Human small-intestinal apolipoprotein B-48 oligosaccharide chains

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Hepatic apolipoprotein (apo) B-100 isolated from human plasma is known to contain *N*-linked oligosaccharides of high-mannose-type and complex-type structures. Sequencing data have revealed that apo B-48 of small-intestinal origin, which represents about 48% of apo B-100 polypeptide from the *N*-terminus, possesses six potential sites for *N*-linked oligosaccharides, of which five are likely to be glycosylated. The characterization of the carbohydrate moiety of apo B-48 is the focus of this study. Apo B-48 was labelled with L-[^{35}S]methionine and D-[^{3}H]glucosamine in organ culture of human small-intestinal explants. *N*-Glycanase treatment resulted in loss of radioactivity from D-[^{3}H]glucosamine-labelled but not L-[^{35}S]methionine-labelled apo B-48 secreted into the medium, and caused no distinct change in mobility of apo B-48 upon electrophoresis on 5% polyacrylamide gel. Analysis of monosaccharide content revealed the presence of 16.8, 17.8, 13.4, 3.4, 2.4 and 2.3 residues of *N*-acetylglucosamine, mannose, galactose, fucose, xylose and *N*-acetylgalactosamine respectively. Small-intestinal apo B-48 from human lymph chylomicrons bound to [^{14}C]concanavalin A, and the binding could be inhibited with methyl α-D-mannoside. In addition, wheat-germ, peanut, *Limulus*, soya-bean and *Ulex* lectins bound apo B-48 specifically. To characterize the carbohydrate moiety further, *N*-linked oligosaccharides were released by *N*-Glycanase treatment and reduced with NaB^3H_4. Labelled oligosaccharides were separated on a concanavalin A–Sepharose column. The majority (78%) were biantennary complex-type structures, 16% were high-mannose type and 6% (not retained by the column) most probably represented higher-branched oligosaccharides. These results suggest the presence of one high-mannose-type and four biantennary complex-type oligosaccharides, as well as probable *O*-linked sugars in apo B-48. By the use of h.p.l.c., exoglycosidase treatments and ion-exchange chromatography, a mixture of high-mannose-type species with predominant $Man_8GlcNAc_2$ as well as monosialylated, disialylated and fucosylated forms of complex-type oligosaccharides were detected.

INTRODUCTION

Apolipoprotein (apo) B-48 is a constituent of triacylglycerol-rich chylomicrons synthesized by the small intestine and secreted into the lymph during active lipid absorption [1]. The same gene that encodes apo B-48 controls the synthesis of apo B-100 in the liver [2,3]. Apo B-100 is a component of plasma very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) and serves as a ligand for LDL uptake by the liver receptor. Amino acid sequencing of apo B-100 has indicated 19 potential glycosylation sites for *N*-linked oligosaccharides, of which 16 were glycosylated [4]. Since apo B-48 is a continuous fragment of 2152 amino acid residues corresponding to the *N*-terminal portion of apo B-100 [2,3], the number of putative sites for *N*-linked oligosaccharides has been determined to be as many as six, of which five are likely to be glycosylated. This assumption derives from the finding that asparagine at position 7 in the putative sequence Asn-Val-Ser is not glycosylated in apo B-100 and apo B-48 [4,5]. The oligosaccharide structures of hepatic apo B-100 from human plasma LDL were reported to consist of 37% high-mannose chains, the rest being mainly biantennary complex oligosaccharides [6–8]. However, the carbohydrate moiety of apo B-48 has not been characterized. Since significant differences in glycosylation pattern of the same polypeptides were found to be tissue-specific [9,10], we have undertaken the present studies to determine the types of oligosaccharide chains in human small-intestinal apo B-48. Characterization of apo B-48 oligosaccharide chains should be helpful in understanding its structural characteristics and interactions, as well as a potential role of the carbohydrate moiety in processing, transport and stability of apo B-48 in health and in some lipid disorders.

EXPERIMENTAL

Metabolic labelling of human small-intestinal explants

Explants of normal human jejunum obtained from surgical procedures were prepared and maintained in organ culture as described previously [11]. The experimental protocol was approved by the institutional Human Investigation Review Committee. The explants were labelled for 4–6 h with 0.1 mCi of L-[^{35}S]methionine (682 Ci/mmol)/ml or with 1 mCi of D-[6-^{3}H]glucosamine (26.4 Ci/mmol)/ml, both from New England Nuclear. Following immunoprecipitation of labelled apo B from the media with affinity-purified goat anti-(human apo B) antibody [11], the antigen–antibody complex was dissociated by boiling in 60 μl of 0.3% SDS containing 60 mM-2-mercaptoethanol. The sample was then diluted with 40 μl of 0.5 M-sodium phosphate buffer, pH 8.6, containing albumin (0.25 mg/ml), 3.15% Nonidet P-40 and 15 mM-phenanthroline [12]. Incubation with or without *N*-Glycanase (Genzyme Corp., Cambridge, MA, U.S.A.) (0.25 unit) was carried out for 18 h at 37 °C. The activity of *N*-Glycanase was tested with fetuin and ribonuclease. The digests were analysed on SDS/PAGE according to the procedure of Laemmli [13].

