Isolation and Characterization of a Family of α-D-Galactosyl-Containing Glycopeptides from Ehrlich Ascites Tumor Cells†

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ABSTRACT: A family of glycopeptides that contain nonreducing terminal α-D-galactosyl residues has been isolated from Pronase digests of delipidated Ehrlich ascites tumor cells. The glycopeptides, which comprise 17.2% of the total plasma membrane hexose, have an average molecular weight of 7500 and are precipitated by *Griffonia simplicifolia* B_4 isolectin, wheat germ agglutinin, and *Ricinus communis* lectin. Exo- and endoglycosidase digestion, periodate oxidation, permethylation analysis, and lectin reactivity provided evidence for a tentative carbohydrate structure for the glycopeptide mixture. The glycopeptides possess tetraantennary branched structures containing a trimannosyl core N-glycosidically linked via an *N,N'*-diacetylchitobiosyl unit to an asparagine residue. Each branch contained repeating →3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→ units resulting in a keratan-like structure, terminated with α-D-Gal*p*-(1→3)-[α-D-Gal*p*-(1→6)]-β-D-Gal*p*- units. The variation in the molecular weight observed for the glycopeptide mixture can be attributed to the variable amounts of →3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→ units found in the branch chains.

The glycoconjugates of Ehrlich ascites tumor cell plasma membranes contain a high density of α-D-galactopyranosyl end groups (Eckhardt & Goldstein, 1979, 1983). *Griffonia simplicifolia* I-B_4 isolectin, a highly specific probe for α-D-galactosyl nonreducing termini, binds to and strongly agglutinates these tumor cells (Eckhardt & Goldstein, 1979, 1983). This lectin is also cytotoxic to Ehrlich cells in vivo (Eckhardt et al., 1982).

In this paper we describe the isolation and characterization from the Ehrlich cell plasma membrane of a family of high molecular weight, complex-type, tetraantennary glycopeptides that are terminated by α-D-galactopyranosyl groups. The oligosaccharide chains of the Ehrlich cell glycopeptides are similar to the keratan-like structures reported for glycopeptides from human erythrocyte band 3 (Fukuda et al., 1979; Krusius et al., 1978; Jarnefelt et al., 1978), Chinese hamster ovary cells (Li et al., 1980), and mouse embryonal carcinoma cells (Muramatsu et al., 1979) in that they contain a variable number of repeating →3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→ units.

Experimental Procedures

Materials

Ferrous 2,4,6-tri-2-pyridyl-*s*-triazine and jack bean hexosaminidase were obtained from Sigma Chemical Co. *Phaseolus vulgaris* lectin was purchased from Difco Laboratories. Pronase, B grade, wheat germ agglutinin, and *Aspergillus niger* β-galactosidase were obtained from Calbiochem-Behring Corp. Coffee bean α-galactosidase, β-nicotinamide adenine dinucleotide, and β-galactose dehydrogenase were purchased from Boehringer Mannheim Biochemicals. Bovine testis β-galactosidase was a gift of Dr. G. W. Jourdian of this University. *Escherichia freundii* endo-β-galactosidase was generously donated by Dr. Y. T. Li of Tulane University. The ovalbumin glycopeptide, $AsnMan_5GlcNAc_5$, was kindly provided by Dr. Rex Montgomery of the University of Iowa. *Ricinus communis* lectin was donated by Dr. Marilynn Etzler of the University of California at Davis. *Griffonia simplicifolia* I lectin mixture and GS I-B_4[1] isolectin were purified as described by Hayes & Goldstein (1974) and Murphy & Goldstein (1977). *Griffonia simplicifolia* lectin II was purified according to Shankar-Iyer et al. (1976). Anti-human blood group B antisera was supplied by the University of Michigan Blood Bank, courtesy of John Judd. All materials were of the highest purity available from commercial sources.

Methods

Preparation of α-D-Galactosyl-Containing Glycopeptides. Ehrlich ascites tumor cell plasma membranes were prepared as described and lyophilized (Eckhardt & Goldstein, 1983). The plasma membrane fraction (115 mg of protein) was extracted with 60 mL of chloroform–methanol (2:1 and 1:2 v/v) for 3 h, each at 25 °C, and lyophilized. The delipidated plasma membranes were suspended in 2 mM calcium chloride (10 mL) in 50 mM Tris buffer, pH 7.9, and treated with 2.4 mg of Pronase, and the digest was incubated with stirring under a toluene atmosphere at 37 °C. Additional Pronase (2.4 mg) was added at 24, 48, 96, 144, and 168 h. Proteolytic digestion was terminated after 192 h by immersion in a boiling water bath for 5 min. The Pronase digest was centrifuged at 27000*g* for 30 min and the supernatant solution removed and saved. The pellet was washed with water (5.0 mL) and centrifuged at 27000*g* for 30 min, and the supernatant was removed and added to the first supernatant solution. The combined supernatant solutions were lyophilized, resuspended in water (2.0 mL), and applied at room temperature to a Bio-Gel P-10 column (1.5 × 105 cm) equilibrated with 0.1 M ammonium bicarbonate at a flow rate of 10 mL/h. Fractions (1.25 mL) were collected and assayed for hexose, absorbance at 280 nm, precipitation with GS I-B_4 by hematocrit tube test, and release of galactose with coffee bean α-galactosidase. Fractions reacting with GS I-B_4 were pooled and chromatographed at a rate of 2.8 mL/h over a GS I–Sepharose column (1.5 × 15 cm) equilibrated with PBS–azide. The column was washed with buffer until the absorbance at 280 nm was zero, as fol-

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[1] Abbreviations: GS, *Griffonia simplicifolia*; GS I-B_4, *Griffonia simplicifolia* I-B_4 isolectin; Tris, tris(hydroxymethyl)aminomethane; PBS, phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.0); Me-α-D-Gal*p*, methyl α-D-galactopyranoside; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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lowed by elution with 40 mL 0.1 M Me-α-D-Gal*p* in PBS–azide. All fractions containing Me-α-D-Gal*p* were pooled, lyophilized, and reapplied to the Bio-Gel P-10 column to separate the α-D-galactosyl-containing glycopeptide from Me-α-D-Gal*p*. Fractions (1.25 mL) were collected and assayed for reaction with GS I-B_4 in the hematocrit tube test. Every fifth fraction was also assayed for Me-α-D-Gal*p* by spotting 125 μL on a TLC plate and developing the plate with butanol–acetic acid–ether–H_2O (9:6:3:1 v/v/v/v). Me-α-D-Gal*p* (R_f 0.217) was detected with a spray containing 5% each of ammonium molybdate, sulfuric acid, and phosphoric acid followed by heating the plate for 10 min at 110 °C (Hannessian & Plessas, 1969). Fractions that reacted with GS I-B_4 were pooled and designated α-D-galactosyl-containing glycopeptides. The elution volume of the α-D-galactosyl-containing glycopeptides from the Bio-Gel P-10 column was used to calculate an approximate molecular weight. The Bio-Gel P-10 column was calibrated with the following oligosaccharides and glycopeptides: dextran 10 (M_n 6200), dextran 8 (M_n 4100), ovalbumin glycopeptide ($AsnMan_5GlcNAc_5$) (M_r 1959), α-(1→2)-*manno*-decose (M_r 1638), α-(1→2)-*manno*-heptose (M_r 1152).

