# Long-Term Ethanol Exposure Impairs Glycosylation of Both *N*- and *O*-Glycosylated Proteins in Rat Liver

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**Carbohydrate residues of glycoproteins play important roles in their functions. We have previously shown that long-term ethanol treatment in rats alters the normal glycosylation pattern of plasma transferrin and apolipoprotein (apo) E. Glycosylation of proteins is a posttranslational process that is regulated by both glycosyltransferases and glycosidases, the resident enzymes of hepatic subcellular organelles. In this investigation using rat transferrin and apo E as model *N*- and *O*-glycosylated proteins, respectively, we have explored the effects of long-term ethanol treatment on the (1) incorporation of various labeled sugar precursors into these specific glycoproteins, (2) activities of mannosyltransferase, galactosyltransferase, and sialyltransferases, and (3) hepatic synthetic rate of N-acetyl glucosamine (GlcNAc) α2,6-sialyltransferase (2,6-ST). The relative ratio of labeled sugar to leucine incorporation (glycosylation index) showed a 43% ($P < .01$) decrease for relative mannosylation of transferrin molecule at both the microsomal and Golgi level in the ethanol group (AN) versus the control group (CN). For apo E, relative mannosylation was reduced by 48.9% ($P < .01$) and 46.9% ($P < .01$), respectively, at the microsomal and Golgi level in the AN versus CN. More importantly, relative sialation of transferrin was reduced by 86% ($P < .001$) in AN as compared with CN. Relative sialation of apo E was reduced by 35% ($P < .01$) in AN as compared with CN. A comparison of the effects of long-term ethanol treatment on key glycosylating enzymes in the liver showed that the activities of mannosyltransferase and galactosyltransferase were decreased by 24.4% ($P < .01$) and 21.1% ($P < .01$), respectively, whereas the activity of 2,6-ST was decreased markedly by 52.9% ($P < .001$) AN versus CN. This inhibitory effect of ethanol was specific for 2,6-ST, since the activities of GlcNAc α-2,3-sialyltransferase and [4,5-$^3$H]-N-acetylgalactosamine (GalNAc) α-2,3-ST were decreased only by 31.6% ($P < .01$) and 4% (NS), respectively, by the same long-term ethanol treatment. It was further shown that this inhibition of 2,6-ST activity was due to a concomitant 48% ($P < .001$) specific inhibition of its synthetic rate caused by long-term ethanol treatment. Thus, our results have clearly established that long-term ethanol treatment leads to a marked specific inhibition of the sialation step of both *N*- and *O*-glycosylated proteins by inhibiting the hepatic synthetic rate of 2,6-ST.**


THE APPEARANCE OF carbohydrate-deficient transferrin in the plasma of chronic alcoholics has been established as a viable marker of long-term alcohol consumption.[1] Similarly, long-term ethanol treatment markedly affects hepatic glycosylation of apolipoprotein (apo) E, an integral component of plasma lipoproteins.[2] Our previous studies have further shown that hepatic sialations of transferrin, a *N*-glycosylated protein, and apo E, an *O*-glycosylated protein, are markedly impaired in ethanol-fed rats as compared with pair-fed controls.[3,4] Therefore, using transferrin and apo E as model *N*- and *O*-glycosylated proteins, we have explored the effects of long-term ethanol treatment on the posttranslational modifications of proteins in rat liver, with special emphasis on their glycosylation at the microsomal and Golgi level.

The liver is the main site of synthesis and posttranslational modifications of transferrin and apo E. The mechanism and the involvement of subcellular organelles, particularly Golgi apparatus and microsomes, in the glycosylation process have been reviewed.[5,6] It has been speculated that glycosylation is a prerequisite for normal secretion of lipoproteins and other secretory proteins.[7-9] Different dietary factors influence the incorporation of various labeled sugars into glycoproteins in various systems studied, including liver.[10-12] Significantly, 85% of plasma transferrin is sialated,[13] whereas the bulk of plasma apo E exists in a nonsialated form.[14] However, the newly secreted transferrin and apo E are highly sialated.

The synthesis of mature transferrin and apo E molecules requires addition of different sugars in a sequential form regulated by specific glycosyltransferases and glycosidases, the resident enzymes of hepatic microsomes, Golgi apparatus, and plasma membranes. Thus, the net amount of sialic acid residues in a mature sialoprotein is governed by the relative actions of sialyltransferase and sialidase. Consequently, both sialyltransferase and sialidase may play important roles in the assembly of a mature glycoprotein molecule. Sialidases from mammalian hepatic lysosomes, cytosol, plasma membranes, and circulating plasma have been reported.[15] However, their properties, including substrate specificities, are obscure.[15] The removal of sialic acid residues catalyzed by sialidase is thought to constitute an important biologic process that may influence the survival of sialoconjugates in the circulation,[16] antigenic expression,[6] and recognition by receptors,[17] and may thus affect the functions of both plasma transferrin and apo E. Our previous studies in rats have shown that long-term ethanol treatment leads to a twofold increase in the activities of both plasma membrane and plasma sialidase.[18]

Glycosylation pathways have been extensively studied and reviewed.[19,20] However, how specific glycosylation steps and the component enzymes are affected under long-term ethanol treatment has not been previously explored. Therefore, to understand the specificity of the steps/sites of glycosylation pathways that are affected by long-term etha-

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nol treatment, we have determined the incorporation of labeled specific sugar precursors relative to the incorporation of labeled leucine into immunoprecipitable hepatic transferrin and apo E molecules and the hepatic activities of the key glycosylating enzymes, mannosyltransferase, galactosyltransferase, and sialyltransferases in long-term ethanol–treated and control rats.