Preparation of apo B-48 from human lymph chylomicrons

Human lymph chylomicrons were obtained from Dr. Ernst Schaefer, Human Nutrition Research Center on Aging at Tufts

Abbreviations used: apo, apolipoprotein; VLDL, very-low-density lipoproteins; LDL, low-density lipoproteins; Con A, concanavalin A.
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University, Boston, MA, U.S.A., and human lymph was from Dr. Patrick Tso, Louisiana State University Medical Center, Shreveport, LA, U.S.A. Chylomicrons were prepared from the lymph by ultracentrifugation in a 50.2 Ti rotor at 39000 rev./min for 18 h at 4 °C. The top layer was collected, resuspended in phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4 and 0.2 g of KH_2PO_4 per litre), pH 7.4, and the ultracentrifugation was repeated. Chylomicrons were delipidated with ethanol/diethyl ether (3:1, v/v) [14] and apo B-48 was isolated by electrophoresis on preparative 5% polyacrylamide gels in the presence of SDS. Human VLDL containing apo B-100 and apo B-48 were used as a standard. The gel was incubated overnight in 0.2 M-KCl at 4 °C for the location of apo B-48 upon precipitation of SDS in the gel [12]. *N*-linked oligosaccharides were released with *N*-Glycanase as reported elsewhere [15].

Monosaccharide composition

Chylomicrons prepared from pooled human lymph were delipidated and apo B-48 was isolated by preparative SDS/PAGE. After strong acid hydrolysis (2 M-HCl at 100 °C, for 6 h), excess acid was removed and the monosaccharide mixture was re-*N*-acetylated [16], followed by reduction with NaB^3H_4 [17]. Radiolabelled monosaccharide alditols were fractionated by h.p.l.c. on a Shodex sugar column (Altech, Dierfield, IL, U.S.A.) maintained at 80 °C [18]. Monosaccharides were eluted with ethanol/water (1:4, v/v) and the identification was based on comparison with retention times of standard alditols. Values were corrected for monosaccharide destruction during hydrolysis and for their differential efficiency of labelling with NaB^3H_4. Glucosamine and galactosamine contents were determined with an amino acid analyser (Picotag system) equipped with a C_{18} reversed-phase column (Millipore Corp., Waters Chromatography Division, Bedford, MA, U.S.A.). Formation of derivatives and the elution programme were carried out according to the manufacturer's instructions.

Lectin blotting

Apo B-48 from human lymph chylomicrons was separated on SDS/PAGE, and electroblotted to poly(vinylidene difluoride) membranes [19] (Millipore Corp.) in the Towbin buffer [20] with no methanol. Specificity of lectin binding was tested first with glycoproteins of known oligosaccharide chains: fetuin, transferrin, ribonuclease and ovalbumin. To prevent non-specific binding of lectins the 3 cm portions of the blots containing apo B-48 were saturated with 3% (w/v) albumin for 1 h. Saturated blots were incubated for 3 h with 0.1 μCi of [^{14}C]concanavalin A (Con A) (Sigma Chemical Co.) (13.9 μCi/μg of protein) in 1 ml of Con A buffer (20 mM-Tris/HCl buffer, pH 7.4, containing 500 mM-NaCl, 1 mM-$MnCl_2$, 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$) at room temperature in the presence or in the absence of 100 mM-methyl α-D-mannoside. The blots were washed three times with Con A buffer, air-dried and cut into 0.5 cm segments, which were counted for radioactivity in a scintillation counter.

The portions (3 cm long) of the blots containing apo B-48 were also used for the binding studies of other lectins. The following lectins conjugated with horseradish peroxidase were employed: wheat-germ agglutinin, 10 μg/ml; peanut agglutinin, 10 μg/ml; *Limulus polyphemus* agglutinin, 50 μg/ml; soya-bean agglutinin, 10 μg/ml; *Ulex europaeus* agglutinin I, 5 μg/ml. All lectin conjugates were purchased from E–Y Laboratories (San Mateo, CA, U.S.A.). Assay of lectin binding and the colour development of the horseradish peroxidase chromogenic substrate were carried out according to the manufacturer's instructions.

Oligosaccharide standards

The oligosaccharide standards, namely asialo biantennary ($Gal_2GlcNAc_2Man_3GlcNAc_2$), disialylated biantennary, asialo biantennary with fucose residue at the core, $Man_9GlcNAc_2$ and $Man_5GlcNAc_2$, were purchased from Dionex Corp. (Sunnyvale, CA, U.S.A.). $Man_8GlcNAc_2$ was prepared by labelling HepG2 cells with [2-^{3}H]mannose [21]. The oligosaccharides were reduced with 10-fold molar excess of NaB^3H_4 (New England Nuclear) [17], except $Man_8GlcNAc_2$, which was reduced with $NaBH_4$.

