# Characterization of recombinant human plasma lecithin: cholesterol acyltransferase (LCAT): N-linked carbohydrate structures and catalytic properties

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**Abstract** The major N-linked carbohydrate structures were determined for recombinant human plasma lecithin:cholesterol acyltransferase (LCAT). The analysis of the structure of oligosaccharides by fast atom bombardment mass spectrometry (FAB-MS) and linkage analysis was preceded by reduction and carboxymethylation of the intact glycoproteins and digestion with trypsin and proline specific endopeptidase. The N-glycans were subsequently released from the glycopeptides by PNGase F digestion and the oligosaccharides were separated using a C18 Sep-pak® cartridge. The data from the combination of FAB spectrometry and linkage analysis show that the N-linked glycans present on recombinant LCAT (rLCAT) were composed primarily of triantennary and tetraantennary structures with and without core fucosylation. A minor population of glycans (less than 5%) contained up to three repeats of N-acetyllactosamine in one or more antennae. The LCAT activities of both recombinant and circulating forms of plasma LCAT were determined using low molecular weight and lipoprotein substrates. The catalytic behavior of these two enzyme forms were found to be very similar if not identical. These findings validate the concept that the recombinant enzyme can serve as an appropriate model for structure/function studies of LCAT and provide the foundation for subsequent structural studies.—**Lacko, A. G., A. J. Reason, C. Nuckolls, B. J. Kudchodkar, M. P. Nair, G. Sundarrajan, P. H. Pritchard, H. R. Morris, and A. Dell.** Characterization of recombinant human plasma lecithin: cholesterol acyltransferase (LCAT): N-linked carbohydrate structures and catalytic properties. *J. Lipid Res.* 1998. **39:** 807–820.

**Supplementary key words** LCAT • glycoprotein structure

Lecithin:cholesterol acyltransferase (LCAT) catalyzes the esterification of plasma cholesterol in mammals and it is a key enzyme in cholesterol homeostasis (1). LCAT is a plasma glycoprotein with unusually high carbohydrate content (~23% by weight; [2, 3]). After the determination of the primary structure of LCAT (4), Yang et al. (5) calculated the hydropathy index for human LCAT indicating that the amino acid composition alone would give rise to an extremely hydrophobic polypeptide structure. Consequently, the rather substantial carbohydrate component (2, 3) of LCAT (~23% by weight) is expected to play a key role in the conformational stability of this enzyme. The elucidation of the representative N-linked carbohydrate structures of the respective recombinant forms of LCAT appears essential for the validation of these respective enzyme species as potential models for structure/function studies of plasma LCAT (6).

The bulk of the oligosaccharides in LCAT are linked to the polypeptide backbone through asparagine residues (7). Recent studies have shown that elimination of specific N-linked glycosylation sites had a profound impact on enzymatic activity (8, 9). Removal of all N-linked glycosylation sites resulted in only trace amounts of marginally active enzyme to be secreted by these recombinant cells (8, 9). Elimination of specific single glycosylation sites resulted in nearly normal secretion of the enzyme and variable alterations of LCAT activity. For instance, the mutagenesis of N→Q are residue 384 resulted in doubling the LCAT activity while alteration of another site (N-84→Q) led to an ~80% reduction in activity (8, 9).

Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein(s); CETP, cholesteryl ester transfer protein; BHK, baby hamster kidney; CHO, Chinese hamster ovary; rLCAT, recombinant LCAT; pLCAT, plasma LCAT; PNGase F, peptide-N-glycosidase F.

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Although recent studies described the glycan structures in plasma LCAT (7), no information has been published on the corresponding recombinant forms of the enzyme. Recent reports include the comparisons of the susceptibility of several recombinant enzyme forms to glycanase digestions (6) but no detailed structural information is available on the glycans of any of the rLCAT species produced. The present study was undertaken to establish the types of carbohydrate structures in recombinant LCAT and to compare the enzymatic properties of plasma LCAT and the recombinant form secreted by a stably transfected BHK cell line (10). These structural and kinetic data strongly suggest that the recombinant form of LCAT secreted by stably transfected BHK cells (10) is likely to be an appropriate model for detailed structural/functional characterization of the enzyme.

## MATERIALS AND METHODS

### Purification and enzymatic studies

*Production of LCAT by the t-BHK cells.* The development of the stable transfected BHK cell lines secreting recombinant LCAT (rLCAT) has been described (10). The BHK cells were cultured on a porous microcarrier, Cultisphere-G (Hyclone Laboratories, Inc., Logan, UT). The cells were initially incubated with 350 mg of Cultisphere-G beads in DMEM for 24 h to allow the attachment of the cells to the solid support. After a 4-day growth period, the medium was changed to Opti-MEM to allow the secretion of LCAT by the cells for a 72-h period. The final yield was $\sim 2 \times 10^9$ cells and $\sim$2 mg of LCAT per 150 ml of medium. Alternatively, the BHK cells were cultured in multilayer flasks (Nunclone Inc.) without beads.

The purification procedure for rLCAT has been previously described (10). Briefly, the medium from the BHK cell cultures was centrifuged to remove insoluble material and then it was applied to a phenyl-Sepharose column (2.5 $\times$ 15 cm) equilibrated with 0.005 m phosphate buffer, 0.3 m NaCl, pH 7.4. The column was washed with the same buffer until the absorbance$_{280}$ decreased to $<$0.01. Subsequently, the LCAT was eluted with deionized water yielding a homogeneous enzyme preparation (10).

Purification of LCAT from the human plasma was achieved by a modification of a previously published procedure (11). Briefly, 1500–1800 ml of cryo-precipitate supernatant (obtained from Carter Blood Center, Ft. Worth, TX) was adjusted to a final polyethylene glycol (PEG 6000) concentration of 7% and the precipitate was removed and discarded. The conductivity of the supernatant was adjusted to $>$25 m MHO with 5 m NaCl and the plasma extract was applied to a previously equilibrated (0.001 m $Na_3PO_4$, 0.3 m NaCl, pH 7.4) phenyl-Sepharose (P-S) column (5 $\times$ 20 cm). Upon loading of the sample, the column was washed with 0.001 m $Na_3PO_4$ 0.3 m NaCl, pH 7.4, until the absorbance$_{280}$ of the effluent was $<$0.08. Subsequently the phenyl-Sepharose column was connected in a sequence to an Affigel-Blue [A-B, 5 cm $\times$ 15 cm] column; and to a heparin-Sepharose [H-S; 5 $\times$ 12 cm] column. The H-S and A-B columns were equilibrated with 0.02 m phosphate buffer, pH 7.4, before connecting the system together. Once the columns were connected, the eluant was changed to deionized water to obtain the LCAT-containing fractions. The conductivity of the eluant was then adjusted to $<$4.0 m MHO and loaded on a DEAE-agarose (Bio-Rad Labs) column (2.5 $\times$ 25 cm) previously equilibrated with 5 mm $NaPO_4$, pH 7.2, containing 0.025% $NaN_3$. The column was eluted with 700 ml of 5 mm $Na_3PO_4$, 0.05 m NaCl, pH 7.2, resulting in the elution of a large protein peak that lacked LCAT activity. Subsequently, the LCAT was eluted from the column by a linear gradient of 1000 ml of 5 mm $NaPO_4$, 0.05 m NaCl, pH 7.2, and 500 ml of 5 mm $Na_3PO_4$, 0.2 m NaCl, pH 7.2. The elution of the bulk of the LCAT activity corresponded with an NaCl concentration of 0.12 m. The fractions containing LCAT were pooled, dialyzed against 1 mm $Na_3PO_4$, pH 7.4, and loaded on a DEAE-MATREX (Amicon Corp.) column (1.5 $\times$ 20 cm), previously equilibrated with 1 mm $Na_3PO_4$, pH 7.4. The enzyme was eluted using a linear gradient of 1000 ml of 1 mm Tris-HCl, 1 mm EDTA, 25 mm NaCl, pH 7.4, and 1000 ml of 10 mm Tris, 5 mm EDTA, 200 mm NaCl, pH 7.4. The LCAT-containing fractions were pooled, concentrated by ultrafiltration, and dialyzed against 1 mm phosphate buffer, pH 7.4. The dialyzed concentrated enzyme was then passed through an Affigel-Blue (Bio-Rad Labs) column (2.5 $\times$ 20 cm) before loading on to the hydroxyl apatite column (1.5 $\times$ 60 cm, previously equilibrated with 4 mm $Na_3PO_4$, 150 mm NaCl, pH 6.9). The LCAT was then eluted with a linear gradient of 250 ml of 15 mm $NaPO_4$, 150 mm NaCl, pH 6.9, and 250 ml of 60 mm $Na_3PO_4$, 150 mm NaCl, pH 6.9. The LCAT activity has consistently been detected in the fractions that coincide with the initial decrease in conductivity. These fractions were pooled, dialyzed against a saturated ammonium sulfate solution, and stored at $-70$°C without significant losses in enzyme activity. The LCAT preparations isolated by this procedure were essentially homogeneous ($>$95% purity) by SDS gel electrophoresis.

