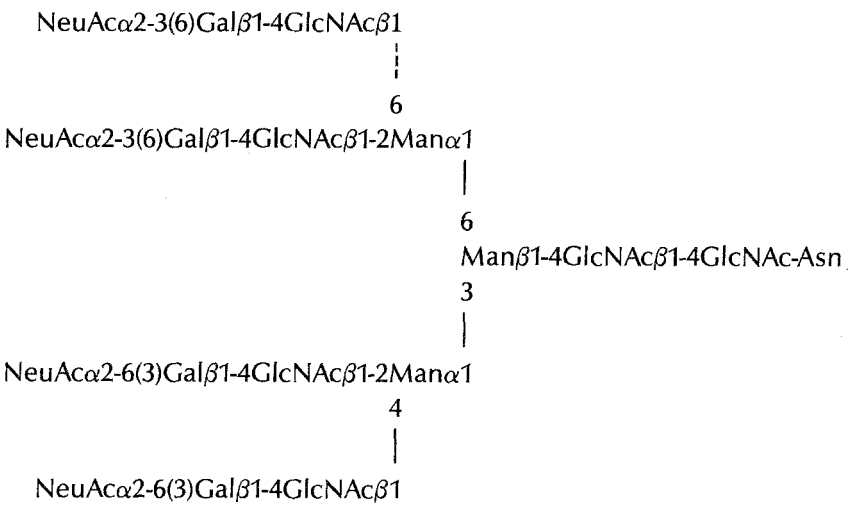
Carbohydrate	Fraction A	Fraction B
	mol/100 mol	
Mannose	20.9	29.8
Galactose	23.8	17.8
N-Acetylglucosamine	33.1	36.6
N-Acetylneuraminic acid	22.2	15.8

Table 2. Substitutions detected in the carbohydrate residues of the glycopeptide fractions A and B isolated from apolipoprotein-B. Molar ratios are expressed in relation to 3 mol of mannose.

Constituent	Substitution	Fraction A	Fraction B
Mannose (total)		3.0	3.0
	2-*O*-	1.1	2.0
	2,4-Di-*O*-	0.7	0.0
	2,6-Di-*O*-	0.2	0.0
	3,6-Di-*O*-	1.0	1.0
	3,4,6-Tri-*O*-	<0.1	0.0
Galactose (total)		3.4	1.8
	none (terminal)	0.2	0.1
	3-*O*-	1.9	0.3
	6-*O*-	1.3	1.4
N-Acetylglucosamine (total)		4.8	3.7
	none (terminal)	1.2	0.4
	4-*O*-	3.6	3.3
N-Acetylneuraminic acid (total)		3.2	1.6
	none (terminal)	3.2	1.6

Data from the methylation analysis of the fraction A sugars suggest that Apo-B contains a complex tri-antennary oligosaccharide of the fetuin-type [20]. This is suggested by the molar ratio of 1.1 : 0.7 : 1.0 for the 2-*O*-, 2,4-di-*O*- and 3,6-di-*O*-substituted mannose residues (Table 2). The chromatographic behaviour of the fraction A structures is also in accordance with that of the fetuin-type tri-antennary chain, which does not bind to concanavalin-A lectin [21, 22]. However, inferred rom the occurrence of 2,6-di-*O*-substituted mannose in fraction A, a tetra-antennary chain may also occur on some Apo-B molecules (Table 2). The relative amounts of *N*-acetylneuraminic acid and galactose (3.2 and 3.4 mol/3 mol of mannose, respectively) in fraction A also support the existence of a

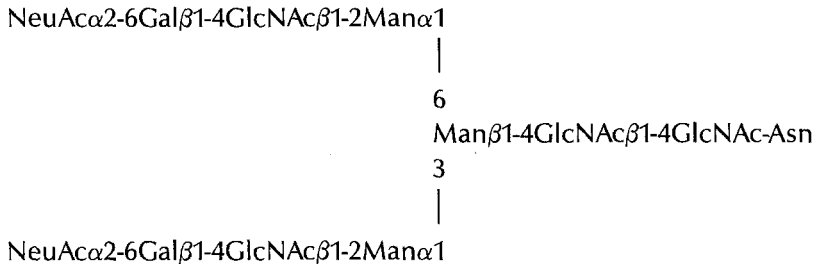
tetra-antennary structure on Apo-B (Table 2). Based on the molar ratio of the 2,4-di-*O*- and 2,6-di-*O*-substituted mannose residues (Table 2), the ratio of tri-antennary to tetra-antennary chains is approximately 4:1. A general structure of a tri- or tetra-antennary-oligosaccharide proposed here for Apo-B would be as follows (the fourth branch is indicated by a dotted line):