Lectin column chromatography

A Con A–Sepharose (Pharmacia, Uppsala, Sweden) column was equilibrated in Con A buffer (see above). The quantity of lectin immobilized was 10–16 mg/ml of gel. After elution of unbound material, the column was eluted with 15 mM-methyl α-D-glucoside and 500 mM-methyl α-D-mannoside in the same buffer to elute biantennary complex and high-mannose oligosaccharides respectively.

QAE-Sephadex chromatography

The columns of quaternary ammonium ethyl (QAE)-Sephadex were equilibrated with 2 mM-Tris base. The unbound oligosaccharide fraction was eluted from the column with 2 mM-Tris and the bound oligosaccharides were eluted with 20–200 mM-NaCl [22].

Exoglycosidase treatments

Oligosaccharides were treated with sialidase (1 unit/ml) from *Streptococcus* (Genzyme Corp.) for 3 h at 37 °C in 100 mM-sodium acetate buffer, pH 6.5, containing 10 mM-$CaCl_2$ in a final volume of 50 μl. Digestion with α-L-fucosidase from *Charonia lampas* (Sigma Chemical Co.) was carried out in 100 mM-citrate/phosphate buffer, pH 4.6, for 18 h at 37 °C with 10 munits in a final volume of 50 μl. The fucosidase cleaves all fucosyl linkages in oligosaccharides [23]. The oligosaccharides were desalted on an Amberlite MB-3 (Sigma Chemical Co.) column before h.p.l.c. analysis. Sialidase activity was tested by incubation with fetuin and the digest was analysed by SDS/PAGE. Digestion with jack-bean α-mannosidase was carried out as described previously [24].

H.p.l.c. of oligosaccharides

Reduced oligosaccharides were analysed by h.p.l.c. on a MicroPak AX-5 column (Varian Associates). The column was pre-equilibrated with acetonitrile/water (13:7, v/v) and elution was performed by a linear gradient to acetonitrile/water (7:13, v/v) for 60 min [15]. The flow rate was 1 ml/min. Fractions (0.8 ml) were collected and counted for radioactivity.

RESULTS

Metabolic labelling of apo B-48 in human small-intestinal explants

We have previously demonstrated the biosynthetic labelling of apo B-48 with L-[^{35}S]methionine in human small-intestinal explants [11]. In order to label the carbohydrate moiety of apo B-48, the explants in organ culture were incubated with D-[^{3}H]glucosamine. Apo B-48 secreted into the medium was immunoprecipitated and incubated with or without *N*-Glycanase followed by SDS/PAGE. Treatment of D-[^{3}H]glucosamine-labelled apo B-48 with *N*-Glycanase resulted in loss of the radioactivity, in contrast with L-[^{35}S]methionine-labelled apo B-48, in which the radioactivity level was not affected by the treatment (Fig. 1). This indicates the cleavage of *N*-linked oligosaccharides from apo B-48. Except for slight broadening of the band, no distinct change in mobility upon electrophoresis on 5% polyacrylamide gel was observed for L-[^{35}S]methionine-

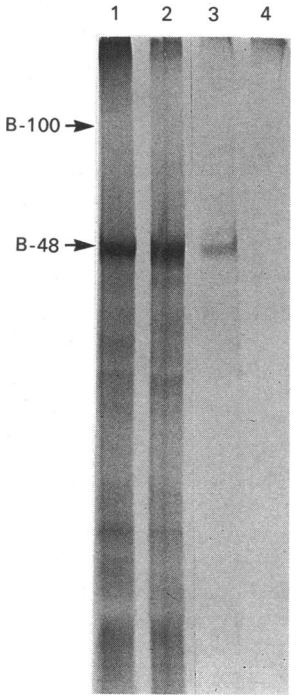

Fig. 1. *N*-Glycanase treatment of apo B-48 from human small-intestinal explants

Apo B-48 secreted into the medium during 6 h of metabolic labelling was immunoprecipitated and incubated with (lane 2 and 4) or without (lane 1 and 3) *N*-Glycanase. After incubation, the samples were diluted with Laemmli sample buffer [13], boiled and analysed by PAGE on 5% polyacrylamide gel. The gel with D-[^{3}H]-glucosamine-labelled apo B-48 was incubated with En^{3}Hance, dried and exposed to the film for 7 days. The gel with L-[^{35}S]methionine was dried and exposed to the film for 20 h. Lanes 1 and 2, L-[^{35}S]-methionine-labelled apo B-48; lanes 3 and 4, D-[^{3}H]glucosamine-labelled apo B-48. Mobilities of standard apo B-48 and apo B-100 are indicated.

Table 1. Monosaccharide composition of small-intestinal apo B-48

Monosaccharide	Composition (mol of residues/mol)$_1$
N-Acetylglucosamine*†	16.8
Mannose†	17.8
Galactose†	13.4
Fucose†	3.4
Xylose†	2.4
N-Acetylgalactosamine*	2.3

* Determined with an amino acid analyser as glucosamine and galactosamine. The amino sugars were well separated from amino acids and eluted before serine.