It will be demonstrated that long-term ethanol treatment of rats preferentially inhibits the sialation of transferrin and apo E by specifically inhibiting the hepatic activity of α2,6-sialyltransferase (2,6-ST) in the Golgi. It will be further shown that this inhibitory effect of long-term ethanol treatment is due to a concomitant specific decrease in hepatic 2,6-ST synthetic rate.

## MATERIALS AND METHODS

L-[U-$^{14}$C]leucine (specific activity, 57.8 Ci/mmol), [4,5-$^{3}$H]leucine (specific activity, 71.2), [2-$^{3}$ H]mannose (specific activity, 20), GDP-$^{14}$C-mannose (specific activity, 25), UDP-$^{14}$C-galactose (specific activity, 12.5), and [4,5-$^{3}$H]*N*-acetylmannosamine were purchased from Amersham (Arlington Heights, IL). Goat antirat transferrin was purchased from Cappel, antiserum to rat apo E was developed in rabbits in our laboratory. Both were proven to be monospecific for their respective antigens as evidenced by Ouchterlony analysis. Other chemicals including authentic standards were from Sigma (St Louis, MO). Hydrofluor scintillant fluid was from National Diagnostics (Manville, NJ). Silica gel H thin-layer chromatography plates were from Analtech (Newark, DE). Organic solvents were from Fisher Scientific (Columbia, MD), and were of analytic grade.

### *Animals*

Weanling male Wistar rats (Charles River, Wilmington, MA) were maintained on normal chow until they reached a body weight of approximately 150 g. They were then divided into two groups and pair-fed the respective control (CN) and long-term ethanol (AN) liquid diets for a period of 8 weeks. The diets were isocaloric, and were formulated according to the modified method reported by Lieber and DeCarli.[21] Accordingly, 40% of the total energy of the AN diet was from fat, 20% from protein, 36% from ethanol, and 4% from carbohydrate. The corresponding isocaloric CN diet had equal amounts of dextrimaltose in place of ethanol.

### *Experiment 1*

Male Wistar rats were pair-fed with the respective AN and CN diets for a period of 8 weeks, following which each rat from both of the groups was perfused and hepatocytes were isolated as described previously.[22] Hepatocytes (100 mg wet weight/mL incubation mixture) were incubated in a shaking water bath (100 excursions/min) with L-[U-$^{14}$C]leucine (25 μCi/flask) coupled with each one of the following carbohydrate precursors: $^{3}$H-mannose (20 μCi/flask), $^{14}$C-galactose (10 μCi/flask), GlcNAc (25 μCi/flask), and [4,5-$^{3}$H]*N*-acetylmannosamine ([ManNAc] 50 μCi/flask) in a 10-mL final volume of Krebs bicarbonate buffer in individual flasks for 2 hours at 37°C. The reaction mixture was immediately centrifuged after 2 hours. The cell pellet thus obtained was washed three times with 10 mmol/L phosphate-buffered saline. A 10% (wt/vol) homogenate of liver cells was made in 0.5 mol/L sucrose solution in sodium phosphate buffer (0.2 mol/L, pH 7.0, containing 10 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Trasylol, and 1% leupeptin), and a known amount of this was processed for isolation of hepatic microsomes and the Golgi apparatus as described later. The cell homogenate, microsomes, and the Golgi fraction were then analyzed for labeled-leucine and labeled-sugar precursor incorporation into immunoprecipitable transferrin and apo E according to our published method.[2] For incorporation into total proteins, hot trichloroacetic acid–precipitable residues were analyzed.

### *Experiment 2*

Rats were pair-fed CN and AN diets as described earlier. After 8 weeks, each rat was killed by aortic exsanguination under pentobarbital anesthesia (50 mg/kg intraperitoneally) and the blood and liver were collected and weighed. A 10% (wt/vol) homogenate of a known amount of liver was made in 0.5 mol/L sucrose solution in sodium phosphate buffer (0.2 mol/L, pH 7.0, containing 10 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Trasylol, and 1% leupeptin). Subcellular organelles were isolated from the liver homogenate as described later. Protein content was measured in these isolated fractions, and their yield and purity were determined. Specific enzyme activities in hepatic subcellular fractions, namely mannosyltransferase in hepatic microsomes and galactosyltransferase and sialyltransferases in hepatic Golgi, were determined according to the published protocols as described later.

### *Experiment 3*

Six each of AN and CN rats were pair-fed their respective diets for 8 weeks as described earlier. At the end of 8 weeks, each rat from both groups was infused intraportally with 30 μCi/100 g body weight [4,5-$^{3}$H]leucine and killed after 60 minutes. The liver was homogenized and processed for isolation of microsomes and Golgi, and labeled leucine incorporation into immunoprecipitable 2,6-ST was determined in each fraction using monospecific antirat liver 2,6-ST (kindly provided by Dr Colley, University of Illinois) as described previously.[2]