Enzymatic desialylation of LCAT was achieved by incubating the enzyme samples with *C. perfringens* neuraminidase (Sigma Chemical Co.). A sample of 2 ml of LCAT (containing $\sim$100 $\mu$g of enzyme) was added to 2

ml of the solution containing 12 units of neuraminidase. After incubation, the desialylated enzyme was purified on phenyl-Sepharose as described above and was found to be essentially free of sialic acids. Sialic acid content of all preparations was determined according to Skoza and Mohos (12) as described previously (13).

*Assay for LCAT activity.* Two hundred μl of proteoliposome (14) substrate containing [$^3$H]cholesterol was incubated with 10-μl aliquots of the enzyme for 6 h at 37°C. The reaction was terminated by the addition of 2 ml of isopropanol, and free and esterified cholesterol were separated by thin-layer chromatography (TLC) followed by isolation of the separated lipid zones for scintillation counting (15).

Alternatively, lipoprotein substrates were used. Lipoproteins were prepared by discontinuous density gradient ultracentrifugation of human plasma (16) yielding 12 fractions. Of these, #7 was designated as $HDL_2$ (d 1.059–1.086 g/ml) and #8–10 as $HDL_3$ (d 1.086–1.23 g/ml). The $HDL_2$ and $HDL_3$ fractions were labeled with [$^3$H]cholesterol as previously described (17).

*Phospholipase activity of LCAT* activity was monitored using a fluorescent water-soluble substrate, 1,2 bis[4-(1-butanoyl-*sn*-glycero-3-phosphatidylcholine (0.125–3 μm) as described by Bonelli and Jonas (18). The excitation wavelength of 332 nm and an emission wavelength of 398 nm were used to monitor the increase in fluorescence over time upon the addition of samples of LCAT (~0.1–0.2 μg).

## Carbohydrate structural studies

*N-glycosidase F digestion of rLCAT.* The rLCAT samples containing 1 μg/digest (100 mm EDTA, 100 mm phosphate, pH 8.6, 1% Nonidet P40, 0.5% mercaptoethanol, 0.1% SDS) were boiled for 10 min. The digestion was initiated by adding various amounts (2–200 mUnits) N-glycosidase F and the reaction was carried out at 37°C for 10 min. The digests were analyzed by SDS-PAGE (7.5%) electrophoresis followed by Western blotting, transferring of protein bands from the gel to the nitrocellulose membrane (0.22 micron, MSI Inc., Westborough, MA). The membranes were subsequently incubated with an anti-human LCAT antibody and the blots were developed with alkaline phosphatase substrate (KPL, Gaithersburg, MD).

*Reduction and carboxymethylation.* The portion of LCAT to be used for carbohydrate analysis was first subjected to reduction and carboxymethylation. This involved treating the preparation with a 4 molar excess of dithiothreitol (DTT) in Tris buffer (0.6 m; pH 8.5). After a 30-min incubation period at 37°C, iodoacetic acid (IAA) was applied in a 5-fold molar excess over the DTT. The excess DTT and IAA were removed by dialysis against deionized water.

*Protease digestion.* After dialysis, the enzyme was lyophilized and digested, first with TPCK-treated trypsin for 6 h at 37°C in 50 mm ammonium bicarbonate (pH 8.4), followed by treatment with a proline-specific protease (Seikagaku Ltd.) for 4 h at 37°C in 100 mm ammonium bicarbonate (pH 7.8). The proteolytic digestion was terminated by lyophilization.

*PNGase F digestion.* The N-glycans were removed from the LCAT glycopeptides by treatment with 0.5 mU of PNGase F in 50 mm ammonium bicarbonate buffer (pH 8.4) for 16 h at 37°C. The sample was lyophilized, dissolved in 5% acetic acid, and loaded onto a C18-Sep-pak® column and eluted with 5% acetic acid followed by 20% propan-1-ol in 5% acetic acid and 60% propan-1-ol in 5% acetic acid. The N-linked oligosaccharides eluted in the 5% acetic acid fraction. Peptides eluted in the subsequent solvent mixtures.

*Desialylation* was carried out using one of two methods. Chemical desialylation was carried out using 200 ml of a 0.025 m solution of sulfuric acid. The reactants were incubated at 80°C for 1 h and the reaction was terminated by lyophilization. Traces of remaining sulfuric acid were removed by dilution with a small amount of deionized water and lyophilization. Otherwise, sialic acid moieties were removed using 20 mU of neuraminidase (Boehringer-Mannheim) in 50 mm sodium acetate (pH 5.5) for 16 h at 37°C. The reaction was terminated by lyophilization.

*β-Galactosidase digestion.* Terminal β-linked galactose residues, exposed by the action of the neuraminidase,