† Relative molar ratio determined by h.p.l.c. as monosaccharide alditols.

labelled apo B-48 digested with *N*-Glycanase (Fig. 1). No change in the mobility was also noticed after incubations with larger quantities of *N*-Glycanase. This can be explained by the large size of apo B-48 (241 kDa before glycosylation) [3] and the relatively small contribution of *N*-linked oligosaccharides (due to their size and/or charge) to the glycoprotein molecular mass.

Monosaccharide composition of apo B-48 purified from human lymph chylomicrons

The following monosaccharides and their relative molar contents (expressed relative to a value of 1.0 for fucose) were found:

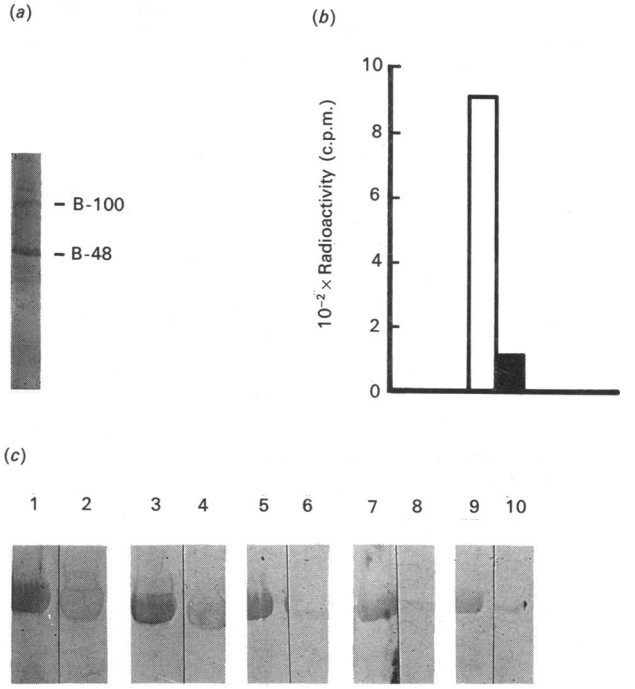

Fig. 2. Lectin-blotting of apo B-48

(*a*) SDS/PAGE of human lymph chylomicrons. Chylomicrons were delipidated with ethanol/diethyl ether, and the protein pellet was dissolved in Laemmli sample buffer and subjected to PAGE on 5% polyacrylamide gel. After electroblotting on to poly(vinylidene difluoride) paper, the proteins were stained with Coomassie Blue. Mobilities of apo B-100 and apo B-48 from human VLDL are indicated. (*b*) [^{14}C]Con A binding to human lymph apo B-48. The incubation was carried out with apo B-48 electroblotted on to poly(vinylidene difluoride) paper in the absence (white column) and presence of 100 mM-methyl α-D-mannoside (black column). After the blots had been washed with Con A buffer, radioactivity content was determined in a liquid-scintillation counter. Poly(vinylidene difluoride) paper saturated with albumin bound no radioactivity above the background. (*c*) Lectin–peroxidase blotting. Apo B-48 electroblotted on to poly(vinylidene difluoride) paper was incubated with the following: lane 1, wheat-germ agglutinin; lane 2, wheat-germ agglutinin with 100 mM-*N*-acetylglucosamine; lane 3, peanut agglutinin; lane 4, peanut agglutinin with 100 mM-lactose; lane 5, *Limulus polyphemus* agglutinin; lane 6, *Limulus polyphemus* agglutinin with 10 mM-sialic acid; lane 7, soya-bean agglutinin; lane 8, soya-bean agglutinin with 50 mM-*N*-acetylgalactosamine; lane 9, *Ulex europaeus* agglutinin I; lane 10, *Ulex europaeus* agglutinin I with 50 mM-fucose. The colour development was achieved by incubation with 0.5% (w/v) diaminobenzidine and 0.25% H_2O_2.

N-acetylglucosamine (4.9), mannose (5.2), galactose (3.9), fucose (1.0) and xylose (0.7). The *N*-acetylgalactosaminitol peak was very small and inconclusive owing to non-carbohydrate interfering materials also eluted in this region. In order to calculate the absolute molar content of individual monosaccharides, the content of amino sugars versus total amino acids was determined with an amino acid analyser. There were 16.8 mol and 2.3 mol of glucosamine and galactosamine per mol respectively. Since the relative molar ratios of glucosamine to other monosaccharides has been determined, the absolute molar proportions of mannose, galactose, fucose and xylose residues have been calculated to be 17.8, 13.4, 3.4 and 2.4 respectively (Table 1). By weight, apo B-48 contains 3.9% carbohydrate, excluding sialic acid residues

Binding of lectins to apo B-48

Human lymph chylomicrons were delipidated in ethanol/diethyl ether and subjected to SDS/PAGE along with human