### *Subcellular Fractionation*

Hepatic microsomal, mitochondrial, lysosomal, Golgi, and plasma membrane fractions were isolated using the continuous sucrose density-gradient ultracentrifugation method.[23,24] Purity of each isolated subfraction was verified by measuring activities of various marker enzymes such as NADPH–cytochrome C reductase for microsomes,[25] galactosyl transferase for Golgi,[26] acid phosphatase for lysosomes,[27] succinate dehydrogenase for mitochondria,[28] and alkaline phosphatase for plasma membrane.[27] Protein content in liver cell homogenate, microsomes, and Golgi-rich fractions was measured according to the Lowry method.[29] Recovery of each subcellular organelle was verified by the protein yield in each fraction relative to the total cell protein. Both purity and recovery of each subcellular fraction were found to be consistent with those reported by earlier investigators.[23,24]

### *Antisera Production*

Rat apo E was purified from rat high-density lipoprotein (HDL) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a preparative scale, and monospecific polyclonal antiserum was produced in rabbits as described previously.[30]

### *Immunochemical Analysis of Transferrin and Apo E*

Dialyzed samples of rat liver homogenate, microsomes, and Golgi were analyzed for immunoprecipitable transferrin and apo E according to our method.[2] Data for incorporation of labeled leucine, labeled mannose, galactose, and ManNAc incorporation into transferrin and apo E of whole liver, hepatic microsomes, and

**Table 1. Effects of Long-Term Ethanol Treatment on Body and Liver Weights and Protein Content of Liver and Its Subcellular Organelles**

| Parameter | CN | AN |
|---|---|---|
| Body weight (g) | 244.50 ± 4.00 | 226.20 ± 6.30 |
| Liver weight (g) | 7.42 ± 0.74 | 9.59 ± 0.53* |
| Liver weight × 100/body weight | 3.00 ± 0.30 | 4.30 ± 0.30† |
| Protein content (mg/100 g body weight) | | |
| Liver | 656.31 ± 31.06 | 1,049.43 ± 196.53* |
| Microsomes | 120.56 ± 11.31 | 140.54 ± 9.64 |
| Golgi | 30.66 ± 2.42 | 35.53 ± 3.72 |

NOTE. Each value is the mean ± SE from 6 rats in each group.
*$P < .05$.
†$P < .001$.

Golgi, have been expressed as disintegrations per minute per kilogram body weight per hour.

*Enzyme Assays*

*GDP-mannose: dolichylphosphate mannosyltransferase.* The assay was performed according to the method reported by Ardail et al.[31] The results are expressed as micromoles of dolichylphosphate-mannose formed per gram of liver per hour.

*UDP-galactose: GlcNAc galactosyltransferase.* This was assayed according to the protocol reported by Brew et al.[26] Radioactivity of transferred galactose in each Golgi reaction was determined in a Beckman liquid scintillation counter (Beckman Instruments, Fullerton, CA). In purified Golgi-rich fractions, the ratio of galactose freely hydrolyzed to that enzymatically transferred was found to be less than 9%. The results are expressed as micromoles of galactose transferred per gram of liver per hour.

*Sialyltransferases.* Hepatic Golgi-rich fraction was prepared from liver homogenate as described earlier. For determination of the activity of sialyltransferases, the modified procedure of Paulson et al[32] was used. Specific acceptor substrates were used for specific sialytransferases,[32] namely asialo α-acid glycoprotein for Gal-β1,4 GlcNAc 2,6-ST, lacto *N*-tetrose for Gal-β1,3[4] GlcNAc α2,3-sialyltransferase, and antifreeze glycoprotein for Gal-β1,3 GalNAc α2,3-sialyltransferase. Enzyme activities are expressed as nanomoles sialic acid residues transferred per gram of liver per hour. The data were statistically analyzed using Student's *t* test for intergroup comparisons.

## RESULTS

*Long-Term Ethanol Feeding and Body and Liver Weights*

Body and liver weight differences between the CN and AN group followed the same pattern as previously reported.[2,30] Mean body weight decreased 7.5% ($P < .05$) by long-term ethanol treatment; mean liver weight increased 29.2% ($P < .05$). More importantly, liver weight as a percentage of body weight increased 30.2% ($P < .001$) by long-term ethanol treatment (Table 1).

**Table 3. Effects of Long-Term Ethanol Feeding on Incorporation of [U-$^{14}$C]leucine Into Total TCA-Precipitable Protein, Immunoprecipitable Transferrin, and Apo E in Hepatic Microsomes and Golgi**

| | [U-$^{14}$]leucine Incorporation (dpm × $10^{-6}$/kg body weight/h) | | |
|---|---|---|---|
| Group | Liver | Microsomes | Golgi |
| Total TCA-precipitable protein | | | |
| CN | 234.6 ± 9.5 | 176.3 ± 8.9 | 98.2 ± 8.8 |
| AN | 287.6 ± 13.2 | 199.8 ± 15.6 | 117.3 ± 20.3 |
| Transferrin | | | |
| CN | 6.89 ± 0.40 | 3.23 ± 0.13 | 2.46 ± 0.09 |
| AN | 7.46 ± 0.47 | 3.75 ± 0.24 | 2.84 ± 0.19 |
| Apo E | | | |
| CN | 4.95 ± 0.07 | 2.61 ± 0.08 | 1.68 ± 0.21 |
| AN | 5.65 ± 0.27 | 3.16 ± 0.23 | 1.84 ± 0.05 |

NOTE. Each value is the mean ± SE from 6 rats in each group.
Abbreviation: TCA, trichloroacetic acid.

*Subcellular Fractionation*

The purity and yield of hepatic microsomes and Golgi-rich fraction were assessed by measuring the yields of their protein content and marker enzyme activities. Based on these results, the recovery of isolated subcellular organelles was consistent with previously reported results[2,23,24] and ranged from 85% to 90%, and purity of the subfractions ranged from 81% to 91% (Table 2).