Assay Procedures. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Neutral sugar was determined by the phenol–sulfuric acid colorimetric assay of Dubois et al. (1956) with Me-α-D-Gal*p* as standard. Sphingosine content of the α-galactosyl-containing glycopeptide was determined according to Naoi et al. (1974) with Fabry's glycolipid as standard. The molecular weight of Fabry's glycolipid was calculated on the basis of C_{18} sphingosine and C_{22} fatty acid. The sialic acid content of glycoconjugates was determined by the method of Warren (1959) after hydrolysis at 80 °C for 1 h in 0.1 N sulfuric acid with *N*-acetylneuraminic acid as standard. The precipitation reaction of α-D-galactosyl-containing glycoconjugates with various lectins and antisera was performed as described by Swift et al. (1943). A solution of the test sample was drawn into a hematocrit tube (1 mm × 7.5 cm) followed by a sample of lectin or antisera. The tube was sealed at the lower end with Critoseal (Scientific Products) and examined after 1 h at room temperature for precipitate formation.

The neutral and amino sugar compositions were determined by the method of Porter (1975) with minor modifications (Eckhardt & Goldstein, 1983). The amino acid composition was determined as described by Spackman et al. (1958). GS I–Sepharose 4B was prepared as described previously (Eckhardt & Goldstein, 1983).

Periodate Oxidation. Oxidation of the α-D-galactosyl-containing glycopeptide with periodate was conducted in the dark at 2 °C in 2 mM sodium metaperiodate–40 mM sodium acetate buffer, pH 5.2, in a volume of 550 μL. The concentration of hexose in the glycopeptide sample was 1.07 mM. The course of the oxidation was followed by removing 10-μL aliquots of the reaction mixture and assaying for periodate with ferrous 2,4,6-tri-2-pyridyl-*s*-triazine as described by Avigad (1969). Me-α-D-Gal*p* (0.46 mM), oxidized with periodate under the same conditions, served as a control. After the oxidation of the glycopeptide had gone to completion (144 h), excess periodate was destroyed by the addition of 50 μL of 36 mM ethylene glycol with incubation in the dark at 2 °C for 24 h. The periodate-oxidized glycopeptide was adjusted to pH 9.0 with 2 N sodium hydroxide and reduced by the addition of 50 μL of 1.8 M sodium borohydride with incubation for 2 h at room temperature. Excess borohydride was destroyed by the addition of 100 μL of glacial acetic acid and the sample concentrated to approximately 100 μL with a stream of nitrogen. The sample was applied to a Bio-Gel P-2 column (0.7 × 15 cm) equilibrated in water, and 0.45-mL fractions were collected. Ten microliters of each fraction was spotted on a TLC plate and sugar detected by spraying with 5% ammonium molybdate, sulfuric acid, and phosphoric acid and heating for 10 min at 110 °C (Hannessian & Plessas, 1969). Fractions containing sugar were pooled and lyophilized. The carbohydrate composition of the salt-free, periodate-oxidized, borohydride-reduced glycopeptide was determined as described previously.

Methylation Analysis. The α-D-galactosyl-containing glycopeptides (2.5 μmol of hexose) were methylated using methylsulfinyl carbanion and methyl iodide as described by Stellner et al. (1973) with two modifications. The preparation of methylsulfinyl carbanion and the separation of the permethylated glycopeptide from reactants by extraction with methylene chloride were performed as described by Jannsson et al. (1976). The permethylated glycopeptide was dried overnight in a vacuum desiccator containing phosphorous pentoxide and methylated a second time to ensure complete methylation. The permethylated glycopeptide was then subjected to acetolysis, hydrolysis, reduction, and acetylation. The partially methylated alditol acetates and 2-deoxy-2-(*N*-methylacetamido)hexitol acetates were separated and tentatively identified by gas chromatography under conditions identical with those used for carbohydrate compositional analysis except that the column temperature was programmed from 160 to 190 °C at 2 °C/min and held at the upper temperature. Peak areas were measured by planimetry. The response factors for the methylated alditol acetates were assumed to be equal. Positive identification of the methylated derivatives was obtained by gas chromatography and mass spectrometry on a Varian MAT 311 spectrometer fitted with an OV-225 column.

Degradation of Glycopeptide with Glycosidases. The α-D-galactosyl-containing glycopeptides (0.36 μmol of hexose) in 0.3 mL 0.05 M sodium citrate buffer, pH 4.0, containing 0.04% sodium azide were incubated at 37 °C for 48 h with either 0.25 unit of bovine testis or *Aspergillus niger* β-galactosidase. The release of galactose from the glycopeptide was followed by removing 60-μL aliquots at various time intervals and adding them to 1.0 mL of 2.7 mM NAD^+–0.9 M Tris-HCl buffer, pH 8.5. Four microliters of galactose dehydrogenase (25 units/mL) was added, and the samples were incubated at room temperature for 1 h. absorption of reduced NAD (NADH) at 340 nm was determined. A standard curve was constructed with galactose as standard. Both β-galactosidases were found to be free of α-galactosidase activity as determined by their failure to hydrolyze *p*-nitrophenyl α-D-galactopyranoside.

The α-D-galactosyl-containing glycopeptide (3.2 μmol of hexose) in 1.7 mL of 0.05 M sodium citrate buffer, pH 5.55–0.04% sodium azide was incubated at 37 °C for 132 h with 2.8 units of coffee bean α-galactosidase. A blank was prepared containing only α-galactosidase and buffer to measure the galactose present in the enzyme. The release of galactose from the glycopeptide and the blank was followed by removing 30-μL aliquots at various time periods and assaying for galactose as described above. The coffee bean α-galactosidase was free of β-galactosidase activity as determined by its failure to hydrolyze *p*-nitrophenyl β-D-galactopyranoside and lactose. The α-galactosidase-treated glycopeptides were tested for precipitation reaction with the fol-

lowing lectins: GS I-B_4, *R. communis*, wheat germ agglutinin, *G. simplicifolia* II, *P. vulgaris*, and concanavalin A. The activity of the coffee bean α-galactosidase after 132 h of incubation was 70% of its initial activity.