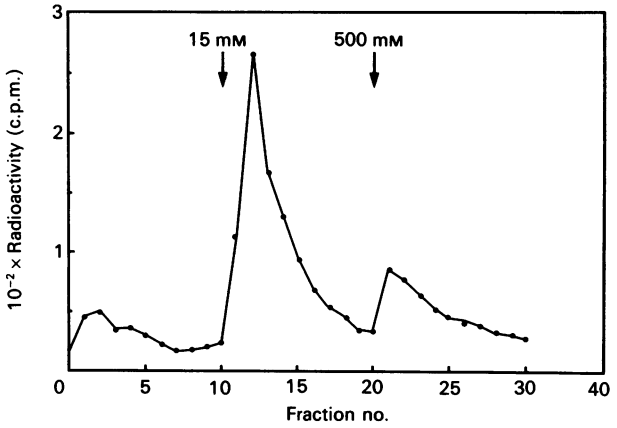

Fig. 3. Con A–Sepharose chromatography of apo B-48 *N*-linked oligosaccharides

Oligosaccharide chains of human lymph chylomicrons apo B-48 were released with *N*-Glycanase and labelled with NaB^3H_4. Oligosaccharides were eluted from the column (1 cm × 5 cm) with Con A buffer alone and then with 15 mM-methyl α-D-glucoside and 500 mM-methyl α-D-mannoside in Con A buffer. Fractions (1 ml) were collected and counted for radioactivity. Recovery of radioactivity from the column was 87%.

VLDL containing apo B-100 and apo B-48 as standards. Proteins from the gel were electroblotted on to poly(vinylidene difluoride) paper. Staining of the paper with Coomassie Blue revealed that apo B-48 was the major protein component (Fig. 2*a*). Apo B-48 blotted onto the paper was incubated with [^{14}C]Con A (Sigma Chemical Co.) in the absence or presence of 100 mM-methyl α-D-mannoside followed by a washing step to remove non-specific binding. Apo B-48 bound [^{14}C]Con A, and the binding was reduced by 80% in the presence of 100 mM-methyl α-D-mannoside (Fig. 2*b*). Albumin bound no radioactivity above background level. Since Con A interacts strongly with α-mannosyl residues of the trimannosidic core [25], these results confirm the presence of high-mannose-type and/or biantennary complex-type *N*-linked oligosaccharide chain(s) in apo B-48. Additional lectin-binding studies were carried out with lectins conjugated to horseradish peroxidase. As shown in Fig. 2(*c*), apo B-48 blotted on to poly(vinylidene difluoride) paper was stained with the following lectins: wheat-germ agglutinin (lane 1), peanut agglutinin (lane 3), *Limulus polyphemus* agglutinin (lane 5), soya-bean agglutinin (lane 7) and *Ulex europaeus* agglutinin I (lane 9); the specific staining was inhibited by the incubation of the lectin conjugates with 100 mM-*N*-acetyl-D-glucosamine (lane 2), 100 mM-lactose (lane 4), 10 mM-sialic acid (lane 6), 50 mM-*N*-acetyl-D-galactosamine (lane 8) and 50 mM-L-fucose (lane 10) respectively. Wheat-germ agglutinin [26] interacts strongly with sialic acid-containing oligosaccharides and with the bisect core, GlcNAc(β1→4)-Man(β1→4)GlcNAc(β1→4)GlcNAc, of oligosaccharides [27]. After sialidase treatment, the reactivity of wheat-germ agglutinin towards apo B-48, although decreased, was still present (results not shown), and was further reduced by the presence of *N*-acetylglucosamine. Thus the presence of the bisect oligosaccharides and/or higher-branched complex-type chains may be responsible for this binding pattern. Specific interactions with *Limulus* lectin [28] and peanut lectin [29] suggest that apo B-48 contains both sialylated and asialo species respectively, whereas the staining with *Ulex* lectin is consistent with the presence of fucosylated chains [30]. Soya-bean lectin, which interacts preferentially with mucin-type oligosaccharides [31] containing *N*-acetylgalactosamine at the reducing end, bound to apo B-48. This observation and the existence of *N*-acetylgalactosamine (Table 1) may indicate the presence of *O*-linked oligosaccharides in apo B-48. In this regard it is important to note that terminal *N*-acetylgalactosamine residues are found on *N*-linked chains of the epidermal-growth-factor receptor in A-431 cells, and the receptor interacts with monoclonal antibodies directed against blood-group determinants contained in *O*-linked oligosaccharides [32]. Thus the presence of *O*-linked chains in apo B-48 is yet to be demonstrated.