*Long-Term Ethanol Treatment and Protein Content of Rat Liver, Hepatic Microsomes, and Golgi Apparatus*

Liver protein was significantly increased by 59.9% ($P < .001$) at the whole-liver level in AN rats (Table 1). Protein content at the microsomal and Golgi levels did not show statistically significant differences in the AN group as compared with the CN group (Table 1).

*Long-Term Ethanol Exposure and Synthetic Rates of Total Protein, Transferrin, and Apo E in Rat Hepatocytes*

Data for incorporation of labeled leucine into TCA-precipitable total proteins and immunoprecipitable transferrin and apo E at the whole-liver, microsome, and Golgi levels are listed in Table 3. Long-term ethanol treatment had no significant deleterious effects on incorporation of labeled leucine into either total protein, immunoprecipitable transferrin, or apo E at any subcellular level in rat liver, although the general pattern of protein synthetic rate showed an increased trend in AN rats.

**Table 2. Distribution of Various Marker Enzymes in Subcellular Fractions Isolated From Hepatocytes of Rats**

| Enzyme | Homogenate | Lysosomes | Mitochondria | Microsome | Golgi | Plasma Membrane |
|---|---|---|---|---|---|---|
| Acid phosphatase | 100 | 80.70 | 11.32 | 4.80 | 2.13 | 1.27 |
| Succinate dehydrogenase | 100 | 3.31 | 86.79 | 1.88 | 2.47 | 5.55 |
| NADPH–cytochrome C reductase | 100 | 1.02 | 4.17 | 89.28 | 1.45 | 4.08 |
| Galactosyl transferase | 100 | 0.26 | 4.08 | 2.86 | 90.58 | 2.22 |
| Alkaline phosphatase | 100 | 3.03 | 1.30 | 6.46 | 5.06 | 84.15 |

*Effect of Long-Term Ethanol Treatment on Relative Ratio of Labeled Sugar to Labeled Leucine Incorporation Into Transferrin and Apo E of Liver Microsomes and Golgi Apparatus of Rats*

To determine the relative glycosylation of transferrin and apo E, it is important to determine the relative incorporation of each labeled sugar to that of labeled leucine into the respective protein. These results are presented in Figs 1 and 2. For transferrin, striking decreases in the ratios were found for Man/Leu, 43.5% ($P < .01$) and 43.5% ($P < .001$), respectively, at the microsomal and Golgi levels, and for ManNAc/Leu, 85.6% ($P < .001$) and 86.1% ($P < .001$), respectively, in whole-liver and Golgi levels in AN rats versus CN rats (Fig 1). For apo E, again, significant decreases were noted in Man/Leu, 46.23% ($P < .01$), 48.9% ($P < .01$), and 46.9% ($P < .01$), respectively, at whole-liver, microsome, and Golgi levels, and for ManNAc/Leu, 35.1% ($P < .01$) and 33.3% ($P < .01$), respectively, at the whole-liver and Golgi level in AN rats versus CN rats (Fig 2). Specific relative ratios in other cases were not affected by long-term ethanol treatment.

*Long-Term Ethanol Feeding and Glycosylation Enzyme Activities*

Our results showed a modest decrease in the activities of mannosyltransferase by 24.4% ($P < .01$) in the hepatic microsomal fraction in AN rats as compared with CN rats (Table 4). Similarly, the activity of galactosyltransferase in liver Golgi fractions of AN animals was decreased by only 21.1% ($P < .01$) as compared with CN rats (Table 4). More importantly, it was found that GlcNAc 2,6-ST activity was significantly decreased by 52.9% ($P < .001$) in hepatic Golgi in the AN group versus CN group (Table 5). In contrast, the same ethanol treatment decreased the activities of GlcNAc α2,3-ST and GalNAc α2,3-ST by only 31.6% ($P < .001$) and 4.2% (NS), respectively, compared with the CN group (Table 5).