The α-galactosidase-treated glycopeptide and the enzyme blank were inactivated by immersion in a boiling water bath for 5 min, and after the samples were cooled, bovine testis β-galactosidase (0.25 unit in 0.1 mL) was added to each sample. The samples were incubated at 37 °C for 48 h and 30-μL aliquots removed and assayed for galactose as described above. The α- and β-galactosidase-treated glycopeptide was tested for precipitation reaction with the lectins used above. After 48 h of incubation, the bovine testis β-galactosidase was determined to have retained 100% of its initial activity.

The α- and β-galactosidase-treated glycopeptides and the blank were immersed in a boiling water bath for 5 min to inactivate the β-galactosidase, and after the samples were cooled, jack bean β-*N*-acetylglucosaminidase (2 units in 60 μL) was added to each. The samples were incubated at 37 °C and 50-μL aliquots removed and assayed for liberated *N*-acetyl-D-glucosamine as described by Reissig et al. (1955). *N*-Acetyl-D-glucosamine was used as standard.

The ability of *E. freundii* endo-β-galactosidase to hydrolyze the α-D-galactosyl-containing glycopeptide was also examined. Four enzymatic digestions were conducted at 37 °C in 0.24 mL of 0.01 M sodium acetate buffer, pH 5.7, containing 0.04% sodium azide as follows: (A) glycopeptide (0.36 μmol of hexose), endo-β-galactosidase (1.7 milliunits), coffee bean α-galactosidase (0.66 unit); (B) glycopeptide (0.36 μmol of hexose), endo-β-galactosidase (1.7 milliunits); (C) glycopeptide (0.36 μmol of hexose), coffee bean α-galactosidase (0.66 unit); (D) endo-β-galactosidase (1.7 milliunits), coffee bean α-galactosidase (0.66 unit). Forty-microliter aliquots were removed at various time intervals and assayed for reduced sugar by the method of Park & Johnson (1949). Galactose was used as standard.

Results

Isolation of α-D-Galactosyl-Containing Glycopeptides. In a typical experiment, plasma membranes (115 mg of protein) containing 7.2 mg of hexose were delipidated by extraction with chloroform–methanol. Following delipidation, the plasma membranes contained 5.9 mg of hexose of which Pronase digestion liberated 5.3 mg or 90% of the total hexose present in the delipidated plasma membrane fraction. Upon gel filtration of the Pronase digest over a Bio-Gel P-10 column, most of the hexose-containing material was eluted as a broad, high molecular weight peak. Assay revealed this fraction to react strongly with the GS I-B_4 isolectin and to contain galactose units that were liberated by digestion with coffee bean α-D-galactosidase (Figure 1). All fractions that formed a precipitate with GS I-B_4 (30–70) were pooled and subjected to affinity chromatography over a GS I-Sepharose 4B column. All material absorbing at 280 nm (peptides and amino acids) passed through the column as did approximately half of the hexose. The unbound hexose-containing components failed to precipitate with GS I-B_4, indicating that the affinity column was not over overloaded. The glycopeptide material eluted from the GS I-Sepharose 4B column with Me-α-D-Gal*p* was rechromatographed over a Bio-Gel P-10 column to remove the Me-α-D-Gal*p*. The glycopeptide eluted as a symmetrical peak that did not absorb at 280 nm (Figure 2). All fractions of the glycopeptide were shown to be free of Me-α-D-Gal*p* by TLC. The yield of α-D-galactosyl-containing glycopeptides was 1.24 mg of hexose or 17.2% of the total plasma membrane hexose.

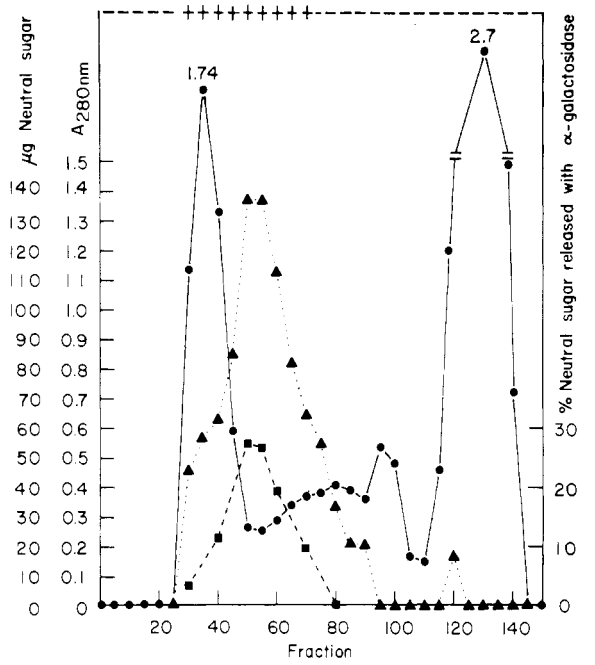


FIGURE 1: Gel-filtration chromatography of Pronase digest of Ehrlich cell plasma membrane on Bio-Gel P-10. Fractions were collected and assayed for hexose (···), absorbance at 280 nm (—), release of galactose with coffee bean α-D-galactosidase (– –), and precipitate formation with GS I-B_4 in the hematocrit tube test. Positive and negative reactions of column fractions with GS I-B_4 are designated by (+) and (−), respectively.