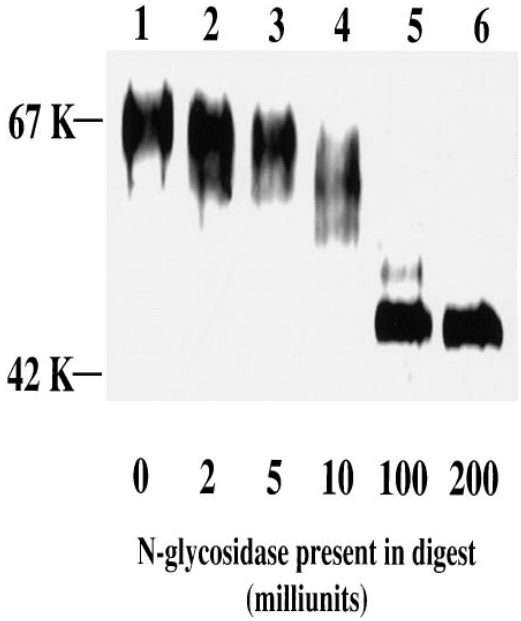


**Fig. 1.** N-glycosidase F digestion of rLCAT with increasing amounts of N-glycosidase (2–200 mUnits/digest).

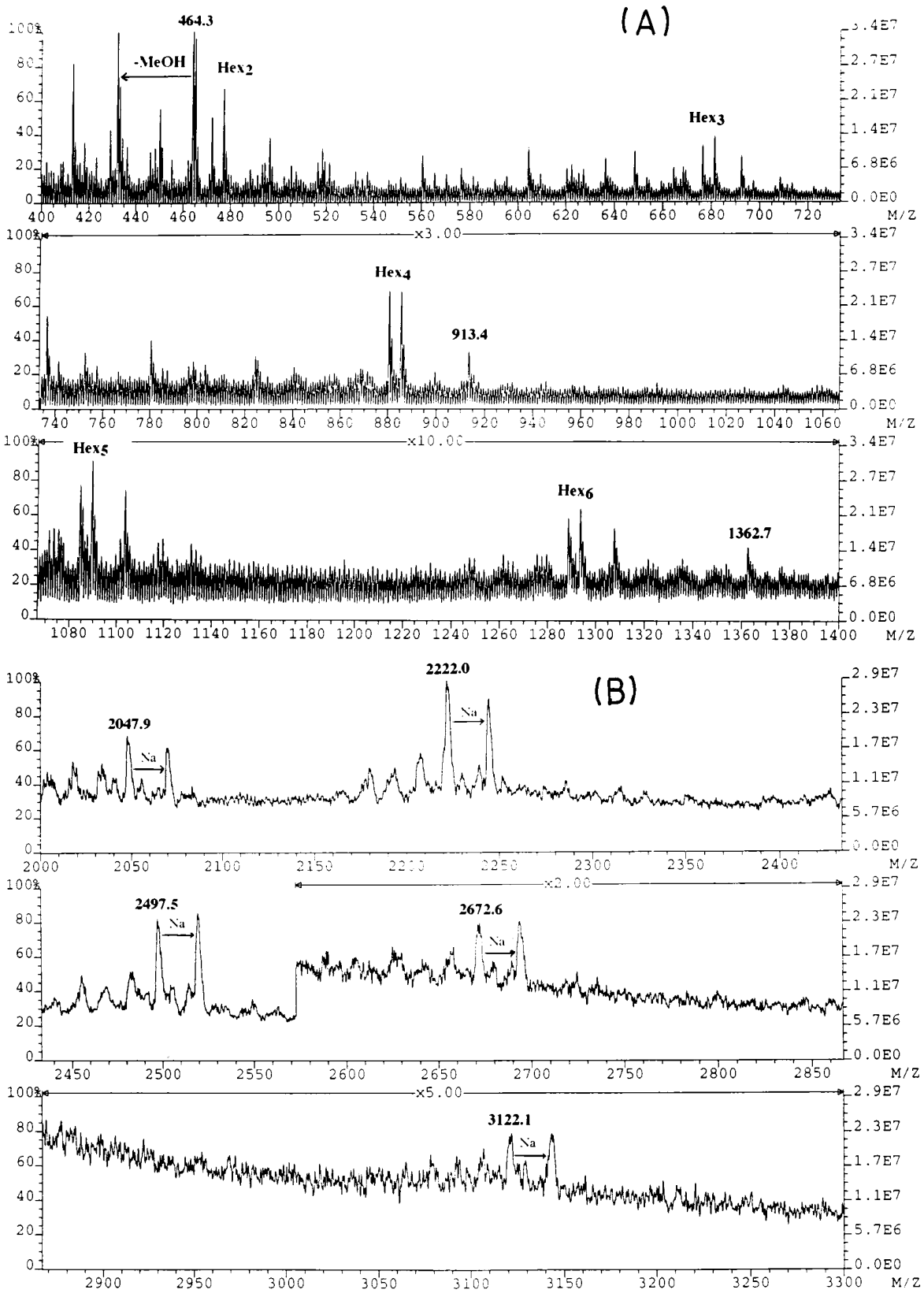


**Fig. 2.**

**Fig. 2.** Partial FAB mass spectra ([A] fragment ion region, [B] molecular ion region) of permethylated N-glycans released from recombinant LCAT. The pairs of signals labeled Hexn are ammonium and sodium cationized molecular ions of glucose oligomers. The signal labeled Hex12 is the ammonium cationized molecular ion of one of a series of contaminating glucose oligomers which are most likely derived from leakage from the dialysis membranes used in desalting steps.

were removed using 0.01 U of exo-β-d-galactosidase from bovine testes (Boehringer-Mannheim) in 200 μl of 5 mm sodium citrate–phosphate buffer, pH 4.6, at 37°C for 48 h.

*β-N-Acetylhexosaminidase digestion*. Terminal β-linked N-acetylhexosaminidase residues exposed by the action of the β-galactosidase were removed using 0.1 U of exo-β-N-acetyl-d-hexosaminidase from bovine kidney (Boehringer-Mannheim) in 200 μl of 5 mM sodium citrate–phosphate buffer, pH 4.6, at 37°C for 48 h.

*Permethylation*. Prior to FAB–MS analysis, oligosaccharides were derivatized in screw-capped tubes by the addition of a NaOH–DMSO slurry (1 ml) made by grinding 8 pellets of NaOH in a dried mortar with dry glass-distilled DMSO (2 ml). This was followed by the addition of methyl iodide, and the mixture was shaken at room temperature for 10 min before quenching slowly with water (2 ml). The derivatized oligosaccharides were extracted into chloroform (1 ml), and the solvent was evaporated in a stream of nitrogen.

*FAB–MS*. FAM mass spectra were acquired on a ZAB-2SE FPD mass spectrometer fitted with a caesium ion gun operated at 25 kV. Spectra from this instrument were computer processed. The matrix was thioglycerol and the derivatized oligosaccharides were dissolved in methanol prior to loading on the target.

*GC–MS analysis*. Partially methylated alditol acetates were prepared from the permethylated oligosaccharides for linkage analysis by GC–MS, according to the method of Albersheim et al. (19). Briefly, the permethylated samples were hydrolyzed with 2 m TFA for 2 h at 121°C, reduced with 10 mg/ml sodium borodeuteride in 2 m ammonium hydroxide at room temperature for 2 h, followed by acetylation with acetic anhydride at 100°C for 1 h. GC–MS analysis was carried out on a Fisons Instruments MD 800 apparatus. The partially

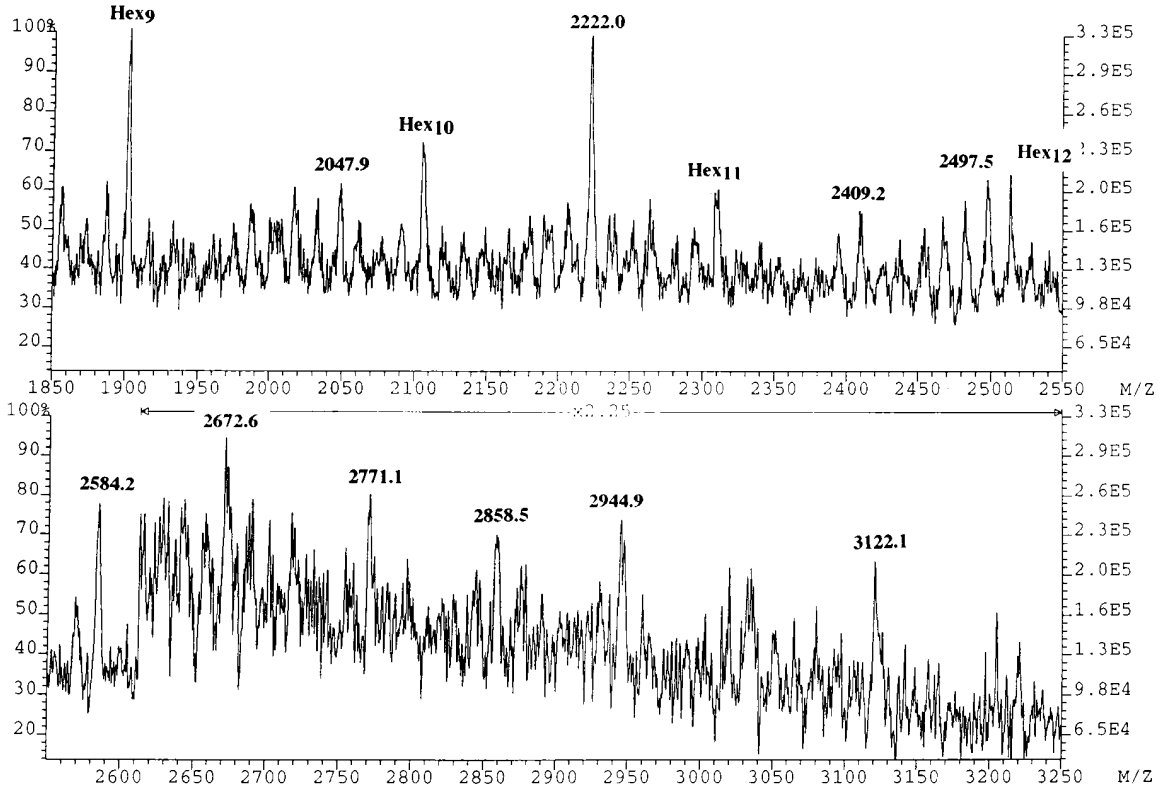


**Fig. 3.** Partial FAB mass spectrum of permethylated N-glycans released from recombinant LCAT. This LCAT sample was isolated from the culture media of cells that were grown in the presence of the sialidase inhibitor 2,3 dehydro-2-deoxy N-acetyl-neuraminic acid. For an explanation of Hex10-12 see legend to Fig. 1.

TABLE 1. Data produced from linkage analysis of recombinant LCAT

| Retention Time | Assignment | Ions Monitored |
|---|---|---|
| *min* | | |
| 12.70 | terminal fucose | 175, 162, 131, 118 |
| 14.28 | terminal galactose | 205, 162, 161, 145 |
| 15.48 | terminal fucose | 190, 161, 130, 129 |
| 15.78 | 3-linked galactose | 234, 161, 129, 118 |
| 16.30 | 6-linked galactose | 233, 189, 162, 129 |
| 16.72 | 2,4-linked mannose | 233, 190, 173, 113 |
| 17.10 | 2,6-linked mannose | 190, 189, 130, 129 |
| 17.28 | 3,6-linked mannose | 234, 189, 129, 118 |
| 19.17 | 4-linked GlcNAc | 233, 159, 117 |
| 20.48 | 4,6-linked GlNAc | 261, 159, 117 |

methylated alditol acetates were dissolved in hexanes prior to on-column injection on a DB-5 (30 m × 0.32 mm internal diameter J&W Scientific) column at 90°C. The oven was held at 90°C for 1 min before being increased to 290°C at a rate of 8°/min.