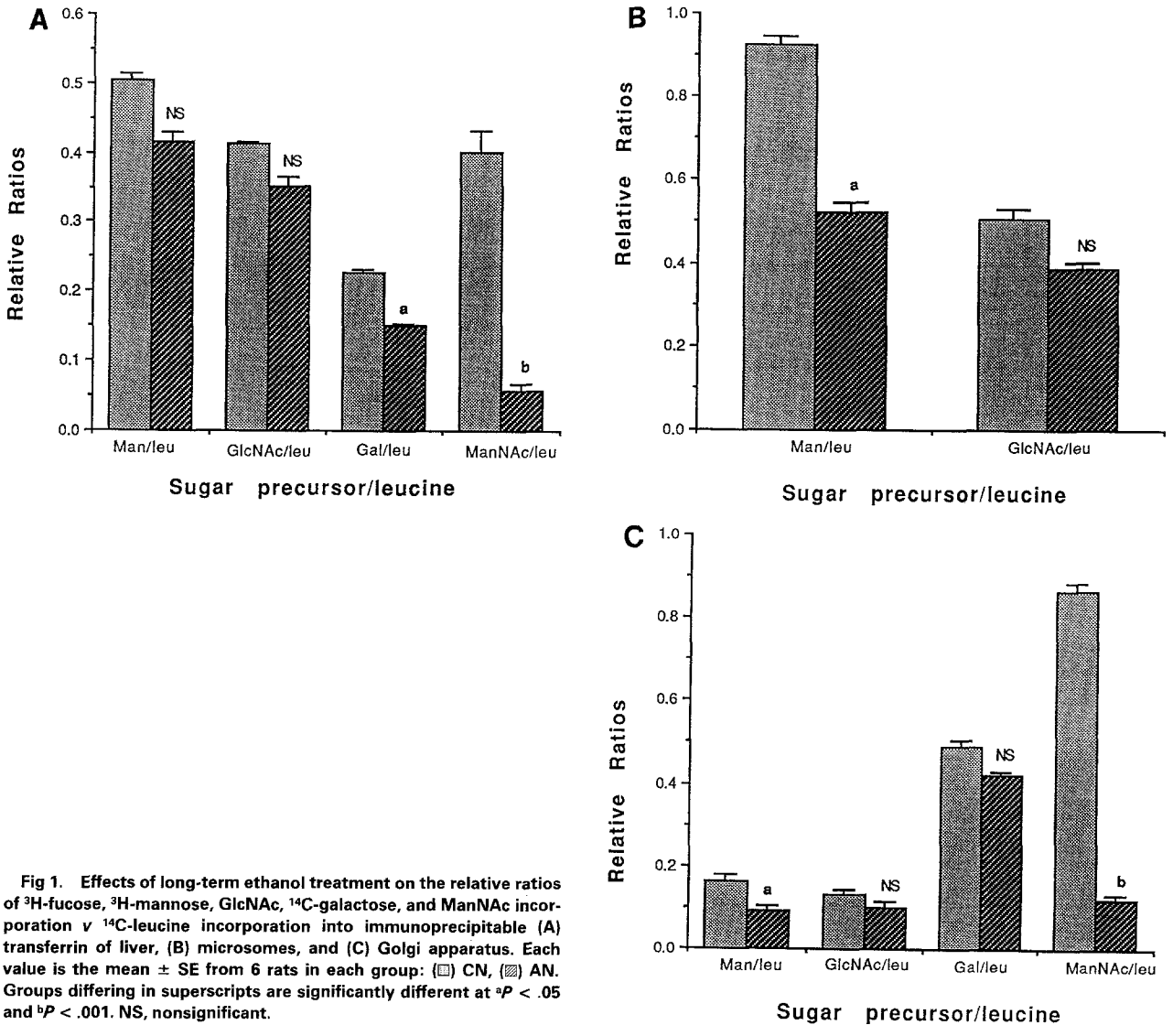


**Fig 1. Effects of long-term ethanol treatment on the relative ratios of $^{3}$H-fucose, $^{3}$H-mannose, GlcNAc, $^{14}$C-galactose, and ManNAc incorporation *v* $^{14}$C-leucine incorporation into immunoprecipitable (A) transferrin of liver, (B) microsomes, and (C) Golgi apparatus. Each value is the mean ± SE from 6 rats in each group: (▫) CN, (▨) AN. Groups differing in superscripts are significantly different at $^{a}P < .05$ and $^{b}P < .001$. NS, nonsignificant.**