Occurrence of terminal *N*-acetylglucosamine and terminal galactose imply that some of the branches of the fraction A chains lack both galactose and *N*-acetylneuraminic acid residues and others lack only *N*-acetylneuraminic acid. Based on the compositional data on fraction A carbohydrates (Table 1) and the amount of monosaccharides in fraction A (about 5% of the total sugars of Apo-B), the number of the fraction A chains on Apo-B in a LDL particle is about 0.5 mol/mol LDL.

The occurrence of terminal *N*-acetylglucosamine and a small amount of 3,4,6-tri-*O*-substituted mannose in the fraction A sugars (Table 2) suggest that some of the carbohydrate chains contain a bisecting *N*-acetylglucosamine residue, which is β(1-4)-linked to the innermost β-mannose of the trimannosyl core of the oligosaccharide [23]. It is logical to find a bisected oligosaccharide in the (non-binding) fraction A as the existence of a bisecting *N*-acetylglucosamine on a complex oligosaccharide has been shown to lower its binding affinity to concanavalin-A [22]. Based on data expressed here it is not possible to propose the structure for the sugar(s) of Apo-B containing a bisecting *N*-acetylglucosamine. Such a structure may be a tri-antennary or a more branched glycan, the first of which was observed on chicken ovotransferrin [24]. A bisected bi-antennary chain may also occur among the fraction A sugars. Such oligosaccharides have been described on rat α_1-fetoprotein [25], ovomucoid [26], human immunoglobulin A [27], human myeloma [28] and bovine [22] immunoglobulin G, glycophorin A [29] and band 3 of red cell membranes [30]. On Apo-B the ratio of 3,4,6-tri-*O*-substituted to 3,6-di-*O*-substituted mannose residue of fractions A and B is less than 1:50. Thus, the existence of a bisecting complex-type carbohydrate on Apo-B is uncommon.

The compositinal data on fraction B oligosaccharides are compatible with a transferrin-type bi-antennary carbohydrate structure:



This structure, which is common on serum glycoproteins [31] is related to the minor (glycopeptide s-GP-II) and the major (GP-II and s-GP-III) complex-type oligosaccharides of Apo-B described by Swaminathan and Aladjem [6]. However, the minor complex-type glycan of Apo-B proposed by these authors has an extra β(1-4)-linked mannose in the core region while the major glycans have in addition an α(1-2)-linked mannose residue in their core as compared to the trimannosyl core of the bi-antennary structure proposed here. Moreover, the minor and major structures described [6] contain only one β(1-4)-substituted *N*-acetylglucosamine in the core region. Calculated from the data presented here (Table 1 and 2), the Apo-B moiety of LDL contains from five to six chains of the transferrin-type, which would thus represent the major complex-type glycan on Apo-B.

The orientation of the complex-type glycans of Apo-B on the surface of LDL was examined by labeling their terminal *N*-acetylglucosamine residues using a galactosyltransferase. The data in Fig. 1 show that Apo-B is efficiently labeled by the transferase. Kinetics of the labeling suggested that on the carbohydrates of Apo-B, there are two to three terminal *N*-acetylglucosamine residues exposed to the galactosyltransferase (Fig. 2). Based on the data from the compositional and structural analysis of the isolated complex-type chains of Apo-B (Table 1 and 2), there are approximately one terminal *N*-acetylglucosamine residue on the tri- or tetra-antennary structure and two residues on the bi-antennary structures. Thus, it is conceivable that the *N*-acetylglucosamine residues on both structures are accessible to galactosyltransferase. Obviously, both the bi-antennary and more branched glycans on Apo-B have a similar hydrophilic orientation on the lipoprotein.