## RESULTS

### Carbohydrate configuration of LCAT

Endoglycosidase F digestion was used to estimate the number of carbohydrate chains linked to the polypeptide portion of LCAT. One μg of recombinant enzyme was incubated with increasing amounts (2–200 mU) of endoglycosidase F (8) for 10 min at 37°C. After the incubation period, the digested samples were subjected to SDS-PAGE and the LCAT bands were visualized by Western blotting utilizing a polyclonal antibody. **Figure 1** shows the electrophoresis pattern resulting from these experiments, indicating the presence of five bands (including the control) and thus the four N-linked carbohydrate chains in recombinant LCAT.

### Analysis of carbohydrate structures

Recombinant lecithin:cholesterol acyltransferase (LCAT) produced by a BHK cell line (rLCAT), was subjected to the following protocol. After reduction and carboxymethylation, the LCAT sample was digested with trypsin and subsequently by proline-specific enzyme to facilitate the release of N-glycans by PNGase F digestion. The N-glycans were separated from the proteolytically cleaved peptides using a C18 Sep-pak® cartridge (20, 21).

*Analysis of the N-glycans released from recombinant LCAT.* The FAB spectra from recombinant LCAT (r-LCAT) (**Fig. 2**) contained $[M + H]^+$ signals at *m/z* 2047.9 ($Hex_5HexNAc_4$), *m/z* 2222.0 ($Fuc1Hex_5HexNAc_4$), *m/z* 2497.5 ($Hex_6HexNAc_5$), *m/z* 2672.6 ($Fuc1Hex_6HexNAc_5$), and 3122.1 ($Fuc_1Hex_7HexNAc_6$; Fig. 1B). These data suggest that the recombinant LCAT contains high levels of tri- and tetraantennary glycans and that core fucosylation is present on many components. Fucosylation of the antennae could be ruled out because A-type fragmentation, at the reducing end of the HexNAc residues in the antennae (22), produced a signal at *m/z* 464 ($HexHexNAc^+$) but not at *m/z* 638 ($FucHexHexNAc^+$) (Fig. 2A). Interestingly, minor A type fragment ions were present at *m/z* 913 ($Hex_2Hex\ NAc_2^+$) and *m/z* 1362 ($Hex_3HexNAc_3^+$) suggesting the presence of lactosanine repeats in a minor population of glycans (see discussion of Fig. 4, below). Surprisingly, none of the signals present in this initial spectrum indicated the presence of sialic acids. Indeed, subsequent chemical analysis of the intact rLCAT revealed only traces of sialic acid (<1% by weight) compared to about 5.5% sialic acid found in pLCAT. As BHK cell lines are known to have the necessary sialyl transferases required for the addition of sialic acid to N-linked glycan structures, it was concluded that sialic acids were probably being removed from the glycan chains by a BHK-derived sialidase analogous to that found in CHO cell lines (23). To test this hypothesis, the recombinant enzyme was isolated in the presence of the sialidase inhibitor 2,3-dehydro-2-deoxy N-acetyl-neuraminic acid resulting in the restoration of the full complement of sialic acids (~5.5%).

The molecular ion region of the FAB spectrum (**Fig. 3**) of permethylated glycans derived from this batch retained all of the signals shown in Fig. 1, i.e., *m/z* 2047.9, *m/z* 2222.0, *m/z* 2497.5, *m/z* 2672.6, and *m/z* 3122.1. However, additional signals were present at *m/z* 2409.2 ($NeuAc_1Hex_5HexNAc_4$), *m/z* 2584.2 ($NeuAc_1Fuc_1Hex_5HexNAc_4$), *m/z* 2771.1 ($NeuAc_2Hex5HexNAc_4$), *m/z* 2858.5 ($NeuAc_1Hex_6HexNAc_5$), and *m/z* 2944.9 ($NeuAc_2Fuc1Hex_5HexNAc_4$). Linkage analysis of this permethylated sample produced the data presented in **Table 1**. These data demonstrated the presence of terminal fucose, terminal galactose, 30 and 6-linked galactose, 2-linked, 2,6-linked, 2,4-linked, and 3,6-linked mannose, as well as 4-linked and 4,6-linked N-acetylglucosamine. In order to unambiguously define the core structures present in this wide range of glycans, the sialic acids were removed to generate a less complex mixture of glycans. After neuraminidase digestion, signals for permethylated glycans were present at the same *m/z* values as the initial non-sialylated sample (**Fig. 4**), i.e., *m/z* 2047.9 ($Hex_5HexNAc_4$), *m/z* 2222.0 ($FucHex_5HexNAc_4$), *m/z* 2497.5 ($Hex_6HexNAc_5$), *m/z* 2672.6 ($FucHex_6HexNAc_5$), and *m/z* 3122.1 ($FucHex_7HexNAc_6OMe^+H^+$). A larger sample was used in this experiment compared with the experiment yielding the data in Fig. 2 resulting in a significant improvement in the sensitivity at high mass, enabling the detection of additional mi-

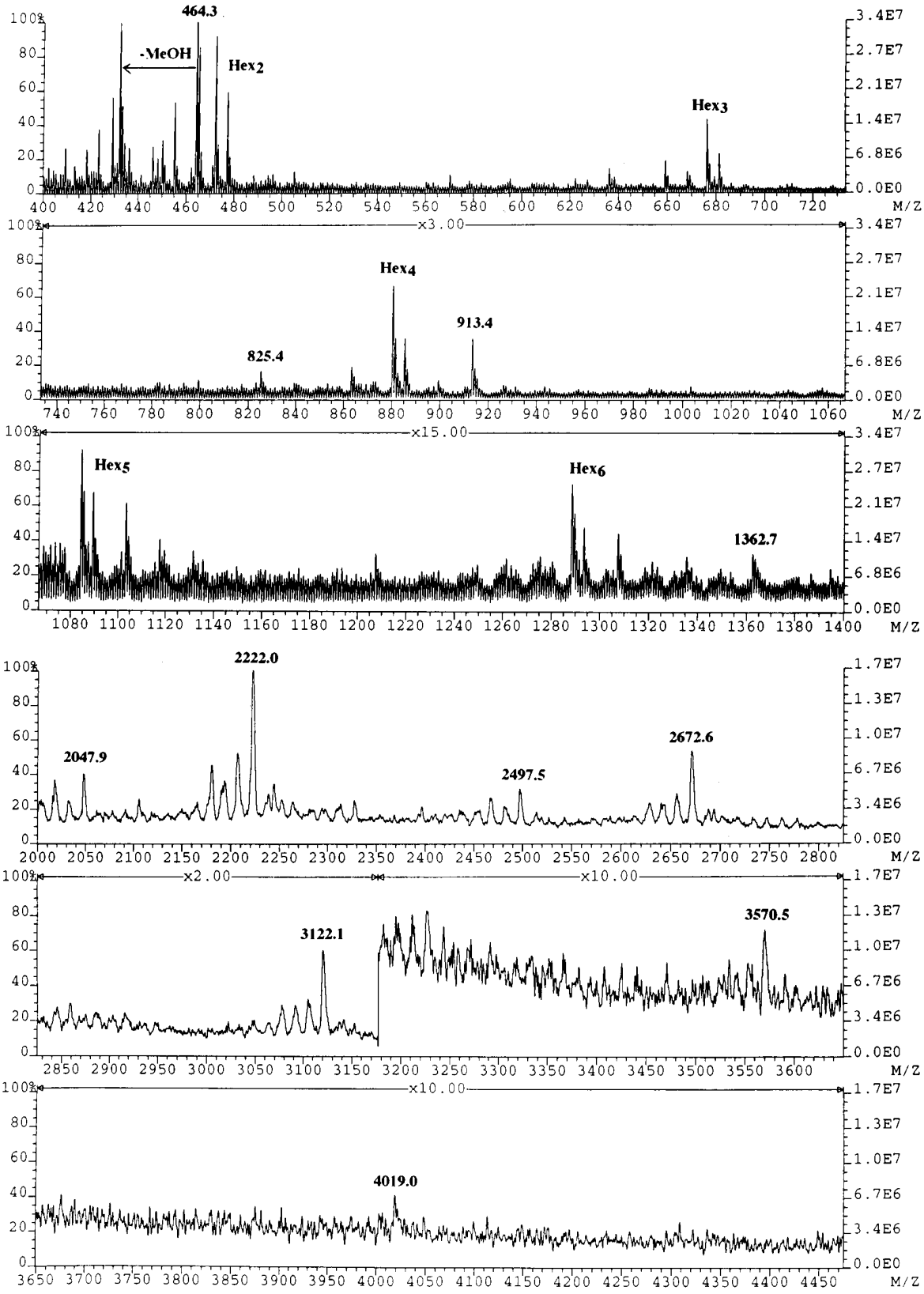


**Fig. 4.** Partial FAB mass spectrum of permethylated N-glycans released from recombinant LCAT after desialylation. For an explanation of Hex2-6 see legend to Fig. 1.