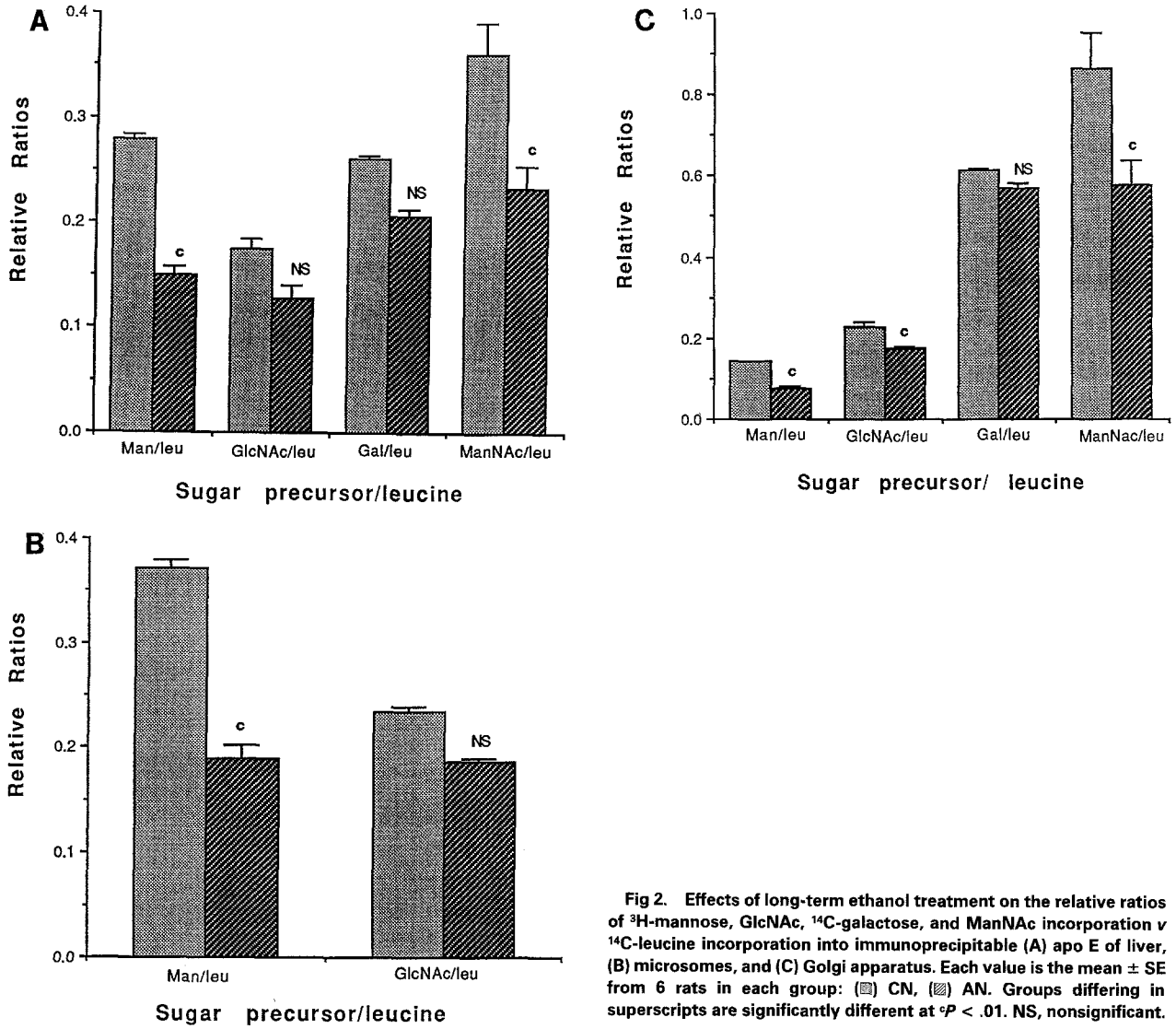


**Fig 2. Effects of long-term ethanol treatment on the relative ratios of $^{3}H$-mannose, GlcNAc, $^{14}C$-galactose, and ManNAc incorporation *v* $^{14}C$-leucine incorporation into immunoprecipitable (A) apo E of liver, (B) microsomes, and (C) Golgi apparatus. Each value is the mean ± SE from 6 rats in each group: (▨) CN, (▨) AN. Groups differing in superscripts are significantly different at $^{c}P < .01$. NS, nonsignificant.**

*Long-Term Ethanol Exposure and Hepatic Synthetic Rate of 2,6-ST*

Incorporation of labeled leucine into total liver proteins and specifically into immunoprecipitable 2,6-ST at the whole-liver, microsome, and Golgi levels is listed in Table 6. It is obvious from the table that the relative hepatic synthetic rate of 2,6-ST is decreased by 44%, 48%, and 39% at the whole-liver, microsome, and Golgi levels, respectively, after long-term ethanol treatment.

**Table 4. Effect of Long-Term Ethanol Treatment on Mannosyltransferase in Hepatic Microsomes and Galactosyltransferase in Hepatic Golgi ($\mu mol \cdot g^{-1} \cdot h^{-1}$)**

| Group | Mannosyltransferase | Galactosyltransferase |
|---|---|---|
| CN | 93.71 ± 4.03* | 8.72 ± 0.75* |
| AN | 70.81 ± 5.28† | 6.87 ± 0.74† |

NOTE. Each value is the mean ± SE from 6 rats in each group. Groups differing in superscripts are significantly different at $P < .05$.

## DISCUSSION

The present studies confirm previous observations[2,30] that although long-term ethanol feeding causes a 7.5% decrease in body weight despite isocaloric consumption of the diet, it causes a 29.2% increase in liver weight per kilogram body weight. This increase is primarily due to significant increases in lipids, as shown previously,[2,30,33] and to increased hepatic proteins as shown in Table 1.

**Table 5. Effect of Long-Term Ethanol Treatment on the Activities of Various Sialyltransferases (nmol/g liver/h) in Hepatic Golgi From Rats**

| Enzyme Type | CN | AN |
|---|---|---|
| Gal-β1,4 GlcNAc 2,6-ST | 16.86 ± 1.26 | 7.93 ± 0.84† |
| Gal-β1,3 GlcNAc α2,3-ST | 9.48 ± 0.53 | 6.48 ± 0.33* |
| Gal-β1,3 GalNAc α2,3-ST | 8.36 ± 0.07 | 8.01 ± 0.05 |

NOTE. Each value is the mean ± SE from 6 rats in each group.
*$P < .05$.
†$P < .001$.

**Table 6. Effect of Long-Term Ethanol Treatment on Hepatic Synthetic Rate of 2,6-ST**

| Fraction | Leucine Incorporation (dpm × $10^{-4}$ $kg^{-1} \cdot h^{-1}$) 2,6-ST | TP | 2,6-ST/TP | % Inhibition | *P* |
|---|---|---|---|---|---|
| Liver | | | | | |
| CN | 9.3 ± 0.4 | 3,356 ± 95 | 0.0028 | | |
| AN | 6.2 ± 0.6 | 3,876 ± 132 | 0.0016 | 43 | <.001 |
| Microsome | | | | | |
| CN | 2.8 ± 0.1 | 1,363 ± 89 | 0.0021 | | |
| AN | 1.7 ± 0.1 | 1,598 ± 156 | 0.0011 | 48 | <.001 |
| Golgi | | | | | |
| CN | 6.3 ± 0.2 | 982 ± 88 | 0.0064 | | |
| AN | 4.1 ± 0.3 | 1,173 ± 203 | 0.0035 | 45 | <.001 |

NOTE. Each value is the mean ± SE from 6 rats in each group.
Abbreviation: TP, total protein.