Fractionation of oligosaccharides by Con A–Sepharose chromatography

To characterize apo B-48 carbohydrates further, *N*-oligosaccharide chains were liberated with *N*-Glycanase. The mild conditions of the procedure and the ability of *N*-Glycanase to cleave to a broad spectrum of *N*-linked oligosaccharides [33] makes it the method of choice. Liberated oligosaccharides were reduced with NaB^3H_4, and the resulting labelled sugar alcohols were separated on a Con A–Sepharose column into three major fractions. About 6% of radioactivity was not retained by the column, whereas 78% of radioactivity was eluted with 15 mM-methyl α-D-glucoside and 16% with 500 mM-methyl α-D-mannoside (Fig. 3). The radioactivity not retained by Con A–Sepharose bound to a benzeneboronic acid column and was eluted with acidic acid (results not shown), suggesting the carbohydrate nature of this material [34]. This fraction may represent higher-branched *N*-linked oligosaccharides; however, it was not analysed further.

Fractionation of oligosaccharides on the basis of their negative charge by QAE-Sephadex chromatography

QAE-Sephadex can be used to separate oligosaccharides on the basis of their negative charge. Neutral species are not retained by the column, and oligosaccharides with one, two, three and four negative charges are eluted with 20 mM-, 70 mM-, 140 mM- and 200 mM-NaCl respectively [16,22]. The oligosaccharide standard asialo biantennary complex did not bind to the QAE-Sephadex column, whereas disialylated oligosaccharide standard did bind and was eluted with 70 mM-NaCl (results not shown). Sialidase treatment of the latter resulted in loss of retention of the oligosaccharide by the QAE-Sephadex. After standardization of the QAE-Sephadex column, the biantennary complex-type oligosaccharide fraction of apo B-48 isolated by Con A–Sepharose chromatography was analysed. About 40% of oligosaccharide species were not retained by the QAE-Sephadex, whereas about 12% and 27% of labelled oligosaccharides were eluted with 20 mM- and 70 mM-NaCl respectively. A small proportion of oligosaccharides was retained by the column with greater affinity and was only eluted with 140 mM- (16%) and 200 mM-NaCl (5%) (Fig. 4). No further radioactivity was then eluted with 1 M-NaCl. After sialidase treatment, about 72% of the oligosaccharides were unbound, and the remaining species were eluted with 20 mM- (11%), 70 mM- (11%), 140 mM- (4%) and 200 mM-NaCl (2%) (Fig. 4). After acid treatment under conditions to effect oligosaccharide desialylation (10 mM-HCl for 1 h at 80 °C) [16,22], 88% of the material was not retained by the QAE-Sephadex column, whereas 6%, 5% and 1% were eluted with 20 mM-, 70 mM- and 140 mM-NaCl respectively (results not shown). Thus the majority of the negative charge in the oligosaccharides is due to the presence of sialic acid residues. The existence of small fractions of oligosaccharides eluted with 140 mM- and 200 mM-NaCl could indicate the presence of additional negatively charged groups, such as sulphate [16] or phosphate [35]. However, we have detected no incorporation of radioactivity into apo B-48 when human jejunal explants in organ culture were incubated with [^{35}S]sulphate, suggesting either the absence of or negligible oligosaccharide sulphation. Since

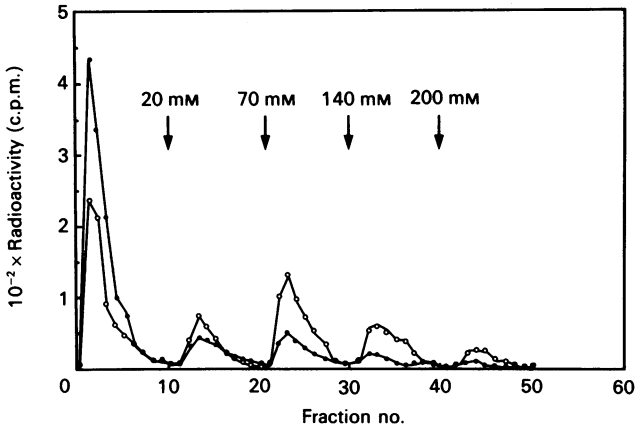

Fig. 4. QAE-Sephadex chromatography of apo B-48 biantennary complex oligosaccharides

The oligosaccharides were isolated on Con A–Sepharose column and incubated with (●) or without (○) sialidase. After being desalted on a Bio-Gel P-2 column, the oligosaccharides were freeze-dried and dissolved in 2 mM-Tris base. The elution of the oligosaccharides from the column (0.6 cm × 4 cm) was carried out with Tris base and 20 mM-, 70 mM-, 140 mM- and 200 mM-NaCl in 2 mM-Tris. No further radioactivity was eluted with 1 M-NaCl. Fractions (1 ml) were collected and counted for radioactivity. Recovery of radioactivity from the column was 82%.