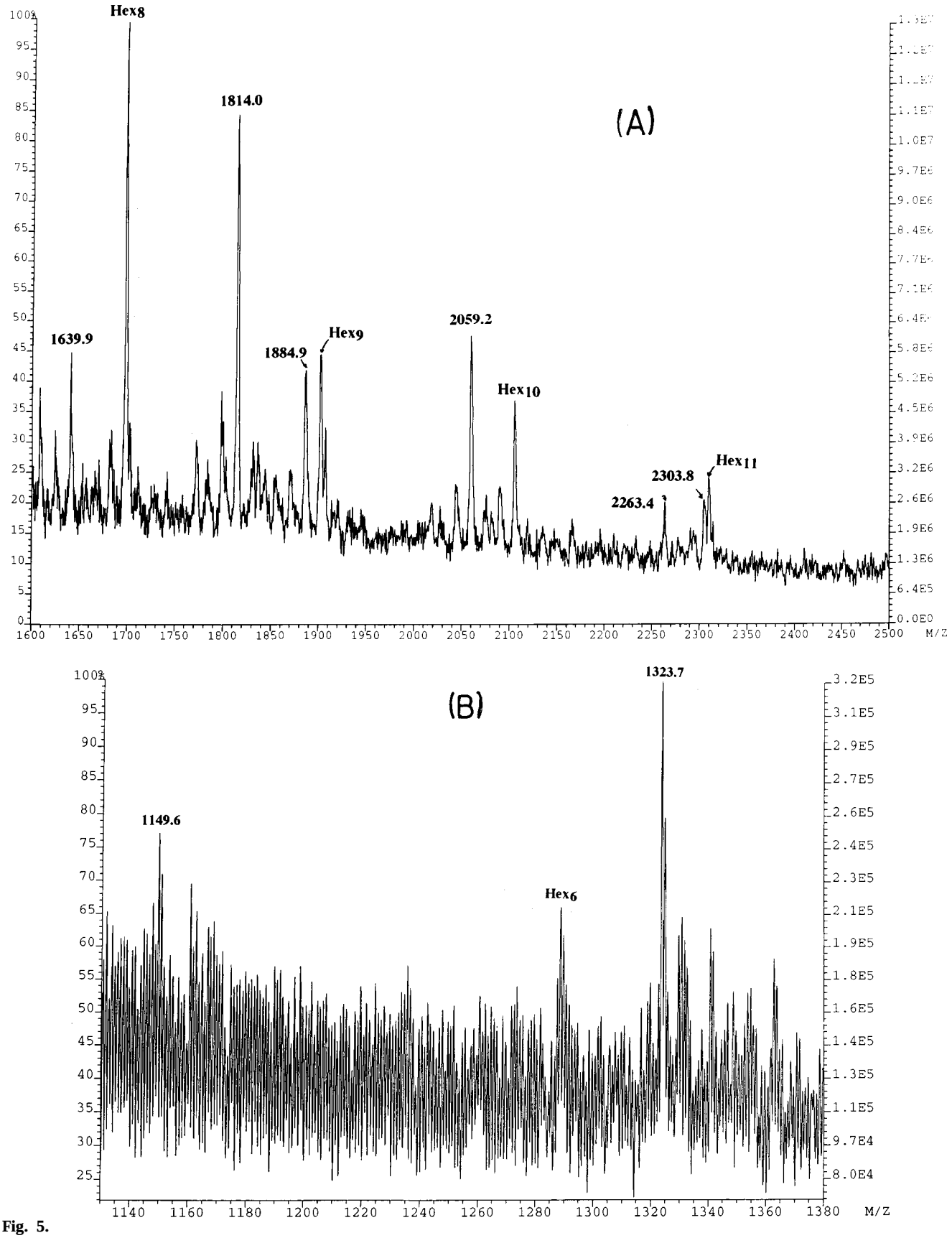


**Fig. 5.**

nor signals at *m/z* 3570.5 ($FucHex_8HexNAc_7\,OMe^+H^+$) and 4019.0 ($FucHex_9HexNAc_8OMe^+H^+$). These two molecular ion compositions are consistent with tri- and/or tetrantennary glycans having up to two (tetraantennary) or three (triantennary) repeats of N-acetyllactosamine. A-type fragment ions are, indeed, present for such antennae: *m/z* 913.4 ($Hex_2HexNAc_2^+$) and 1362.7 ($Hex_3HexNAc_3^+$) [compare to Fig. 2]. After desialylation, β-galactosidase was used to unambiguously define whether the signals at *m/z* 2497.5, *m/z* 2672.6, and *m/z* 3122.1 arose from tri- and tetraantennary structures or from structures with fewer antennae but with N-acetyllactosamine repeats. The molecular ion region of the spectrum obtained from FAB–MS analysis of a portion of the permethylated products is shown in **Fig. 5A**. After the digest, the signals at *m/z* 2047.9 and *m/z* 2222.0 shifted to *m/z* 1639.9 and *m/z* 1814.0, respectively (loss of two Ga1 residues), the signals at *m/z* 2497.5 and *m/z* 2672.6 shifted in mass to *m/z* 1884.9 and *m/z* 2059.2, respectively, (loss of three Ga1 residues) and that at *m/z* 3122.1 to *m/z* 2303.8 (loss of four Ga1 residues). These data show that *m/z* 2047.9 and 2222.0 are molecular ions of biantennary structures, *m/z* 2497.5 and 2672.6 are molecular ions of triantennary structures, and *m/z* 3122.1 is the molecular ion of a tetraantennary structure. The remainder of the products from this digest were subjected to digestion with bovine kidney exo-β-acetyl-d-hexosaminidase followed by FAB–MS to probe the nature of the core structures. The permethylated products gave two molecular ions at *m/z* 1149.6 ($Hex_3HexNAc_2OMe^+H^+$) and 1323.7 ($FucHex_3\,HexNAc_2OMe^+H^+$) (Fig. 5B). The latter was the most abundant molecular ion confirming that a major portion (estimated from molecular ion abundances to be about 70%) of the glycans present in rLCAT are core fucosylated. Taken together, the FAB–MS and linkage data from the intact and enzyme digested samples showed that the major structures on rLCAT are bi- and triantennary glycans with and without core fucosylation and tetraantenary glycans with core fucosylation (**Fig. 6**). In addition, a minor population of glycans (estimated from molecular ion abundances to be less than 5%) contained up to three repeats of N-acetyllactosamine in one or more antennae.

### LCAT activity of recombinant and plasma enzyme preparations

Because of the marked differences observed in glycan structure between the recombinant and plasma LCAT, it was important to examine the catalytic properties of these enzyme forms. The data in **Table 2** show the reactivity of both rLCAT and pLCAT with lipoprotein substrates. These studies were conducted with enzyme preparations that were standardized based on their reactivity with a proteoliposome substrate in order to avoid discrepancies in specific activity due to differential inactivation during storage. The $V_{max}$ and $K_m$ values are essentially the same for the pLCAT and the sialylated rLCAT preparations while the $K_m$ was 2- to 30-times higher for the desialylated recombinant enzyme (−S/rLCAT).

The data in **Table 3** show similar results with the fluorescent substrate, 1,2 bis[4-(1-pyreno)-butanoyl-*sn*-glycero-3-phosphatidylcholine (18). Here the $K_m$ values of the recombinant and plasma enzymes were very similar and desialylation of both enzymes gave rise to a nearly 3-fold increase in $K_m$. During the phospholipase activity studies, with the fluorescent substrate, apoA-I was found to enhance the activity of LCAT at low substrate concentrations (**Fig. 7**). Serum albumin and apoD had no such effect (data not shown). When the effects of apoA-I on pLCAT and rLCAT were compared, the results once again were similar in the catalytic behavior of both enzymes (**Fig. 8**). Thus, both the native and recombinant forms of the intact (sialylated) enzyme were activated by apoA-I to similar extents and the desialylated rLCAT, both in the presence and absence of apoA-I, showed lower activity against the phospholipase substrate.

In order to compare the effects of in vitro desialylation on rLCAT and pLCAT, respectively, both purified enzymes were digested by neuraminidase. Desialylation of pLCAT and rLCAT resulted in modest decreases in activity (23% for pLCAT and 10% for rLCAT) against $HDL_3$ (Table 2). In contrast, the activity increased for both enzymes when assayed with the liposome substrate (data not shown).