Fatty liver and protein accumulation caused by long-term ethanol feeding may be due to a lack of concomitant changes in liver secretory protein synthesis, secretion, or both.[34] Our previous report[30] showed that this was due to defective hepatic secretion of export proteins. Underglycosylation of plasma lipoproteins resulting in their impaired secretion has been reported under various experimental conditions.[11,35] Earlier studies by Sorrell and Tuma[36] in rat liver slices have suggested a selective impairment of glycoprotein metabolism by ethanol, and suggested that impaired secretion of glycoproteins is the major manifestation of the altered metabolism. The function of carbohydrate moieties of glycoproteins is currently an area of intense investigation.[37] The present study has explored the possible effects of long-term administration of ethanol on glycosylation of liver proteins using transferrin and apo E as model *N*- and *O*-glycosylated proteins, respectively.

Studies by Tuma and Sorrell[38] have demonstrated that although protein synthesis and degradation are altered by ethanol administration, posttranslational events in protein metabolism are especially vulnerable to the actions of ethanol in hepatocytes. Our results clearly establish the fact that the two crucial steps in apo E glycosylation, namely mannosylation and sialation of the protein molecule, are significantly impaired by long-term ethanol treatment (Figs 1 and 2). Studies on the activities of corresponding enzymes also support these facts (Tables 4 and 5). The functional significance of apo E glycosylation is not well understood. It is possible that glycosylation may protect apo E from proteolytic degradation. Carbohydrate residues may also be important for the relative association of apo E with HDL or very-low-density lipoprotein (VLDL) molecules. Thus, decreased relative glycosylation of apo E caused by long-term ethanol treatment may impede its association with HDL and may increase its association with VLDL. This might explain our previous finding that plasma apo E is decreased in HDL under alcoholic conditions.[39] Furthermore, the decreased association of apo E with HDL may affect its ability to perform its reverse cholesterol transport (RCT) function. The loss of apo E from HDL could result in its impaired clearance, and the net result would be increased plasma HDL and yet defective RCT leading to increased accumulation of lipids.

Apo E undergoes posttranslational modifications enroute different from those of transferrin. There is no evidence showing involvement of the lipid-sugar complex in the attachment of core sugar in *O*-glycosylated proteins. *N*-acetylgalactosamine is the core sugar residue in *O*-glycosylated proteins. The presence of *N*-acetylgalactosaminetransferase in smooth endoplasmic reticulum (ER) and the Golgi apparatus is known.[40] It is interesting to find incorporation of labeled mannose into the newly synthesized apo E molecule in this investigation, although the presence of mannose in a mature apo E molecule has been debated.[35,40,41] Our earlier experiments have also shown incorporation of labeled mannose into newly synthesized apo E.[2] There is no known role of GalNAc transferase in the initial *N*-glycosylation process in the ER. It seems likely that *O*-glycosylated proteins add GalNAc as the initial sugar residue catalyzed by GalNAc transferase, followed by mannose, in the ER, a prerequisite for its transport to Golgi, where it undergoes further modifications. That the first step in the glycosylation of *O*-glycosylated proteins also occurs in hepatic microsomes is strongly supported by the following evidence: (1) mannose incorporation into apo E was found at the microsomal level, and (2) the presence of GalNAc transferase in the ER was reported by others.[40] Signal proteins may also play an important role in this transport process.[43] At the Golgi level, mannose residues can be completely removed from the molecule at the medial Golgi compartment. Thus, it appears that the presence of mannose in apo E at the Golgi level may be primarily due to its existence in the *cis*-compartment of Golgi, ie, at a stage before mannosidase action. Although the residence of glycosyltransferases within microsomes and Golgi are known, the exact site of each enzyme within the compartments of each subcellular organelle is still obscure.

Hepatic microsomes as the first glycosylation site for *N*-glycosylated proteins are well established.[19] Glycosylation is a sequential additive process, and impairment in any of the steps may lead to alteration in the structural maturation of the molecule. Mannosylation of the dolichol molecule is the first crucial step in the glycosylation process of the transferrin molecule. *N*-linked glycosylation of transferrin is a complex multistep process. Based on the typical topography of a *N*-glycosylation reaction, biosynthesis of precursors of protein *N*-glycosylation is initiated by formation of dolichol-P-P-$GlcNAc_2Man_9Glc_3$. The immediate precursor sugars in these reactions are corresponding nucleotide sugars. This initial reaction takes place in the lumen of ER.[19] Later, dolichol monosaccharide derivatives act as immediate sugar donors in the ER compartment. Further glycosylation of the polypeptide chain, ie, attachments of *N*-acetylglucosamine, galactose, and sialic acid, occur in the Golgi compartment where the protein takes its final form to be secreted. A 24% decrease in the activity of mannosyltransferase under long-term ethanol treatment thus suggests an impairment in initiation of glycosylation of transferrin molecule. It seems likely that this inhibition may lead to a concomitant decrease in the intracellular concentration of Dol-P-mannose, which may not be optimal for subsequent glycosylation steps. Furthermore, long-term