In summary, it is suggested that the Apo-B moiety of LDL synthesized in the liver cells contains three main types of carbohydrate, i.e. on the average less than one tri- or tetra-antennary chain, five or six transferrin-type bi-antennary chains, and about five high-mannose chains described earlier [10]. Chains containing a bisecting *N*-acetylglucosamine structure are also found as a minor component. The absence of terminal mannose on the complex-type glycans excludes the existence of hybrid oligosaccharides on Apo-B. It is probable that the different types of glycans have incompletely synthesized branches and thus heterogeneity in their structures. Based on the available data it is not possible to show the relative distribution of these chains on individual lipoprotein particles, which may vary both in the amount and type of their oligosaccharides.

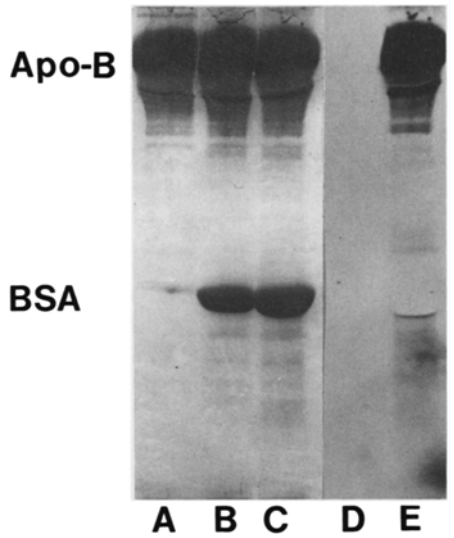


Figure 1. Polyacrylamide gel electrophoresis and fluorography of [^{14}C]-galactose-labeled apolipoprotein-B. Labeling of Apo-B with UDP-[^{14}C]-galactose and galactosyltransferase was carried out as described under Materials and Methods. 25 μg of Apo-B were analyzed on a 7.5% polyacrylamide gel [19] and the bands containing ^{14}C-radioactivity were detected by fluorography. A; control, 25 μg of Apo-B in native LDL. B; control, 25 μg of Apo-B in LDL incubated for 1 h with UDP-[^{14}C]-galactose without galactosyltransferase. C; 25 μg of Apo-B in LDL incubated for 1 h with UDP-[^{14}C]-galactose and galactosyltransferase. D; fluorography of lane B. E; fluorography of lane C. BSA; bovine serum albumin.

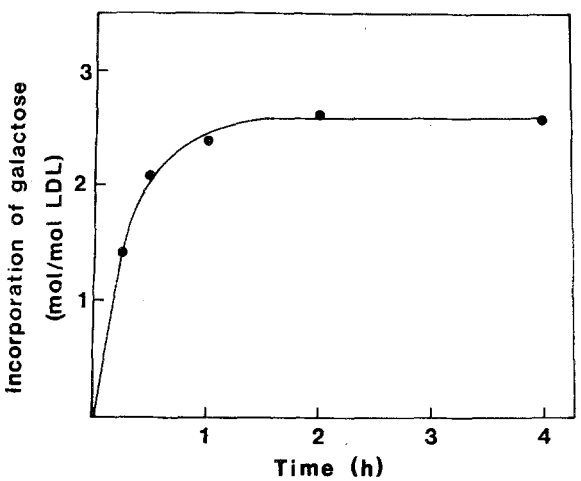


Figure 2. Kinetics of labeling of apolipoprotein-B in native low-density lipoproteins with [^{14}C]-galactose. Apo-B was labeled with UDP-[^{14}C]-galactose and galactosyltransferase for different times as indicated. Experimental details are described under Materials and Methods. Incorporation of galactose into the oligosaccharides of Apo-B is calculated as mol/mol LDL. Each point represents the mean of two experiments, carried out in duplicate.

Recent reports [32, 33] on the partial amino acid sequences of Apo-B inferred from studies on its mRNA suggest that Apo-B has characteristics of an integral membrane protein, which would thus possess a secondary structure different from that of the other amphipathic apolipoproteins [34]. The overall oligosaccharide composition of Apo-B is also reminiscent in some respects of a membrane protein rather than a soluble serum protein, since (i) Apo-B contains terminal *N*-acetylglucosamine, mannose, and galactose residues, which are detected only in low amounts on serum glycoproteins because of the rapid removal of asialoglycoproteins and terminal mannose- and *N*-acetylglucosamine-containing glycoproteins from plasma by specific receptor-mediated mechanisms [35]; (ii) Apo-B contains both complex-type glycans and high-mannose chains [36, 37]; (iii) Apo-B contains a relatively high amount of oligomannosidic carbohydrate [10, 36].