## DISCUSSION

The elucidation of the pathways of lipoprotein metabolism is likely to require an in-depth understanding of glycoprotein structure and function, as apolipoproteins and catalytic components of lipid transport (LCAT, lipoprotein lipase, and cholesteryl ester transfer protein) have been shown to have significant carbohydrate content (2, 3, 24, 25). Studies on the structure

**Fig. 5.** (A) Partial FAB mass spectrum of permethylated products of β-galactosidase digestion of the desialylated rLCAT N-glycans. (B) Partial FAB mass spectrum of the permethylated products of β-N-acetyl hexosaminidase digestion of the glycans present in (A). For an explanation of Hex6-10 see legend to Fig. 1.

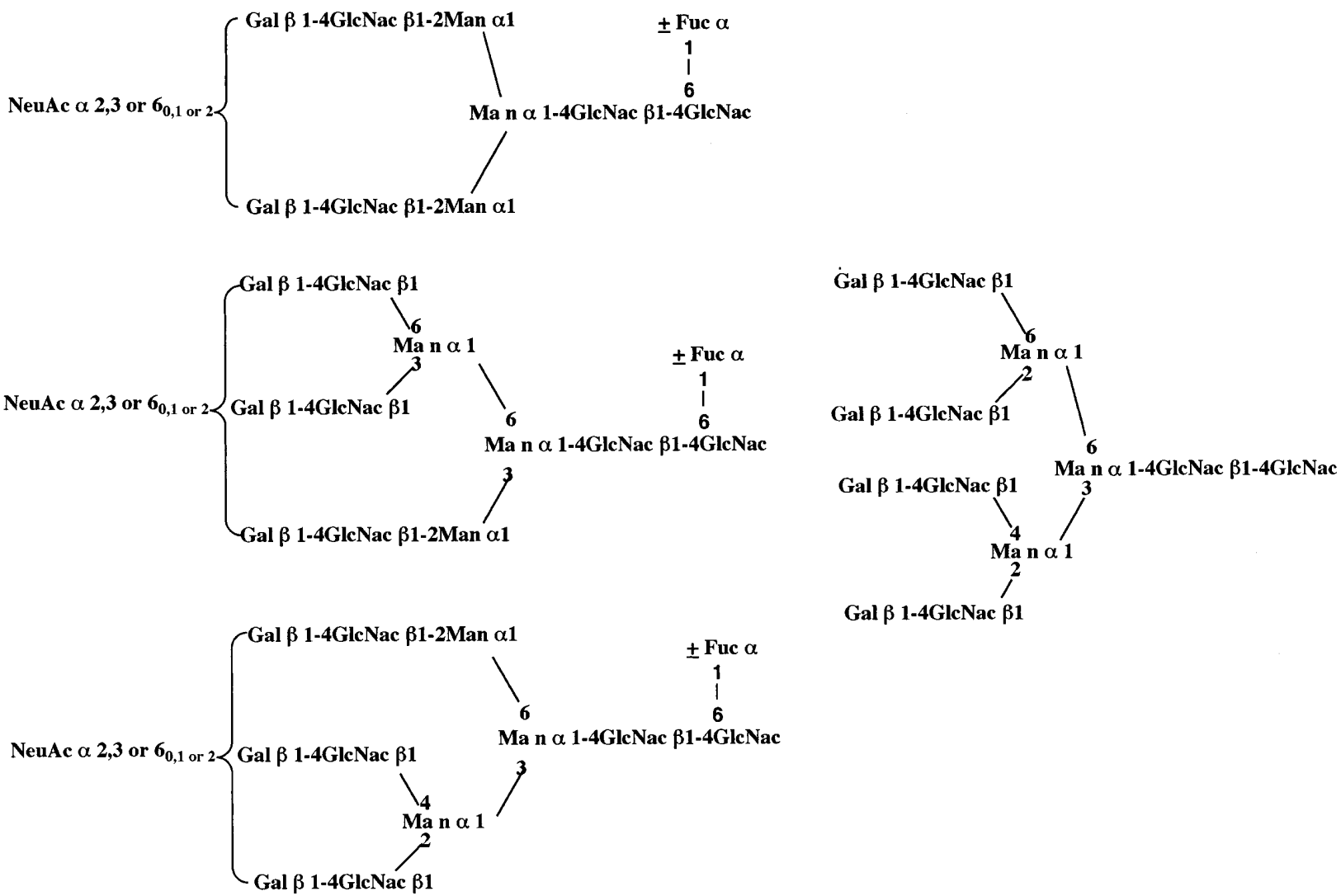


**Fig. 6.** N-glycans present on recombinant LCAT expressed in a BHK cell line.

TABLE 2. Effects of desialylation on the LCAT activity of plasma and recombinant enzyme preparations with lipoprotein substrates

| | Enzyme Source | | | | | |
|---|---|---|---|---|---|---|
| | $HDL_3$ | | | $HDL_2$ | | |
| | pLCAT | rLCAT | rLCAT−S | pLCAT | rLCAT | rLCAT−S |
| $K_m$ ($10^{-6}$ m) | 7.44 | 10.1 | 20.5 | 6.66 | 9.46 | 19.5 |
| $V_{max}$ (nmol/ml/h) | 2.20 | 1.75 | 3.02 | 1.25 | 0.86 | 1.83 |

Values represent duplicate determinations (average error <5%); p, plasmal r, recombinant; −S, devoid of sialic acid.

and function of LCAT have opened the way for a detailed investigation of this enzyme system (4, 5, 26). Although cloning and expression of the enzyme have been achieved (4, 6, 10), the precise role of the carbohydrates in the structure/function of LCAT is yet to be defined. The N-linked oligosaccharides have been shown to be essential for the secretion of active LCAT from the CHO and COS cells (8, 9). In addition, the hydropathy index for LCAT (5) is as hydrophobic as any of the plasma apolipoproteins. These findings (5) strongly suggest that the carbohydrate component makes an important contribution to the conformational stability of the enzyme in an aqueous environment. Consequently, the oligosaccharide chains of LCAT are likely to play an important role in the maintenance of its active conformation as well as its ability to interact with substrates (6).

Currently, there is only limited information available on the role of oligosaccharides in LCAT. Two separate investigations provided surprisingly similar patterns when the impact of carbohydrate depletion on the activity of the enzyme secreted from recombinant cells was tested (8, 9). Although the carbohydrates of LCAT have been shown to be essential for the secretion of the enzyme (8, 9), no information is yet available on the specific roles that the oligosaccharide structures play in determining the structure/function of LCAT.

The data presented in this paper show that recombinant LCAT contains four N-glycosylated chains. The linkages of these oligosaccharides to the polypeptide chain are very likely to be in the same respective positions as described for other systems (8). We have also shown that rLCAT contains bi-, tri-, and tetraantennary saccharides. These structures appear to be representative glycoprotein components as disialylated biantennary oligosaccharides were shown to be the dominant structures in many circulating human serum glycoproteins, including transferrin (27), Factor VIII (28), and interferon-β1 (29). On the other hand, sialylated tri- and tetraantennary oligosaccharides are the major structures on other circulating glycoproteins including human erythropoietin (30) and tissue plasminogen activator (31, 32). The first phase of the structural analysis for LCAT revealed N-glycans ranging from biantennary to tetraantennary structures with and without core fucosylation. The amount of core fucosylation increased with the number of antennae. Thus, non-fucosylated tetraantennary structures were almost undetectable while non-fucosylated biantennary structures gave prominent signals. The molecular ion abundances suggest that the total amount of tri- and tetraantennary glycans was comparable to that of the biantennary glycans (Fig. 2). Interestingly, none of these rLCAT preparations were sialylated. The lack of sialylation was attributed to be the action of a soluble sialidase (23). Inclusion of the sialidase inhibitor 2,3-dehydro-2-deoxy N-acetylneuraminic acid into the extraction procedure restored the sialylation of the glycan population. These sialic acid moieties were attached to galactose via α2,3 and α2,6 linkages with α2,6 being the predominant

TABLE 3. Effects of desialylation on the phospholipase activity of plasma and recombinant LCAT preparations

| | Enzyme Source | | | |
|---|---|---|---|---|
| | pLCAT | pLCAT−S | rLCAT | rLCAT−S |
| $K_m$($10^{-6}$ m) | 0.14 ± 0.04 | 0.34 ± 0.11 | 0.19 ± 0.09 | 0.55 ± 0.11 |
| $V_{max}$ δfluorescence units/ml | 27.8 ± 2.2 | 32.0 ± 1.8 | 8.5 ± 1.5 | 10.7 ± 1.3 |

These studies were carried out with a fluorescent phosphatidylcholine substrate (1,2 bis[4-(1-pyrenol)-butanoyl-*sn*-glycero-3-phosphatidylcholine; p, plasma; r, recombinant; −S, devoid of sialic acid. The data represent a series of five determinations and are given as means ± SD.