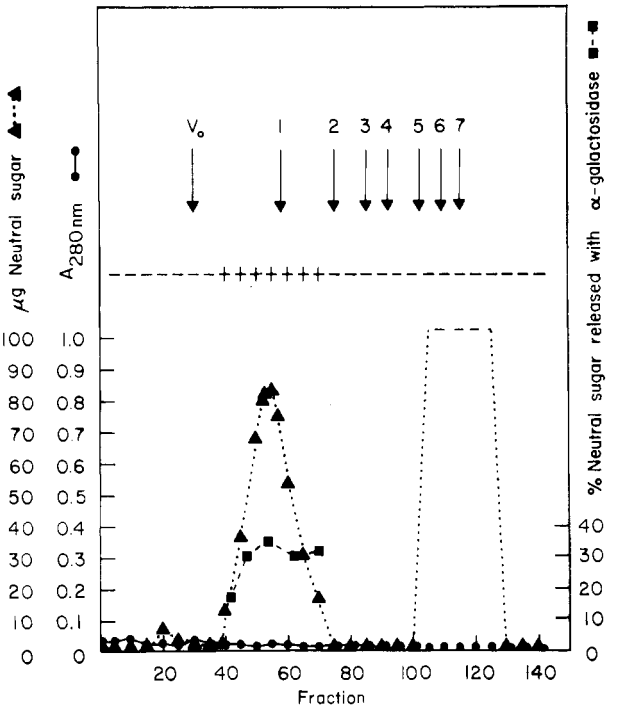


FIGURE 2: Gel-filtration chromatography of GS I bound Pronase digest of Ehrlich cell plasma membrane on Bio-Gel P-10. Molecular weight standards: (V_0) blue dextran; (1) dextran 10 (M_n 6200); (2) dextran$_8$ (M_n 4100); (3) AsnMan$_5$GlcNAc$_2$ (M_r 1959); (4) Man$_{10}$ (M_r 1638); (5) Man$_7$ (M_r 1152); (6) stachyose (M_r 666); (7) Me-α-D-Gal*p* (M_r 194).

The α-D-galactosyl-containing glycopeptides had a molecular weight of approximately 7500 as determined by gel-filtration chromatography over the calibrated Bio-Gel P-10 column. A second Pronase digestion of the purified α-D-galactosyl-containing glycopeptides did not change their molecular weight.

Five fractions of the α-D-galactosyl-containing glycopeptide

Table I: Chemical Properties of α-D-Galactosyl-Containing Glycopeptides Chromatographed on Bio-Gel P-10

fraction	compositions			Gal/Man	Gal/GlcNAc	mol of galactose released by α-D-galactosidase/mol of hexose	calcd M_r
	Gal	GlcNAc	Man				
41–42	34.8	29.1	3	11.6	1.20	0.167	12031
46	31	19	3	10.3	1.63	0.301	9365
56	16.8	9	3	5.6	1.87	0.349	5035
63	12	7.5	3	4.0	1.6	0.300	3952
70–71	11.1	12	3	3.7	0.93	0.320	4720

peak eluted from the Bio-Gel P-10 column (Figure 2) were subjected to carbohydrate compositional analysis. Analysis of fractions 41–42, 46, 56, 63, and 70–71 by GLC revealed marked differences in carbohydrate composition between fractions. All of the glucosamine in the glycopeptide fractions was assumed to be N-acetylated. The tracings (normalized to mannose) demonstrated a decrease in the amount of galactose relative to mannose when proceeding from early eluting fractions 41–42 (galactose/mannose ratio = 11.6) to late eluting fractions 70–71 (galactose/mannose ratio = 3.7). The galactose content relative to *N*-acetylglucosamine also varied, being at a maximum in intermediate fraction 56 (galactose/*N*-acetylglucosamine ratio = 1.87) and decreasing to minima in either early eluting fractions 41–42 (galactose/*N*-acetylglucosamine ratio = 1.20) or late eluting fractions 70–71 (galactose/*N*-acetylglucosamine ratio = 0.93). The carbohydrate compositional data for each fraction, normalized to three mannosyl residues, are summarized in Table I. The composition for the earliest eluting fraction 41–42 was galactose/*N*-acetylgucosamine/mannose 34.8:29.1:3 with a calculated molecular weight of 12031. The carbohydrate composition for the intermediate fraction 56 was galactose/*N*-acetylglucosamine/mannose 16.8:9:3 with a calculated molecular weight of 5035, and the composition for fraction 70–71 was galactose/*N*-acetylglucosamine/mannose 11.1:12:3 with a molecular weight of 4720. The amount of galactose released by coffee bean α-galactosidase from the glycopeptides in each fraction was fairly constant, ranging from 30–34.9% of the total hexose in fractions 46, 56, 63, and 70–71 but only 16.7% in fraction 41–42. All of the fractions gave strong precipitation reactions with GS I-B_4 in the hematocrit tube test. These data indicated that the α-D-galactosyl-containing glycopeptide was not a homogeneous species but rather a heterogeneous mixture representing a spectrum of glycopeptides of varying molecular weights. Due to insufficient glycopeptide material in any one fraction, all fractions (40–74) from the Bio-Gel P-10 column with α-D-galactosyl-containing glycopeptide were pooled for carbohydrate structural determination.

Structural Studies on the α-D-Galactosyl-Containing Glycopeptide Mixture. The carbohydrate composition of the α-D-galactosyl-containing glycopeptide mixture, normalized to three mannosyl residues, was galactose/*N*-acetylglucosamine/mannose/sialic acid in a molar ratio of 20:17:3:0.3–0.5. All of the glucosamine was assumed to be N-acetylated. *N*-Acetylgalactosamine, fucose, and glucose were not detected (Table II). The major amino acid was aspartic acid with 0.9 mol/mol of glycopeptide, suggesting the carbohydrate was N-glycosidically linked to an asparagine residue. There were also significant amounts of othe neutral and acidic amino acids that were resistant to two rounds of Pronase digestion, probably due to their proximity to the oligosaccharide chain(s). The calculated molecular weight of the glycopeptide mixture on the basis of composition was 7596. This was in good agreement with the molecular weight of 7500 obtained for the glycopeptide by gel-filtration chromatography over Bio-Gel P-10.

Table II: Composition of α-D-Galactosyl-Containing Glycopeptide Mixture[a]

residue	mol/mol of glycopeptide
galactose	20
N-acetylglucosamine	17
mannose	3
sialic acid	0.3–0.5
aspartic acid	0.9
threonine	0.5
glycine	0.3
serine	0.2
glutamic acid	0.2
alanine	0.2
isoleucine	0.1
leucine	0.1
sphingosine	<0.018

[a] Calculated molecular weight 7596.

The glycopeptide mixture formed precipitates with wheat germ agglutinin and *R. communis* lectin in addition to GS I-B_4. The glycopeptide did not react with *G. simplicifolia* II lectin, which suggested the absence of terminal nonreducing *N*-acetylglucosaminyl residues and the probability that wheat germ agglutinin was binding to internal 1→4, 1→6, or 1→4,1→6 β-linked *N*-acetylglucosaminyl residues. The glycopeptide mixture also failed to form a precipitate with PHA and concanavalin A. The glycopeptide mixture was also unreactive with anti-blood group B or P_1 antisera, which suggested α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→R) and α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→R) were unlikely structures for the nonreducing terminal portion of the α-D-galactosyl-containing glycopeptde. Sphingosine was not detected in the glycopeptide mixture, ruling out glycolipids as possible contributors of α-D-Gal*p* residues.