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References

1 Kane JP, Hardman DA, Paulus HE (1980) Proc Natl Acad Sci USA 77:2465-69.
2 Goldstein JL, Brown MS (1977) Annu Rev Biochem 46:897-930.
3 Brown MS, Goldstein JL (1983) J Clin Invest 72:743-47.
4 Ehnholm C, Garoff H, Renkonen O, Simons K (1972) Biochemistry 11:3229-32.
5 Lee O, Breckenridge WC (1976) Can J Biochem 54:42-49.
6 Swaminathan N, Aladjem F (1976) Biochemistry 15:1516-22.
7 Triplett RB, Fischer WR (1978) J Lipid Res 19:478-88.
8 Attie AD, Weinstein DB, Freeze HH, Pittman RC, Steinberg D (1979) Biochem J 180:647-54.
9 Shireman RB, Fischer W (1979) Biochim Biophys Acta 572:537-40.
10 Vauhkonen M, Viitala J, Parkkinen J, Rauvala H (1985) Eur J Biochem 152:43-50.
11 Krusius T (1976) FEBS Lett 66:86-89.
12 Hatch FT, Lees RS (1968) Adv Lipid Res 6:1-68.
13 Folch J, Lees M, Sloane-Stanley GA (1957) J Biol Chem 226:497-509.
14 Bhatti T, Chambers RE, Clamp JR (1970) Biochim Biophys Acta 222:339-47.
15 Rauvala H, Finne J, Krusius T, Kärkkäinen J, Järnefelt J (1980) Adv Carbohydr Biochem 38:389-416.
16 Hakomori S (1964) J Biochem (Tokyo) 55:205-8.
17 Finne J, Rauvala H (1977) Carbohydr Res 58:57-64.
18 Viitala J, Finne J (1984) Eur J Biochem 138:393-97.
19 Laemmli UK (1970) Nature 227:680-85.
20 Nilsson B, Nordén NE, Svensson S (1979) J Biol Chem 254:4545-53.
21 Krusius T, Finne J, Rauvala H (1976) FEBS Lett 71:117-20.
22 Baenziger JU, Fiete D (1979) J Biol Chem 254:2400-7.

23 Carver JP, Grey AA (1981) Biochemistry 20:6607-16.
24 Dorland L, Haverkamp J, Vliegenthart JFG, Spik G, Fournet B, Montreuil J (1979) Eur J Biochem 100:569-74.
25 Bayard B, Kerckaert J-P (1981) Eur J Biochem 113:405-14.
26 Bayard B, Montreuil J (1973) in Colloques Internationaux du Centre National de la Recherche Scientifique, No 221, 2nd Int Symp Glycoconjugates, CNRS, Paris, p 209-18.
27 Baenziger J, Kornfeld S (1974) J Biol Chem 249:7260-69.
28 Kornfeld R, Keller J, Baenziger J, Kornfeld S (1971) J Biol Chem 246:3259-68.
29 Yoshina H, Furthmayr H, Kobata A (1980) J Biol Chem 255:9713-18.
30 Tsuji T, Irimura T, Osawa T (1981) J Biol Chem 256:10497-502.
31 Montreuil J (1982) in Comprehensive Biochemistry 19B, part II, eds. Neuberger A, van Deenen LLM, Elsevier, Amsterdam, p 1-188.
32 Wei C-F, Chen S-H, Yang C-Y, Marcel YL, Milne RW, Li W-H, Sparrow JT, Gotto AM Jr, Chan L (1985) Proc Natl Acad Sci USA 82:7265-69.
33 Knott TJ, Rall SC Jr, Innerarity TL, Jacobson SF, Urdea MS, Levy-Wilson B, Powell LM, Pease RJ, Eddy R, Nakai H, Byers M, Priestley LM, Robertson E, Rall LB, Betsholtz C, Shows TB, Mahley RB, Scott J (1985) Science 230:37-43.
34 Mahley RW, Innerarity TL, Rall SC Jr, Weisgraber KH (1984) J Lipid Res 25:1277-94.
35 Neufeld EF, Ashwell G (1980) in The Biochemistry of Glycoproteins and Proteoglycans, ed. Lennarz WJ, Plenum Press, New York, p 241-66.
36 Finne J, Krusius T (1979) Eur J Biochem 102:583-88.
37 Pollack L, Atkinson PH (1983) J Cell Biol 97:293-300.