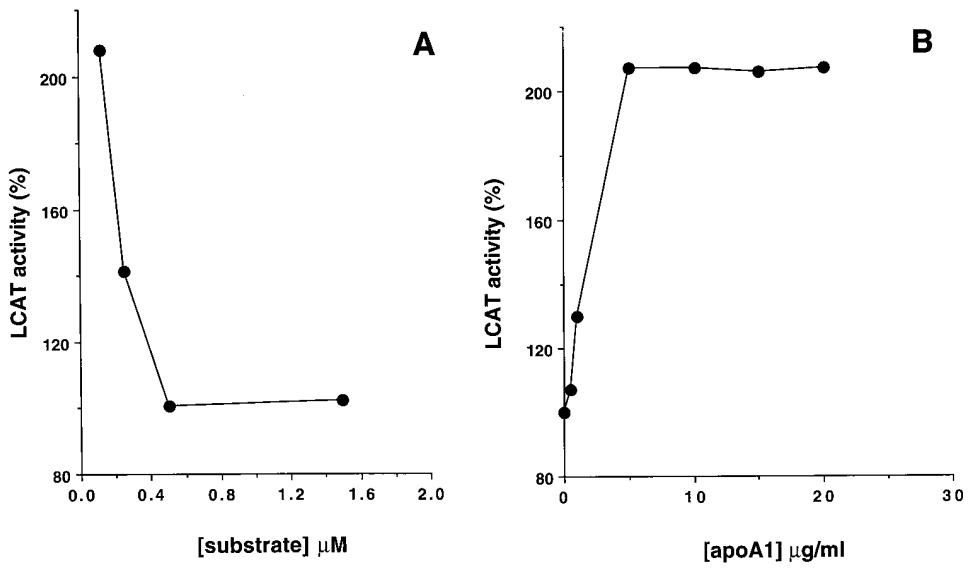


**Fig. 7.** Enhancement of the phospholipase activity of LCAT by apoA-I, monitored by the hydrolysis of 1,2 bis[4-(1-pyreno)-butanoyl-*sn*-glycero-3-phosphatidylcholine (100% represents activity without apoA-I). A: The effect of substrate concentration (with 5 μg/ml apoA-I added); B: The effect of substrate concentration on activity.

linkage. The recombinant enzyme contained a minor population of glycans having one or more antennae with up to three repeats of N-acetyllactosamine. These structures are characteristic of membrane glycoproteins (33) but have also been found at low levels in those circulating glycoproteins that carry tri- and tetraantennary oligosaccharides (30, 31). Thus the presence of such structures in recombinant LCAT is not surprising in view of the high levels of glycans with three or four antennae.

The types of N-linked oligosaccharides found on recombinant LCAT are broadly similar to those reported by Schindler et al. (7) for human plasma LCAT. Thus both glycoproteins have complex-type bi-, and tri-, and tetraantennary structures in which the only capping sugar identified is sialic acid. However, there are two notable differences between the recombinant and natural forms of the enzyme. First, the extent of core fucosylation is considerably greater in recombinant LCAT. Our data suggest that about 70% of the oligosaccharides of recombinant LCAT are core fucosylated with fucose being present on bi-, tri-, and tetraantennary structures. In contrast, the plasma enzyme is fucosylated only on triantennary structures and no more than about 30% of such structures carry fucose (7). Second, recombinant LCAT has comparable amounts of tri- and tetraantennary structures while plasma LCAT is predominantly triantennary with tetraantennary oligosaccharides being almost undetectable.

Desialylation of the enzyme resulted in only modest changes in LCAT activity. A larger impact on $K_m$ than on $V_{max}$ was noted (Tables 2 and 3) perhaps reflecting alterations in the binding properties of the enzyme toward its substrates associated with the removal of sialic

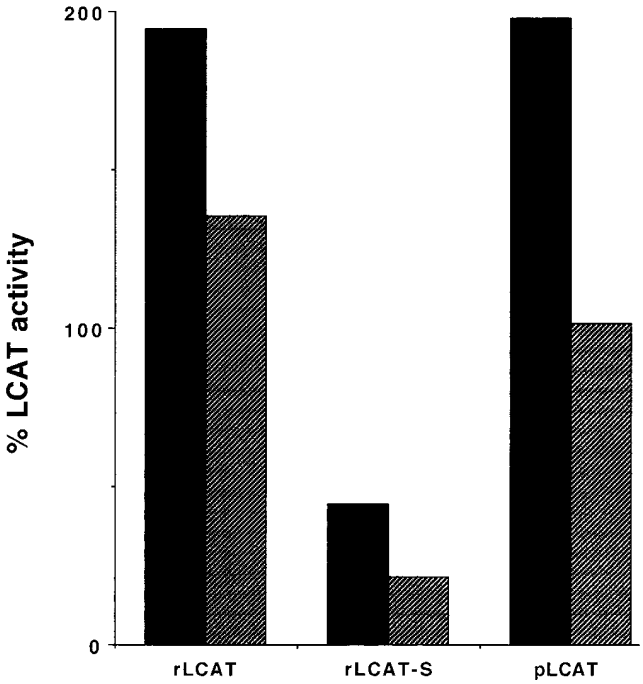


**Fig. 8.** The effect of apoA-I (5 μg/ml) on the phospholipase activity of LCAT preparations assayed by a fluorescent substrate ($2 \times 10^{-7m}$). The desialylated rLCAT preparations in this experiment were isolated directly from the culture supernatants of BHK cells; (■) with apoA-I; (▨) without apoA-I.

acids. Similar findings were reported earlier by Doi and Nishida (34). The effect of apoA-I on the activity of the enzyme against the low molecular weight, fluorescent phospholipase substrate is intriguing. The enhancement of this type of phospholipase activity of LCAT by apoA-I is restricted to lower substrate concentrations (Fig. 6). Perhaps structural rearrangement of the substrate at these lower concentrations favorably influences the affinity for LCAT and thus results in the increase in the activity of the enzyme.

The impact of that nature of the glycans present on the structure and function of LCAT may be assessed from several lines of available evidence. First, the elimination of specific glycosylation sites by site-directed mutagenesis gave rise to very similar findings regarding the LCAT activity of the modified structures secreted by COS (8) and CHO cells (9). Although some structural differences were expected between the glycoproteins secreted by these different cell lines, the impact of such differences on enzymatic activity appeared to be negligible. Second, comparison of the catalytic parameters of the recombinant and plasma enzymes (that exhibit differences in their oligosaccharide components) have functional properties that are essentially indistinguishable (Tables 2 and 3, Fig. 8). These observations include enzyme assays conducted with high molecular weight substrate (HDL) for transesterification as well as a low molecular weight substrate to monitor the phospholipase reaction (18). Finally, recent studies by Miller et al. (6) revealed that the carbohydrate component of human LCAT, derived from HepG2 cells and via a transfected baculovirus vector, are likely to be markedly different from that of the plasma enzyme. The carbohydrate structural differences were accompanied by differences in enzymatic activity against reconstituted HDL and phospholipase substrates. These latter studies (6) further accentuate the need for detailed carbohydrate structural studies in order to elucidate the functional differences observed between the different forms of LCAT.

In this communication, we report, for the first time, on the structural components of the carbohydrate moiety of a recombinant form of LCAT. These studies revealed that the recombinant and naturally occurring forms of LCAT possess considerably different carbohydrate moieties regarding the configuration of the oligosaccharide chains. Nevertheless, the catalytic behavior of these two enzyme species was very similar if not identical, thus validating the concept that the recombinant enzyme from BHK cells can serve as an appropriate model for structure/function studies of LCAT. The wild type as well as the mutant recombinant enzyme forms (10, 35) should provide the foundation for additional studies in order to elucidate the functional consequences of the structural modifications in LCAT.