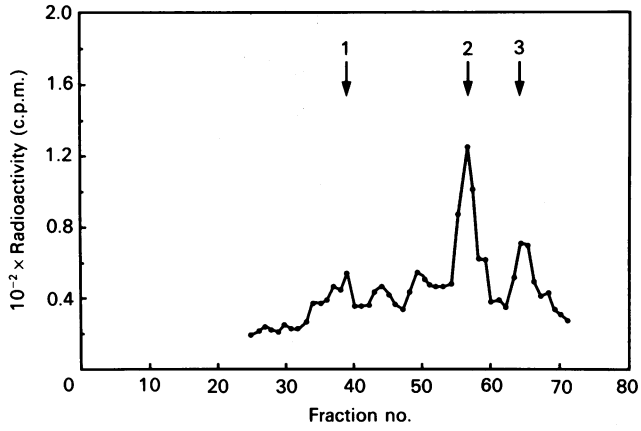

Fig. 5. H.p.l.c. separation of apo B-48 high-mannose-type oligosaccharides

The oligosaccharides were released with *N*-Glycanase, labelled by reduction with NaB^3H_4 and separated on a Con A–Sepharose column. The oligosaccharides (eluted from Con A–Sepharose with 500 mM-methyl α-D-mannoside) were desalted on Amberlite MB-3, and analysed on a MicroPak AX-5 column with a linear gradient of acetonitrile/water, starting with a 13:7 (v/v) mixture and ending with a 7:13 (v/v) mixture in 60 min. The flow rate was 1 ml/min and 0.8 ml fractions were collected. Arrows indicate the elution positions of standards: 1, $Man_5GlcNAcGlcNAcol$; 2, $Man_8GlcNAcGlcNAcol$; 3, $Man_9GlcNAcGlcNAcol$.

phosphate groups are contained on oligosaccharides of lysosomal enzymes [36], they are not expected to reside on apo B-48 oligosaccharides.

Identification of oligosaccharides by h.p.l.c.

After reduction with NaB^3H_4, labelled oligosaccharide fractions separated by Con A–Sepharose chromatography were analysed by h.p.l.c. on a MicroPak AX-5 column. Each sample was desalted, freeze-dried and dissolved in acetonitrile/water (13:7, v/v). High-mannose oligosaccharides (eluted from a Con A–Sepharose column with 500 mM-methyl α-D-mannoside) revealed a major species corresponding to $Man_8GlcNAcGlcNAcol$. Smaller quantities of $Man_9GlcNAcGlcNAcol$ as well as shorter oligomannosyl chains were also present (Fig. 5).

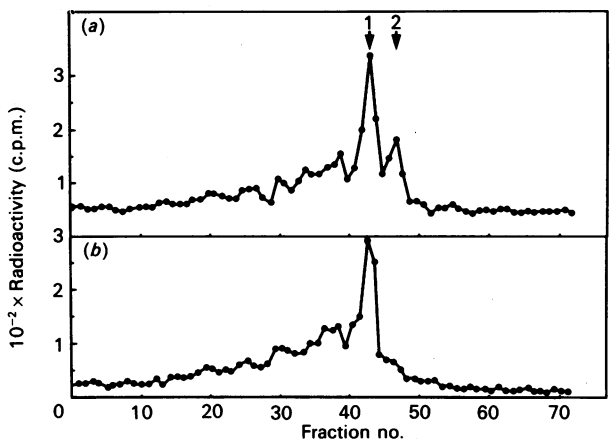

Fig. 6. H.p.l.c. separation of apo B-48 biantennary complex-type oligosaccharides

The oligosaccharides were released with *N*-Glycanase, labelled by reduction with NaB^3H_4 and separated on a Con A–Sepharose column. The oligosaccharides (eluted from Con A–Sepharose with 15 mM-methyl α-D-glucoside) were desalted, incubated in the absence (*a*) or in the presence (*b*) of α-L-fucosidase, and injected on to a MicroPak AX-5 column. The column was eluted with a linear gradient of acetonitrile/water, starting with a 13:7 (v/v) mixture and ending with a 7:13 (v/v) mixture in 60 min. The flow rate was 1 ml/min and 0.8 ml fractions were collected. Arrows indicate the elution position of standard oligosaccharides: 1, asialo biantennary complex ($Gal_2GlcNAc_2Man_3GlcNAcGlcNAcol$); 2, asialo biantennary complex with a fucose residue in the core.

The processing of high-mannose oligosaccharides entails the removal of three glucose as well as some mannose residues [27]. However some high-mannose chains in a variety of mature glycoproteins were found to contain one or two glucose residues. Since the h.p.l.c. analysis cannot distinguish between mannose and glucose residues, mannosidase digestion was performed in order to detect the presence of glucose residue(s), as glucose-containing oligosaccharides are resistant to complete α-mannosidase digestion. The oligosaccharides were digested with α-mannosidase to a mixture consisting of $Man_{1-3}GlcNAc$-GlcNAcol (results not shown), indicating the absence of glucose residues and the lack of hybrid-type structures [27].