The α-D-galactosyl-containing glycopeptide mixture consumed 0.742 mol of periodate/mol of hexose or 17 mol of periodate/mol of glycopeptide (Figure 3). As a control, Me-α-D-Gal*p* consumed 1.94 mol of periodate/mol of glycoside under identical conditions, in excellent agreement with the theoretical value of 2.0 mol of periodate/mol of Me-α-D-Gal*p*. The carbohydrate composition of the glycopeptide after periodate oxidation, reduction with sodium borohydride, and acid hydrolysis was galactose/*N*-acetylglucosamine/mannose 13:14:2.6, demonstrating the destruction by periodate of 7 galactosyl residues, 3 *N*-acetylglucosaminyl residues, and 0.4 mannosyl residues. The periodate-oxidized glycopeptide mixture formed a strong precipitate with wheat germ agglutinin but no longer reacted with GS I-B_4 or *R. communis* lectin, indicating the destruction of terminal galactosyl residues (Table III).

Treatment of the α-D-galactosyl-containing glycopeptide mixture with β-galactosidase from *A. niger* or bovine testis failed to release any galactose. Digestion of the glycopeptide with coffee bean α-galactosidase released 7.3 residues of ga-

Table III: Lectin Reactivity of α-D-Galactosyl-Containing Glycopeptide Mixture after Periodate Oxidation and Glycosidase Digestion

treatment	lectin reactivity[a]				
	GS I-B_4	GS II	Con A	*R. communis*	WGA
untreated glycopeptide	+	−	−	+	+
periodate oxidation	−	−	−	−	+
bovine testis β-D-galactosidase or *A. niger* β-D-galactosidase	+	−	−	+	+
coffee bean α-D-galactosidase, followed by β-D-galactosidase	−, −	−, +	−, −	+, −	+, +

[a] GS II, *G. simplicifolia* II; Con A, concanavalin A; WGA, wheat germ agglutinin.

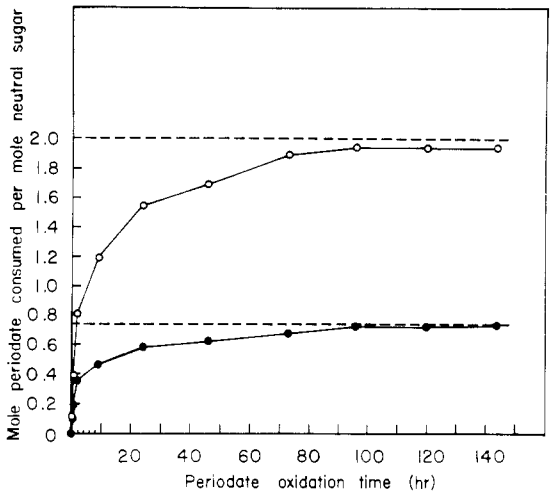


FIGURE 3: Periodate oxidation of α-D-galactosyl-containing glycopeptide. The oxidation was conducted in the dark at 2 °C in 2 mM sodium metaperiodate–40 mM sodium acetate buffer, pH 5.2. Aliquots were removed (10 μL) and assayed for periodate. The concentration of the glycopeptide (—) was 46.5 μM. Me-α-D-Gal*p* (O), 0.46 mM, oxidized with periodate under the same conditions, served as a control.

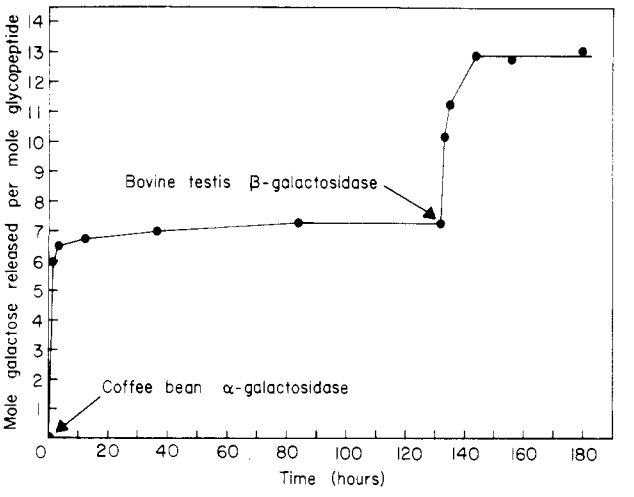


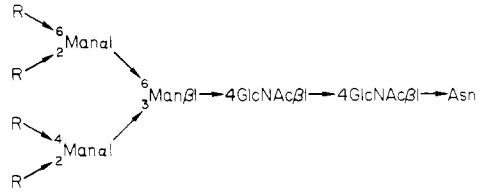
FIGURE 4: Digestion of α-D-galactosyl-containing glycopeptide with coffee bean α-D-galactosidase and bovine testis β-galactosidase. Incubation conditions are described under Methods. Liberated galactose was determined by NAD-linked β-galactose dehydrogenase assay.

lactose and abolished reactivity of the glycopeptide with GS I-B_4 (Figure 4). The α-galactosidase-treated glycopeptide still formed a strong precipitate with *R. communis* lectin and wheat germ agglutinin. Digestion of the α-galactosidase-treated glycopeptide with bovine testis β-galactosidase liberated 5.5 mol of galactose and totally abolished reactivity of the glycopeptide with *R. communis* lectin. The glycopeptide after treatment with both α- and β-galactosidases still formed a strong precipitate with wheat germ agglutinin and also reacted for the first time with the *G. simplicifolia* II lectin. These results suggested digestion of the glycopeptide with both α- and β-galactosidase-exposed *N*-acetylglucosaminyl residues. Incubation of the α,β-galactosidase-digested glycopeptide with jack bean β-*N*-acetylglucosaminidase for 142 h at 37 °C released only 0.85 residue of *N*-acetylglucosamine.

The intact α-D-galactosyl-containing glycopeptide was found to be resistant to hydrolysis by *E. freundii* endo-β-galactosidase at an enzyme concentration of 0.0068 unit/mL. The enzyme hydrolyzes the galactosidic bond in glycoprotein and glycolipid structures containing internal β-D-Gal*p*-(1→4)-β-D-GlcNAc (or Glc) sequences (Fukuda & Matsumura, 1976; Fukuda et al., 1978). The presence of branch points near or at the susceptibile galactosyl residues inhibits enzymatic hydrolysis (Fukuda & Matsumura, 1976), but Fukuda et al. (1978) claimed this resistance may be overcome with a high concentration of endo-β-galactosidase (1.25 unit/mL). Digestion of the α-D-galactosyl-containing glycopeptide with coffee bean α-galactosidase generated a product that was slowly hydrolyzed by 0.0068 unit/mL endo-β-galactosidase, demonstrating the presence of internal β-D-Gal*p*-(1→4)-β-D-GlcNAc units in the glycopeptide mixture and providing an explanation for reaction of the glycopeptide with wheat germ agglutinin.