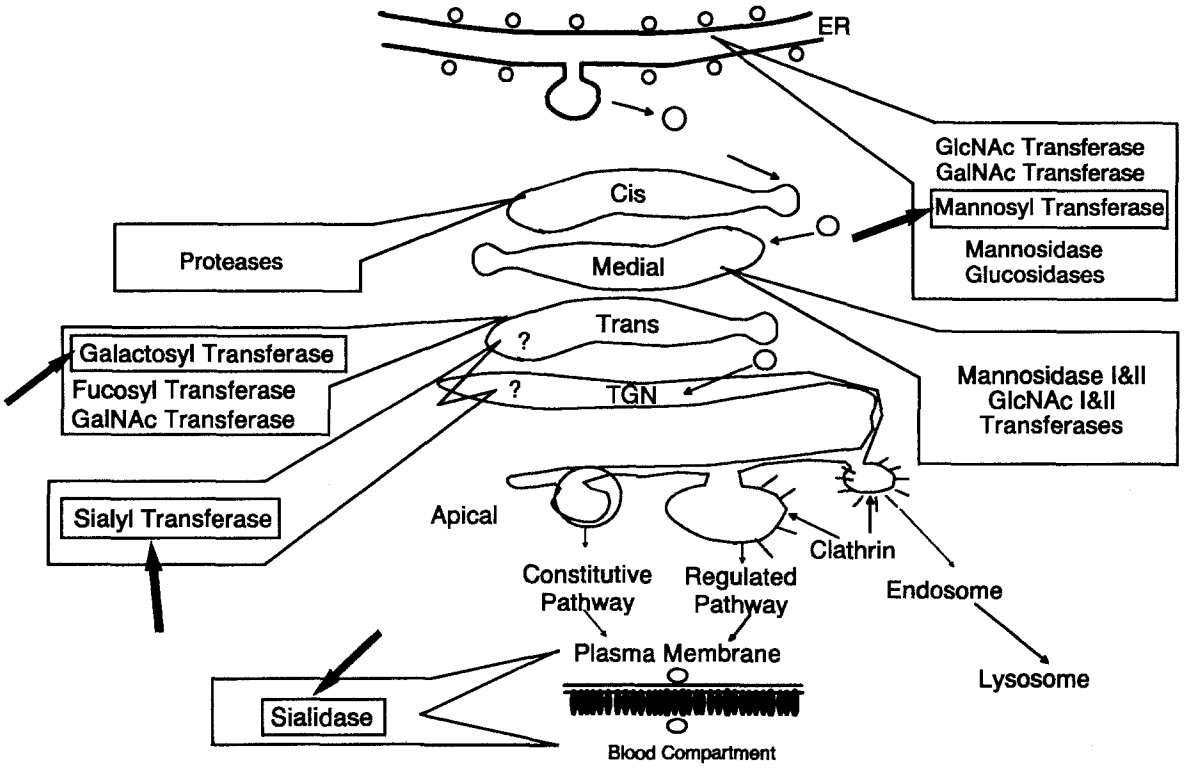


**Fig 3. Schematic representation of protein glycosylation cascade with the possible sites sensitive to long-term ethanol treatment. After its synthesis in the ER, the protein undergoes *N*- or *O*-glycosylation initially in the ER, followed by the vesicular process of its translocation through various compartments of the Golgi apparatus. Various further modifications of the glycoprotein occur in different Golgi compartments such as proteolysis, addition of sugar residues like GlcNAc, GalNAc, galactose (Gal), fucose (Fuc), and sialic acid catalyzed by the respective glycosyltransferases. Finally, depending on the recognition markers on the glycoprotein, it is directed to follow different pathways such as the constitutive, regulated, or endosomal. Secretory proteins are secreted via the plasma membrane. Possible sites of action of ethanol are indicated by arrows based on the present study. Thus, mannosyltransferase in the ER and galactosyltransferase in Golgi are marginally affected by ethanol, whereas sialyltransferase in the Golgi is markedly inhibited. In addition, plasma membrane sialidase is stimulated by ethanol. The net result is the decreased rate of sialation coupled with increased rate of desialation, leading to the emergence of a sialic acid–deficient glycoprotein under alcoholic conditions.**

ethanol treatment in rats also caused decreased activities of galactosyltransferase (21%) and 2,6-ST (58%) in the Golgi fraction. Thus, all these changes caused by long-term ethanol exposure seem to be responsible for decreased sialic acid content in the newly formed transferrin molecule. Transferrin in plasma is normally 85% sialated. Whether glycosylation is essential for secretion of the molecule remains to be answered.

In general, most terminal glycosyltransferases compete for common acceptor substrates.[44] However, based on the one enzyme–one linkage concept,[45] differential expression of sialyltransferase has been reported.[44] Thus, the types of sialyltransferase expressed by a cell should influence the types of terminal sequences of the carbohydrate groups it produces. In vitro, any of the sialyltransferases can completely resialate asialoglycoproteins that contain di-, tri-, and tetra-branched oligosaccharides. Gal 2,6-ST has been shown to have considerable branch specificity.[46] Structural analyses have shown that *N*-linked oligosaccharides of some glycoproteins have exclusively the 2,6-Gal linkage, whereas others have only 2,3-Gal linkages, and still others have mixtures of both linkages on alternate branches of the same carbohydrate group.[37] Transferrin is known to have its terminal sialic acid residues attached to the oligosaccharide chain through 2,6-Gal linkage.

The complete cascade for the glycosylation pathway including the involvement of hepatic microsomes and Golgi as the major subcellular organelles in glycosylation of proteins is well documented[5,6,15,18,19] and is presented in Fig 3. Based on our results, the possible sites of action of ethanol are as follows: First, mannosyltransferase in ER and galactosyltransferase in the *trans*-Golgi compartment are marginally sensitive to ethanol. In contrast, 2,6-ST activity in the *trans*-Golgi compartment is sensitive to ethanol inhibition due to a concomitant decreased rate of its hepatic synthesis. Surprisingly, sialidase in the plasma membrane compartment is stimulated by long-term treatment with ethanol.[18]

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