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## REFERENCES

1. Glomset, J. A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid. Res.* **9:** 155–167.
2. Chung, J., D. A. Abano, G. M. Fless, and A. M. Scanu. 1979. Isolation, properties, and mechanism of in vitro action of lecithin:cholesterol acyltransferase from human plasma. *J. Biol. Chem.* **254:** 7456–7464.
3. Chong, K. S., M. Jahani, S. Hara, and A. G. Lacko. 1983. Characterization of lecithin:cholesterol acyltransferase from human plasma III: Chemical properties of the enzyme. *Can. J. Biochem.* **61:** 875–881.
4. McLean, J., C. J. Fielding, D. Drayna, H. Dieplinger, B. Baer, W. Kohr, W. Henzel, and R. Lawn. 1986. Cloning and expression of human lecithin:cholesterol acyltransferase cDNA. *Proc. Natl. Acad. Sci. USA.* **83:** 2335–2339.
5. Yang, C. Y., D. Manoogian, Q. Pao, R. D. Knapp, A. M. Gotto, and H. J. Pownall. 1987. Lecithin:cholesterol acyltransferase. Functional regions and a structural model for the enzyme. *J. Biol. Chem.* **262:** 3086–3091.
6. Miller, K. R., J. Wang, M. Sorci-Thomas, R. A. Anderson, and J. S. Parks. 1996. Glycosylation structures and enzyme activity of lecithin:cholesterol acyltransferase from human plasma. HepG2 cells and baculoviral and Chinese hamster ovary cell expression systems. *J. Lipid. Res.* **37:** 551–561.
7. Schindler, P. A., C. A. Settineri, X. Collett, C. J. Fielding, and A. L. Burlingame. 1995. Site-specific detection and structural characterization of the glycosylation of human plasma lecithin:cholesterol acyltransferase and apolipoprotein D using HPLC/electrospray mass spectrometry and sequential glycosidase digestion. *Prot. Sci.* **4:** 791–803.
8. O, K., J. S . Hill, X. Wang, R. McLeod, and P. H. Pritchard. 1993. Lecithin:cholesterol acyltransferase: role of N-linked glycosylation on the enzyme function. *Biochem. J.* **294:** 879–884.
9. Francone, O., L. Evangelista, and C. J. Fielding. 1993. Lecithin-cholesterol acyltransferase: effects of mutagenesis at N-linked oligosaccharide attachment sites on acyl acceptor specificity. *Biochim. Biophys. Acta.* **1166:** 301–304.
10. Hill, J., K. O, X. Wang, S. Paranjape, D. Dimitrijevich, A. G. Lacko, and P. H. Pritchard. 1993. Expression and characterization of recombinant human lecithin:cholesterol acyltransferase. *J. Lipid. Res.* **34:** 1245–1251.
11. Chong, K. S., L. Davidson, R. Huttash, and A. G. Lacko. 1981. Characterization of lecithin:cholesterol acyltransferase from human plasma. *Arch. Biochem. Biophys.* **211:** 119–124.
12. Skoza, I., and S. Mohos. 1976. Stable thiobarbituric acid chromophore with dimethyl sulphoxide. Application to

sialic acid assay in analytical de-O-acetylation. *Biochem. J.* **159:** 457–462.
13. Park, Y. B., and A. G. Lacko. 1986. Isolation and characterization of lecithin-cholesterol acyltransferase from hog plasma. *Biochim. Biophys. Acta.* **877:** 179–190.
14. Batzri, S., and E. D. Korn. 1973. Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta.* **298:** 1015–1019.
15. Lacko, A. G., H. L. Rutenberg, and L. A. Soloff. 1972. Recovery of labeled cholesterol and cholesterol esters from thin-layer chromatograms. *Clin. Chim. Acta.* **39:** 506–510.
16. Terpstra, A. H. M., C. J. H. Woodward, and F. J. Sanchez-Muniz. 1991. Improved technique for the separation of serum lipoproteins by density gradient ultracentrifugation: visualization by prestaining and rapid separation of serum lipoproteins from small volume of serum. *Anal. Biochem.* **111:** 149–157.
17. Jahani, M., and A. G. Lacko. 1982. Study of the lecithin:cholesterol acyltransferase reaction with liposome and high density lipoprotein substrates. *Biochim. Biophys. Acta.* **113:** 504–511.
18. Bonelli, F., and A. Jones. 1992. Continuous fluorescence assay for lecithin:cholesterol acyltransferase using water-soluble phosphatidylcholine. *J. Lipid. Res.* **33:** 1863–1869.
19. Albersheim, P., D. J. Nevins, P. D. English, and A. Karr. 1967. A method for the analysis of sugars in plant cell wall polysaccharides by gas–liquid chromatography. *Carbohydr. Res.* **5:** 340–345.
20. Dell, A., K-H. Khoo, M. Panico, R. A. McDowell, A. T. Etienne, A. J. Reason, and H. R. Morris. 1993. *In* Glycobiology: a Practical Approach. M. Fukuda, editor. IRL Press, Oxford, 187–222.
21. Dell, A., A. J. Reason, K-H. Khoo, M. Panico, R. A. McDowell, and H. R. Morris. 1993. Mass spectrometry of carbohydrate containing bipolymers. *Methods Enzymol.* **230:** 108–132.
22. Dell, A. 1987. F.A.B.—mass spectrometry of carbohydrates. *Adv. Carbohydr. Chem. Biochem.* **45:** 19–72.
23. Warner, T. G., J. Chang, J. Ferrari, R. Harris, T. McNerney, G. Bennett, J. Burnier, and M. B. Sliwkowski. 1993. Isolation and properties of sialidase from the culture fluid of Chinese hamster ovary cells. *Glycobiology.* **3:** 455–464.
24. Stevenson, S. C., S. Wang, L. Deng, and A. R. Tall. 1993. Human plasma cholesterol transfer protein consists of a mixture of two forms reflecting variable glycosylation at asparagine 341. *Biochemistry.* **32:** 5121–5126.
25. Ben-Zeev, O., M. H. Doolittle, R. C. Davis, J. Elovson, and M. C. Schotz. 1992. Maturation of lipoprotein lipase. Expression of full activity requires glucose trimming but not translocation of the *cis*-Golgi compartment. *J. Biol. Chem.* **267:** 6219–6227.
26. Albers, J. J., C. H. Chen, and A. G. Lacko. 1986. Isolation, characterization and assay of lecithin-cholesterol acyltransferase. *Methods Enzymol.* **12:** 763–782.
27. Spik, G., B. Bayard, B. Fournet, G. Strecker, S. Bouquelet, and J. Montreuil. 1975. Studies of glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferrin. *FEBS Lett.* **50:** 296–299.
28. Hironaka, T., K. Furukawa, P. C. Esmon, M. A. Fournel, S. Sawada, M. Kato, T. Minaga, and A. Kobata. 1993. Comparative study of the sugar chains of factor VIII, purified from human plasma and from the culture media of baby hamster kidney cells. *J. Biol. Chem.* **267:** 8012–8020.
29. Kagawa, Y., S. Takasaki, J. Utsumi, K. Hosoi, H. Shimizu, N. Kochibe, and A. Kobata. 1988. Comparative study of aspargine-linked sugar chains of natural human interferon-beta 1 produced by three different mammalian cells. *J. Biol. Chem.* **263:** 17508–17515.
30. Sasaki, H., B. Bothner, A. Dell, and M. Fukuda. 1987. Carbohydrate structure of erythropoietin expressed in a Chinese hamster ovary cell line by a human erythropoietin cDNA. *J. Biol. Chem.* **262:** 12059–12076.
31. Spellman, M. W., L. J. Basa, C. K. Leonard, J. A. Chakel, J. V. O'Connor, S. Wilson, and J. van Halbeek. 1989. Carbohydrate structures of human tissue plasminogen activator expressed in Chinese hamster ovary cells. *J. Biol. Chem.* **264:** 14100–14111.
32. Parekh, R. B., R. A. Dwek, J. R. Thomas, G. Opdenakker, T. W. Rademacher, A. J. Wittwer, S. C. Howard, R. Nelson, N. R. Siegel, M. G. Jennings, N. K. Harakas, and J. Feder. 1989. Cell-type specific N-glycosylation of type I and type II human tissue plasminogen activator. *Biochemistry.* **28:** 7644–7662.
33. Fukuda, M. 1985. Cell surface glyconjugates as onco-differentiation markers in hematopoietic cells. *Biochim. Biophys. Acta.* **780:** 119–150.
34. Doi, Y., and T. Nishida. 1983. Microheterogeneity and physical properties of human lecithin-cholesterol acyltransferase. *J. Biol. Chem.* **258:** 5840–5846.
35. O, K., J. S. Hill, X. Wang, and P. H. Pritchard. 1993. Recombinant lecithin:cholesterol acyltransferase containing the Thr 123→Ile mutation esterifies cholesterol in low density lipoproteins but not in high density lipoproteins. *J. Lipid. Res.* **34:** 81–88.