Wheat germ agglutinin is capable of forming a precipitate with isolated (1→4)-*N*-acetyl-β-D-glucosaminyl residues such as occur in keratan and cell wall polysaccharide (Carlsson et al., 1976). The endo-β-galactosidase reaction was still incomplete after 46 h. The resistance of the α-D-galactosyl-containing glycopeptide to the action of endo-β-galactosidase prior to removal of the α-D-galactosyl end groups parallels the observation made by Fukuda & Matsumura (1976) in their study of type AB blood group substance. These investigators reported AB blood group substance to be completely resistant to *E. freundii* endo-β-galactosidase (1 unit/mL). Removal of peripheral sugars by Smith degradation generated a product that was hydrolyzed by endo-β-galactosidase.

Permethylation studies of the intact glycopeptide gave 7.3 mol of 2,3,4,6-tetra-*O*-methylgalactose, 8.1 mol of 2,4,6-tri-*O*-methylgalactose, and 4.5 mol of 2,4-di-*O*-methylgalactose per mol of glycopeptide. Glucosamine was present solely as its 3,6-di-*O*-methyl derivative. The mannose units all gave dimethyl derivatives (3,6-di-*O*-methylmannose, 0.6 residue; 3,4-di-*O*-methylmannose, 0.7 residue; 2,4-di-*O*-methylmannose, 0.7 residue), indicating their involvement as branch points in the oligosaccharide chain. The methylation products obtained for the mannose residues were identical with that expected for a tetraantennary branched structure similar to the one present in α_1-acid glycoprotein (Fournet et al., 1978) in which the mannose residues are linked in the core region in the following manner:



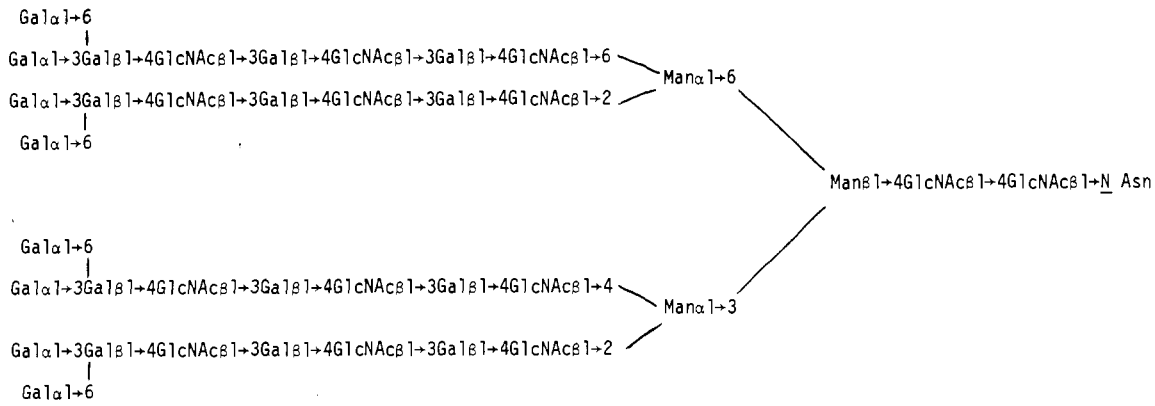


FIGURE 5: Tentative structure for average α-D-galactosyl-containing glycopeptide from Ehrlich ascites tumor cell plasma membrane.

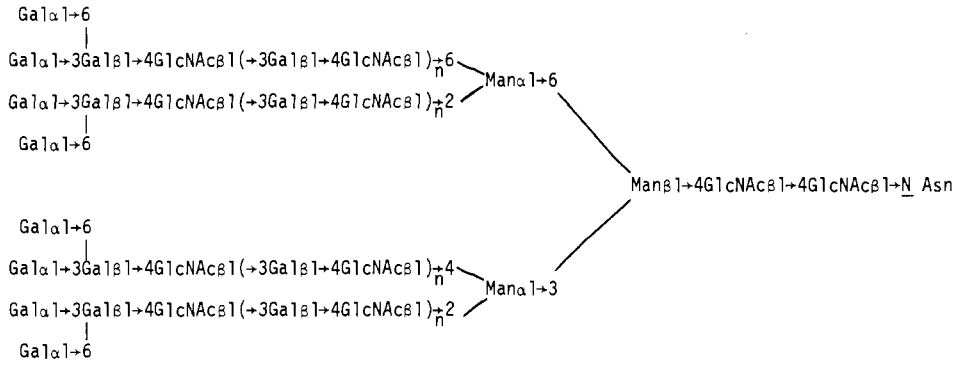


FIGURE 6: Tentative structure for family of α-D-galactosyl-containing glycopeptides from Ehrlich ascites tumor cell plasma membrane. The number of β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→3) units per branch (*n*) can vary between 0 and 6.

All glycopeptides containing the mannosyl core shown above have been shown to be N-glycosidically linked via an *N,N'*-diacetylchitobiosyl unit to an asparagine residue. The presence of mannose with the indicated linkages together with the observation that aspartic acid was the major amino acid in the glycopeptide strongly suggested that the oligosaccharide portion of the α-D-galactosyl-containing glycopeptide was linked to the peptide through an *N*-glycosidic linkage.

The finding of seven to eight nonreducing terminal galactosyl residues by methylation analysis in conjunction with the release of seven to eight galactosyl units with coffee bean α-galactosidase established that all of the chain-terminating galactosyl residues were of the α-configuration. Four and one-half of the galactose residues were substituted at both the C-3 and C-6 positions, which would generate eight to nine ends in the oligosaccharide chain and would account for the seven to eight terminal galactose residues detected by methylation analysis. The remaining eight galactosyl units are linked solely at the C-3 position. A tentative structure for the α-D-galactosyl-containing glycopeptide mixture that takes into account the above experimental evidence is presented in Figure 5.