The biantennary complex-type oligosaccharides (eluted from Con A–Sepharose with 15 mM-methyl α-D-glucoside) were first desialylated with sialidase to eliminate differences in the number of sialic acid residues (see Fig. 4), and desialylated oligosaccharides were analysed by h.p.l.c. on a MicroPak column (Fig. 6). The major peak (peak 1) corresponded to Gal_2-$GlcNAc_2Man_3GlcNAcGlcNAcol$, and the smaller one (peak 2) to the fucose-containing $Gal_2GlcNAc_2Man_3GlcNAcGlcNAcol$ (Fig. 6*a*). The fucose-containing species represented about 15% of total complex-type oligosaccharides. α-L-Fucosidase treatment resulted in the disappearance of the minor (fucose-containing) species (peak 2) but not the major oligosaccharide (peak 1) (Fig. 6*b*).

DISCUSSION

We have shown here that apo B-48 of human lymph chylomicrons possesses predominantly (78%) biantennary complex-type oligosaccharides, of which about half are sialylated. The ratio of monosialylated to disialylated chains was about 1:2.

These structures are typical for oligosaccharide chains of transferrin and many other secretory proteins [37]. About 15% of these oligosaccharides also contain a fucose residue as judged by fucosidase digestion and interaction of apo B-48 with *Ulex* lectin. In addition, apo B-48 possesses 16% of high-mannose oligosaccharides with $Man_8GlcNAc_2$ as the predominant species, and probably 6% of higher-branched complex-type oligosaccharides. In comparison, no fucosylated chains and no xylose residues have been found in hepatic apo B-100 from human plasma [7,8]. Because xylose is sometimes a contaminating sugar, it is important to demonstrate the presence of xylose residues in apo B-48 oligosaccharides. The presence [7] and the absence [8] of higher-branched oligosaccharides were reported for LDL apo B-100. Similar disagreement exists concerning the presence of the hybrid-type and the bisect oligosaccharides in apo B-100 [7,8]. The other oligosaccharide structures in apo B-48 have been found in apo B-100, except that apo B-100 contains proportionately twice as much high-mannose oligosaccharides (37%) [6,8] as does apo B-48. In addition, either a shorter $Man_5GlcNAc_2$ [6] or a longer $Man_9GlcNAc_2$ [8] was a predominant species in apo B-100. The carbohydrate content of about 4% in apo B-48 is similar to that reported for LDL apo B-100 (4.4%) [7]. A relatively small carbohydrate moiety in apo B-48 may explain the lack of a detectable shift in mobility on SDS/PAGE after *N*-Glycanase treatment.

Apo B-48 is a polypeptide fragment of approx. 48% of the *N*-terminal apo B-100. Thus the differences in the glycosylation pattern may be due to the organ-specificity and/or to an effect of the polypeptide moiety on the processing mechanism. Furthermore, differences in intracellular transport of the proteins and in assembly of the protein/lipid particles may also play a role. The higher proportion of high-mannose oligosaccharides in apo B-100 may be a result of the prevalence of high-mannose oligosaccharides in the *C*-terminal portion of the molecule, as a high degree of site-specific glycosylation has been reported [9,38]. Direct amino acid sequencing of apo B has suggested that asparagine-7 (in Asn-Val-Ser sequence) is not glycosylated [4,5]. Thus the number of *N*-linked oligosaccharide chains in apo B-48 is estimated to be as many as five. From the monosaccharide composition and the proportion of different types of *N*-linked oligosaccharides, it could be calculated that on average apo B-48 possesses one high-mannose-type and four complex-type chains. The site of high-mannose oligosaccharide is unknown. It is likely that, along with *N*-linked oligosaccharides, apo B-48 contains *O*-linked chains. This conclusion derives from lectin blotting studies, the presence of *N*-acetylgalactosamine and a higher content of galactose residues than expected for *N*-linked oligosaccharides. However, a definitive proof for the presence of *O*-linked sugars is at present lacking, and further study is required.

The role of the carbohydrate moiety in apo B is unknown. Studies carried out with the inhibitor tunicamycin have shown that glycosylation is not required for apo secretion in chick [39] and rat [40] liver, or for the assembly of the protein with its major lipid constituents [40]. A cluster of oligosaccharides has been localized to the putative LDL receptor domain and to some heparin-binding sites of human apo B-100 [4], and it is possible that oligosaccharides protect the precise function of this region of the molecule. In Watanabe hyperlipidaemic rabbits, apo B-100 oligosaccharides were poorly sialylated, supporting a hypothesis that altered surface charge of LDL particles may play a role in atherosclerotic development [41]. In chylomicron-retention disease, which is characterized by malabsorption and low concentrations of plasma lipids and cholesterol, there are no detectable circulating chylomicrons. However, apo B-48 accompanied by the accumulation of lipid droplets is present in the small-intestinal cells of patients, suggesting that chylomicron assembly and/or secretion may be defective. Interestingly, incorporation of mannose into intracellular chylomicrons was reduced by 80% in patients as compared with normal subjects [42]. It appears that the glycosylation deficiency in chylomicron-retention disease is confined to apo B-48, as this is the only apolipoprotein present in chylomicrons that contains *N*-linked oligosaccharides.

As more data concerning the structural characteristics of apo B-48 become available, it should be feasible to delineate the complex mechanisms of interactions and to understand processes underlying apo B function in health and disease.

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