The tentative structure has a composition of Gal/GlcNAc/Man/Asn 20:14:3:1, which is in good agreement with the observed composition. The model glycopeptide would react with GS I-B_4 and *R. communis* lectin at the terminal α-D-galactosyl residues and with wheat germ agglutinin at the internal *N*-acetyl-β-D-glucosaminyl residues. The eight terminal α-D-galactosyl residues would consume 16 mol of periodate, and the 2,6-linked mannose would consume 1 mol of periodate for a total of 17 mol of periodate, which is identical with the observed value. The sugar composition of the model glycopeptide after periodate oxidation would be Gal/GlcNAc/Man 12:14:2, which is in close agreement with the experimental results. α-Galactosidase digestion would liberate 8 mol of galactose, and additional incubation with β-galactosidase would liberate 4 mol of galactose, exposing *N*-acetyl-β-D-glucosaminyl end groups. Permethylation would yield eight 2,3,4,6-tetra-*O*-methylgalactose residues, eight 2,4,6-tri-*O*-methylgalactose residues, and four 2,4-di-*O*-methylgalactose residues. All the *N*-acetyl-D-glucosaminyl residues bear substituents at the C-4 position and would therefore be resistant to periodate oxidation. The tentative structure also contains the branched mannosyl core, which would generate the tetraantennary structure.

The α-D-galactopyranosyl stubs were glycosidically-linked via 1→6 linkages to penultimate galactosyl units to account for the failure of the glycopeptide to react with anti-blood group B antisera. If the α-(1→6)-linked D-galactosyl groups were situated on internal galactosyl residues other than the penultimate galactose, the glycopeptide would have eight branches all terminated with α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→R. A glycopeptide with this structure would be expected to react with anti-blood group B antisera (Schiffman & Marcus, 1964). The tentative structure also contains internal -β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*- units, which would be susceptible to hydrolysis by endo-β-galactosidase.

The tentative structure predicts that four *N*-acetyl-β-D-glucosaminyl residues would be liberated on treatment of the glycopeptide with α- and β-galactoside followed by β-*N*-acetyl-D-glucosaminidase. Experimentally, only 0.85 *N*-acetylglucosamine residue was released. Because the α- and β-galactosidase-treated glycopeptide precipitated with *G. simplicifolia* II lectin, there should be at the minimum two exposed *N*-acetylglucosamine residues in order to form the lattice required for precipitate formation. This unexpected low release of *N*-acetylglucosamine by β-*N*-acetyl-D-glucosaminidase remains unexplained.

To account for the heterogeneity in the carbohydrate com-

Table IV: Chemical Properties for a Hypothetical Tetraantennary Branched Glycopeptide[a] Containing a Variable Number of β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3) Units vs. the Observed Chemical Properties for the α-D-Galactosyl-Containing Glycopeptide Mixture[b]

	composition						corresponding glycopeptide fraction eluted from Bio-Gel P-10 column (Table I)
n[c]	Gal	GlcNAc	Man	Gal/Man	Gal/GlcNAc	calcd M_r	
6	36 (34.8)	30 (29.1)	3 (3)	12.0 (11.6)	1.20 (1.19)	12408 (12031)	41–42
5	32	26	3	10.7	1.23	10948	
4	28 (31)	22 (19)	3 (3)	9.3 (10.3)	1.27 (1.63)	9488 (9365)	46
3	24	18	3	8.0	1.33	8028	
2	20	14	3	6.67	1.43	6568	
1	16 (16.8)	10 (9)	3 (3)	5.33 (5.6)	1.60 (1.87)	5108 (5035)	56
0	12 (12)	6 (7.5)	3 (3)	4.0 (4.0)	2.0 (1.6)	3648 (3952)	63

[a] Hypothetical values are not enclosed in parentheses. [b] Observed values for α-D-galactosyl-containing glycopeptide fractions are enclosed in parentheses. [c] n, number of β-D-Galp(1→4)-β-D-GlcNAcp-(1→3) units per branch of a tetraantennary branched glycopeptide.

position of the α-D-galactosyl-containing glycopeptide described above (Table I), the modified structure shown in Figure 6 is proposed. This structure retains all of the features of Figure 5, but contains a variable number of β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3) units, which vary from 0 to 24 mol/mol of glycopeptide. This repeating β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3) sequence would lead to a glycopeptide with keratan-like chains terminated with α-D-galactosyl units. Increasing the number of β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3) units when proceeding from low to high molecular weight glycopeptide fractions, would generate a family of glycopeptides that would have chemical properties very similar to the various glycopeptide fractions eluted from the Bio-Gel P-10 column and tabulated in Table I. A comparison can be made of the chemical properties for the different fractions eluting off the Bio-Gel P-10 column with the hypothetical properties of a glycopeptide family containing increasing numbers of β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3) units. These data are presented in Table IV.

In the variable-structure model, the setting of n equal to 6, 4, 1, or 0 will generate glycopeptides with carbohydrate compositions and molecular weights that correspond very closely with the observed properties of fractions 41–42, 46, 56, and 63, respectively. The model breaks down for the low molecular weight fractions (71–72), and this may reflect incomplete synthesis in these oligosaccharide chains. The variable-structure model was presented with the number of β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3) units per branch being identical, but there is no evidence to support this assumption. The number of β-D-Galp-(1→4)-β-D-GlcNAcp-(1→3) units could vary greatly from branch to branch within the same glycopeptide.

Discussion

The oligosaccharide chains of the Ehrlich cell α-D-Galp-containing glycopeptides have a number of structural features in common, as well as several differences, with the megaloglycopeptides isolated from Chinese hamster ovary cells, human erythrocytes, and murine embryonal carcinoma cells. All of the megaloglycopeptides are found on the cell surface, have a high content of galactose and *N*-acetylglucosamine, and have high molecular weights ranging from 4000 to 13 000. Each of the glycopeptides has repeating →3)-β-D-Galp-(1→4)-β-D-GlcNAcp-(1→ units linked to a trimannosyl-*N*,*N*′-diacetylchitobiose core that is linked to an asparagine residue (Li et al., 1980; Krusius et al., 1978; Muramatsu et al., 1979).

Methylation analysis of the Ehrlich cell and human erythrocyte megaloglycopeptides produced similar results with *N*-acetylglucosamine existing mainly as the 4-O-substituted derivative and the presence of terminal galactose, 3-O-substituted galactose, and 3,6-di-O-substituted galactosyl residues (Krusius et al., 1978; Jarnefelt et al., 1978). Methylation analysis of Chinese hamster ovary megaloglycopeptide indicated all of the *N*-acetylglucosamine was 4-O substituted. However, galactose was predominantly 3-O substituted with a small number of terminal galactose residues and no disubstituted galactose residues (Li et al., 1980). The nonreducing termini of the Chinese hamster ovary megaloglycopeptide contained solely β-D-Galp residues, whereas the Ehrlich cell megaloglycopeptide contained solely α-D-Galp residues. The nonreducing termini of the erythrocyte megaloglycopeptide were found to be very heterogeneous, terminated by β-*N*-acetyl-D-glucosamine, β-D-galactose, α-*N*-acetylneuraminic acid, or blood group A or B determinants (Krusius et al., 1978).

The Ehrlich cell keratan-like α-D-Galp-containing glycopeptides as isolated were also found to be very heterogeneous in size, which we attribute to variable amounts of →3)-β-D-Galp-(1→4)-β-D-GlcNAcp-(1→ units. The glycopeptide mixture was isolated from a delipidated Ehrlich cell plasma membrane fraction that was shown to contain a number of α-D-galactosyl-terminated glycoproteins (Eckhardt & Goldstein, 1983). It is not known whether the megaloglycopeptides from a particular glycoprotein has a fixed number of →3)-β-D-Galp-(1→4)-β-D-GlcNAcp-(1→ units, resulting in a unique carbohydrate sequence, or the length of the repeating unit for any individual glycoprotein is variable, resulting in a heterogeneous structure. An α-D-Galp-containing glycopeptide was obtained from the major glycoprotein (M_r 130 000) of the Ehrlich cell plasma membrane. The carbohydrate composition of this glycopeptide was galactose/*N*-acetylglucosamine/mannose/fucose in the molar ratio of 9.4:11.2:3.0:0.5. The calculated molecular weight of this glycopeptide was 4364 (Eckhardt & Goldstein, 1983). These results would indicate that this glycopeptide falls on the low molecular weight end of the mixture of Ehrlich cell plasma membrane megaloglycopeptides (average molecular weight 7500). This would tend to support a somewhat fixed number of repeating units in the keratan-like glycopeptide for this particular glycoprotein.

The Ehrlich cell α-D-Galp-containing megaloglycopeptides were isolated from an enriched plasma membrane fraction. It is not know if other internal organelles of the Ehrlich cell contain glycoproteins with α-D-Galp residues or repeating →3)-β-D-Galp-(1→4)-β-D-GlcNAcp-(1→ units. The function of these megaloglycopeptides on the cell surface is still unknown. However, a blood group I active, biantennary ceramide decasaccharide (Hanfland et al., 1981) and a ceramide pentadecasaccharide (Dabrowski & Hanfland, 1982) were re-

cently isolated from rabbit erythrocytes. These glycosphingolipids both contain α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNAc-terminated chains similar to the structures postulated for the Ehrlich cell membrane glycopeptides. It is probable that an α-(1→3)-galactosyltransferase, similar to the one described in Ehrlich cell microsomes (Blake & Goldstein, 1981), is also present in rabbit erythrocytes.

Registry No. α-D-Galactopyranose, 3646-73-9.

References

Avigad, G. (1969) *Carbohydr. Res. 11*, 119–123.

Blake, D. A., & Goldstein, I. J. (1981) *J. Biol. Chem. 256*, 5387–5391.

Carlsson, H. E., Lonngren, J., Goldstein, I. J., Christner, J. E., & Jourdian, G. W. (1976) *FEBS Lett. 62*, 38–40.

Dabrowski, J., & Hanfland, P. (1982) *FEBS Lett. 142*, 138–142.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Eckhardt, A. E., & Goldstein, I. J. (1979) in *Glycoconjugate Research* (Gregory, J. D., & Jeanloz, R. W., Eds.) Vol. 2, pp 1043–1045, Academic Press, New York.

Eckhardt, A. E., & Goldstein, I. J. (1983) *Biochemistry* (preceding paper in this issue).

Eckhardt, A. E., Malone, B. N., & Goldstein, I. J. (1982) *Cancer Res. 42*, 2977–2979.

Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliengenthart, J. F. G., Binette, J. P., & Schmid, K. (1978) *Biochemistry 17*, 5206–5214.

Fukuda, M. N., & Matsumura, G. (1976) *J. Biol. Chem. 251*, 6218–6225.

Fukuda, M. N., Watanabe, K., & Hakomori, S. (1978) *J. Biol. Chem. 253*, 6814–6819.

Fukuda, M., Fukuda, M. N., & Hakomori, S. (1979) *J. Biol. Chem. 254*, 3700–3703.

Hanfland, P., Egge, H., Dabrowski, U., Kuhn, S., Roelcke, D., & Dabrowski, J. (1981) *Biochemistry 20*, 5310–5319.

Hannessian, S., & Plessas, N. R. (1969) *J. Org. Chem. 34*, 1035–1044.

Hayes, C. E., & Goldsten, I. J. (1974) *J. Biol. Chem. 246*, 1904–1914.

Jannson, P. E., Kenne, L., Liedgren, H., Lindberg, B., & Lonngren, J. (1976) *Chem. Commun. 8*, 1–75.

Jarnefelt, J., Rush, J., Li, Y., T., & Laine, R. A. (1978) *J. Biol. Chem. 253*, 8006–8009.

Krusius, T., Finne, J., & Rauvala, H. (1978) *Eur. J. Biochem. 92*, 289–300.

Li, E., Gibson, R., & Kornfeld, S. (1980) *Arch. Biochem. Biophys. 199*, 393–399.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Muramatsu, T., Gachelin, G., Damonneville, M., Delarbre, C., & Jacob, F. (1979) *Cell (Cambridge, Mass.) 18*, 183–191.

Murphy, L. A., & Goldstein, I. J. (1977) *J. Biol. Chem. 252*, 4739–4742.

Naoi, M., Lee, Y. C., & Roseman, S. (1974) *Anal. Biochem. 58*, 571–577.

Park, J. T., & Johnson, M. J. (1949) *J. Biol. Chem. 181*, 149–151.

Porter, W. H. (1975) *Anal. Biochem. 63*, 27–43.

Reissig, J. L., Reinhold, V., & Leloir, L. F. (1955) *J. Biol. Chem. 217*, 959–966.

Schiffman, G., & Marcus, D. M. (1964) *Prog. Hematol. 4*, 97–116.

Shankar-Iyer, P. N. S., Wilkinson, K. D., & Goldstein, I. J. (1976) *Arch. Biochem. Biophys. 177*, 330–333.

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.

Stellner, K., Saitg, H., & Hakomori, S. (1973) *Arch. Biochem. Biophys. 155*, 464–472.

Swift, H. F., Wilson, A. T., & Lancefield, R. C. (1943) *J. Exp. Med. 78*, 127–133.

Warren, L. (1959) *J. Biol. Chem. 234*, 1971–1975.